Alma Mater Studiorum - Università di Bologna

DOTTORATO DI RICERCA IN
SCIENZE E TECNOLOGIE AGRARIE, AMBIENTALI E ALIMENTARI
XXVI CICLO

Settore Concorsuale di afferenza: 07/E1
Settore Scientifico Disciplinare: AGR/13

Land use change to perennial energy crops in Northern Italy: Effects on soil organic carbon sequestration and distribution, soil enzyme activities and microbial communities

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Esame finale anno 2014

“Venite amici, che non è tardi per scoprire un nuovo mondo.
Io vi propongo di andare più in là dell’orizzonte,
e se anche non abbiamo l’energia che in giorni lontani mosse la terra e il cielo,
siamo ancora gli stessi.
Unica, eguale tempra di eroici cuori, indeboliti forse dal fato,
ma con ancora la voglia di combattere, di cercare, di trovare...e di non cedere.”
(A.L. Tennyson)

...a me stessa.

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GENERAL ABSTRACT

Soil is considered the second major biologically active pool of carbon after the oceans. Its capacity to act as a carbon sink depends on a variety of factors such as existing soil carbon, soil type, climate, crop types and management practices. Loss of organic carbon from soil has been estimated at about 42-78 Gt of carbon over the last century, due to anthropic influences. Moreover, since the 1960's, there has been a decline of grassland and forests in Europe, with an increase of cropland and arable areas. In this scenario, practical measures are primary to enhance soil organic carbon sequestration in agricultural soil.

A promising solution pursued in North European countries is the use of perennial crops, such as *Miscanthus* and giant reed, to restore soil carbon stocks and fertility, while concurrently producing a valuable bioenergy feedstock. Indeed, recent research has shown a high carbon sequestration potential across Europe. These studies are available under Atlantic and Continental environments of North-Central Europe, while there is a substantial lack of information for Southern Europe.

Therefore, this research was conducted in the South-Eastern Po Valley (Northern Italy), in a Mediterranean-temperate climatic zone. This agro-ecosystem is characterised by low average soil organic carbon levels (<10 g C kg^{-1} up to 0.30 m), largely due to anthropic practices.

The aim was to assess the factors influencing soil carbon sequestration and its distribution through soil layers and within soil fractions, after a 9-year old conversion from two annual crop systems, continuous wheat and maize/wheat rotation, to *Miscanthus* (*Miscanthus × giganteus*) and giant reed (*Arundo donax*), respectively.

The ^{13}C natural abundance, in three layers up to 0.60 m, was used to evaluate the total amount of soil organic carbon in annual and perennial species, and determine the portion of carbon derived from perennial crops. Soil organic carbon was significantly higher under perennial (average, 91 Mg C ha^{-1}) than

annual species (average, 56 Mg C ha^{-1}), with a stronger accumulation in the topsoil (0-0.15 m). After 9 years of *Miscanthus* plantation, the amount of C$_4$-derived carbon was 18.7 Mg ha^{-1}, mostly stored at 0-0.15 m, whereas the amount of C$_3$-derived carbon, under giant reed, was 34.7 Mg ha^{-1}, more evenly distributed through soil depths.

To better investigate the fate of newly sequestered carbon, physical soil fractionation was combined with ^{13}C natural abundance analysis. The newly derived-carbon from perennial crops was mainly found as particulate organic matter (68% and 71% in *Miscanthus* and giant reed, respectively), i.e. it was in a labile state with short turnover rate. Under giant reed, organic matter associated with the microaggregates and the mineral fraction explains the main difference in soil organic carbon, with respect to *Miscanthus*. Indeed, under giant reed, microaggregates and mineral fraction played a relevant role, storing much more newly derived-carbon than under *Miscanthus*. Conversely, this latter appeared to preserve more old derived carbon.

A molecular approach based on denaturing gradient gel electrophoresis (PCR-DGGE) was used to evaluate changes occurred on microbial community structure, after the land conversion to perennial energy crops. Functional aspects were investigated through the determination of three soil enzymes involved in nutrient cycles (β-glucosidase, urease and alkaline phosphatase). Introduction of perennial energy crops positively stimulated the three soil enzymes, especially in the topsoil (0-0.15 m), where accumulation of carbon and nitrogen was stronger. The DGGE profiles revealed some differences between land use system and soil microbial communities. Community richness was higher in perennial than in annual crops, but no effect of soil depth was observed. In contrast, Shannon index of diversity was not influenced by land use system, but only by soil depth, being enhanced in the topsoil (+32%).

In conclusion, this research shows a remarkable potential of *Miscanthus* and giant reed to enhance soil organic carbon sequestration, also in Mediterranean conditions. It is perceived that reduced soil disturbance, that characterises perennial crops management, contributes to maintain more carbon in the stable soil fractions, positively reflecting on microbial communities.

LIST OF ACRONYMS

ANOVA	Analysis of variance
ARPA	Agenzia Regionale per la Protezione dell' Ambiente
a.s.l.	Above sea level
C_{mic}	Microbial biomass carbon
DGGE	Denaturing Gradient Gel Electrophoresis
FAO	Food and Agriculture Organization of the United Nations
GHG	Greenhouse gas
GLU	β-glucosidase
H'	Shannon's diversity index
IALF	Intraggregate light fraction
IPCC	Intergovernmental Panel on Climate Change
LSD	Least significant difference
LUC	Land use change
MF	Mineral fraction
P_{AL}	Alkaline phosphatase
P_{AVAIL}	Available phosphorus (Olsen method)
PCR	Polymerase chain reaction
PDB	Pee Dee formation belemnite carbonate standard
POM	Particulate organic matter

r	Pearson’s product moment correlation coefficient
R	Community richness
RDW	Root dry weight
SNK	Student - Newman-Keuls test
SOC	Soil organic carbon
SOM	Soil organic matter
TN	Soil total nitrogen
UR	Urease
WRR	Water Resources Research

GENERAL INTRODUCTION

Since the beginning of the industrial revolution, greenhouse gas (GHG) emissions have significantly increased and the burning of fossil fuels and cement manufacturing have been the major contributors (Dumanski, 2004). However, nowadays agriculture and land use changes (LUCs) for agricultural, forestry, residential and industrial purposes, appear to have important effects on GHG emissions and carbon stocks in soil (Feddema et al., 2005) with a contribution of about 20% of the anthropic emissions. Carbon sequestration has been recognised in the Kyoto Protocol (Article 3.4) as one of the two major strategies in the agricultural context to reduce direct emissions of CO_2 from soil, along with the use of biomass for energy production to mitigate carbon emissions from fossil fuels. These strategies are strongly interlinked (Anderson-Teixeira et al., 2009) and this complexity cannot be overlooked in the development of GHG mitigation policies.

In general, soil carbon sequestration is defined as the long-term incorporation of atmospheric CO_2 into the soil, in the form of stable organic compounds (Zimmermann, 2012). The rate of this process depends on the input of the net primary production and on its fraction being recycled into the soil. Loss of carbon is determined by organic matter mineralisation and loss of topsoil by erosion (Freibauer et al., 2004). When the input of carbon is larger than the output, soil carbon sequestration occurs.

Whether agricultural soils act as sink or source of carbon depends on management practices used (IPCC, 2000). For example, burning of crop residues, grassland degradation, low water use efficiencies, soil organic matter (SOM) and fertility loss, excessive tillage, etc. are practices that promote net carbon emissions. Instead, well-managed lands under good conservation and nutrient management, conservation or zero tillage, well managed fallows, preservation of wetlands, etc. enhance carbon sink activity (Dumanski, 2004). Increasing the level of soil organic carbon (SOC) and SOM can provide considerable environmental and agricultural co-benefits (Wood et al., 2000). Increased SOM normally improves soil aggregation, which, in turn, improves soil aeration, infiltration, surface and groundwater quality and reduces soil erosion. Moreover, the increased SOM enhances soil water storage capacity, providing some degree of mitigation against crop failure in dry years (Hudson. 1994).

The increase of SOM, through carbon sequestration, also increases nutrient cycling by stimulating soil biology and biodiversity. Thereby, soil decomposition rate and nutrient supply are stimulated and soil fertility improves because less chemical fertilisers are required (Dumanski, 2004).

Since soil is an heterogeneous and dynamic ecosystem, especially in the agricultural context, where it is continuously subjected to land management and use change, soil carbon sequestered in arable soils is non-permanent as it is lost more rapidly than it accumulates (Smith et al., 1996). As affirmed by Lal (2004), land use and soil management play an important role in the global carbon budget, leading to a depletion of up to two thirds of the original SOC contents in agricultural soils, equivalent to a loss of 30 to 40 Mg C ha^{-1}. The phenomenon is ever-increasing, with an expectation of important land use changes in Europe in the coming decades (WRR, 1992; Rounsevell et al., 2005). Land use types differ in the content of carbon stored in soil and vegetation (Arrouays et al., 2001; Rodriguez-Murillo, 2001; Lettens et al., 2004; Bellamy et al., 2005): SOC stocks under cropland are lower than under pasture, perennial crops or forest. Thereby, conversion of forest or pasture to cropland is found to decrease SOC stocks; Lal (2004) assumed that the conversion of natural to agricultural ecosystems led to a SOC depletion of 60% in temperate regions, and up to 75% in tropical ones. On the contrary, conversions to forestry, grassland or natural regeneration usually lead to an increase SOC stocks (Guo & Gifford, 2002; Lettens et al., 2005; Falloon et al., 2006).

Variation in SOC stocks and decomposition rates are also due to environmental factors, such as soil texture, climate conditions, vegetation type and root architecture. As demonstrated by Christensen (1996), clay soils accumulate carbon relatively quickly, while sandy soils may accumulate practically no carbon even after 100 years of high carbon inputs. Similarly, soils in colder climates, where decomposition is slower, may accumulate carbon more rapidly than soils in warmer regions.

Calculating the global carbon mitigation potential is difficult: apart from assessing the full potential of measures increasing soil carbon sequestration, restraints regarding land use change as well as socio-economic variables, need to be taken into consideration (Smith, 2004). Tans et al. (1990), Ciais et al. (1995) and Fan et al. (1998) have assessed that, over the past decades, soils have stored between 1 and 2 Pg yr^{-1} in the Northern hemisphere. On the contrary, little estimate of agricultural soil carbon sequestration potential for Europe is available (Freibauer et al., 2004). Smith (2004) calculated the biological potential for carbon storage in European (EU-15) cropland around the order of 90-120 Mg C yr^{-1}, with a range of options available, including reduced and zero tillage, perennial energy crops and deep rooting crops, more efficient use of organic amendments (animal manure, sewage sludge, compost...), improved rotations and conversion of arable land to grassland or woodland. Considering only constraints on land use and amounts of available land, the estimate of the sequestration potential is up to 45 Mg C yr^{-1}. Because of several interacting variables and constraints of different nature, it is right to assume that the realistic potential of agricultural soils in Europe may be considerably lower than the biological potential, with a realistically feasible potential estimated to be about 20% of the biological potential.

PERENNIAL ENERGY CROPS

Energy crops are produced with the express purpose of using their biomass energetically (Lewandowski et al., 2003). The replacement of fossil fuels by renewable bioenergy fuel sources and energy crops is one of the main strategies to achieve the aims of the Kyoto Protocol (Clifton-Brown et al., 2007). As proposed by Smith et al. (2000), energy crops are identified as the agricultural land use change with the greatest potential for carbon mitigation across Europe, although, within the scientific community, there is controversial discussion about this proposal. Recent research suggests that, under certain conditions, bioenergy production can lead an increase in food prices, due to direct and indirect competition. To avoid conflict with food production, energy crops need to be planted on lower grade land or marginal land, unsuitable for arable crops such as wheat (Fargione et al., 2008). Also, the conversion of native, semi-natural or generally more diverse ecosystems to large monocultures can lead to a loss of biodiversity (Cook et al., 1991; Koh, 2007; Koh and Wilcove, 2008). Therefore, if the development of energy crops is not properly regulated with regard to land allocation and use of the most suitable crop species, the environmental and social benefits of biofuels may be substantially diminished (Zatta et al., 2013). Inappropriate choice of land types and crop types may even increase GHG emissions from soil such that the environmental benefits of growing energy crops are negated (Fargione et al., 2008; Hillier et al., 2009; Frische et al., 2010; Powlson et al., 2011). Belowground biomass is the primary vehicle for soil carbon storage (Kuzyakov, 2002; Nguyen, 2003; Kell, 2011); therefore, energy grasses, especially perennial species, are expected to increase SOC and to slow down mineralisation processes thanks to minimal soil tillage and deeper root system (Ma et al., 2000a; Monti and Zatta, 2009). There are many ecological benefits expected from the production and use of perennial grasses, that make them more suitable than annual grasses. Unlike annual crops, the need for soil tillage in perennial grasses is limited to the year in which the crops are established. The reduced disturbance by tillage leads to decreased risk of soil erosion, less aeration, lower plant residues-decomposition rates and better carbon stabilisation for longer periods (Schneckenberger et al., 2007).

Furthermore, due to the recycling of nutrients by their rhizome systems and to a slower decomposition of plant residues (stubbles, leaves and roots), that increases C:N ratio, perennial grasses have a low demand for nutrient inputs (Christian et al., 1997). Since they have few natural pests, they may also be produced with little or no pesticide use (Lewandowski et al., 2000). Studies of fauna show that, due to long-term lack of soil disturbance, the late harvest of perennial grasses in winter to early spring and the insecticide-free production, an increase of abundance and activity of different species, especially birds, mammals and insects occurs (Jodl et al., 1998; Hoffman et al., 1995). Perennial crops can therefore contribute to ecological values in agricultural production: they can function as elements in landscape management and as habitat for different animals (Lewandowski et al., 2003). The increase in stored carbon in soil results also from the relatively large quantities of rootstocks belowground, as well as enhanced SOM content. As assumed by Monti and Zatta (2009), the root apparatus is likely the most responsible source of soil carbon and therefore root growth determination ought to be a reliable indicator of the potential carbon accumulation.

European research on perennial grasses for bioenergy production began with the interest shown in the hybrid *Miscanthus × giganteus* (Greef and Deuter), which was introduced as ornamental plant about 50 years ago (Lewandowski et al., 2003). The first field trials of *Miscanthus* were established in the late 1980's in different countries of Northern Europe, as Denmark, Germany, Ireland and the UK. In 1993, a large project was set up under the European AIR program, to extend the distribution of field trials into Southern Europe, including Greece, Italy and Spain (Walsh, 1997). Nationally funded projects in Denmark, Netherlands, Germany, Austria and Switzerland supported research on propagation and establishment, management practices, harvest and handling of *Miscanthus*. Results showed a very vigorous growth of this crop, and the advantage of concentrating the biomass harvest in one cut in a delayed harvest system, allowing it to dry out. By this, major limitations were identified, as the narrow genetic base (only the hybrid *Miscanthus × giganteus* is suitable for bioenergy production), the poor overwintering in Northern Europe and the need to propagate it vegetative because of its high establishment costs (Lewandowski et al., 2003). For these reasons, it seemed to be worth identifying other perennial grasses which could be established by seeding at lower costs and that were more adapted to climatic conditions at different sites in Europe. Many indigenous grasses have been evaluated at various Southern and

Northern European sites (Mediavilla et al., 1997; Wellie-Stephan, 1998). Among these, giant reed (*Arundo donax* L.) and reed canarygrass (*Phalaris arundinacea* L.) were found to offer good bioenergy characteristics. There is another perennial grass, firstly used in the US as promising bioenergy crop, that has been recently introduced into Central and Southern Europe for the same purpose and it is switchgrass (*Panicum virgatum* L.).

PERENNIAL ENERGY CROPS AND CLIMATIC CONDITIONS

The main reason for developing different perennial energy grasses as bioenergy crops is the need to establish appropriate crop types for different ecological/climatic zones, because climatic conditions influence biomass production and, therefore, SOM input. The main climatic limitations to be overcome in Europe are the low winter temperatures in Northern Europe and the dry summer periods in Southern Europe (Lewandowski et al., 2003). These meteorological factors can be an advantage/disadvantage for different perennial crops depending on their photosynthetic pathway (C_3 and C_4 plants). Low winter temperatures and short vegetation periods are major limitations to the growth of C_4 grasses, like *Miscanthus* or switchgrass, in Northern Europe, making C_3 plants, like reed canarygrass, the best choice for countries like Sweden or Finland. With increasing temperatures, towards Central and Southern Europe, the productivity of C_4 grasses and therefore their biomass yields and competitiveness increase (Lewandowski et al., 2003).

Many surveys about the establishment and the use of perennial crops for bioenergy production or SOC storage increasing are available but they largely refer to different climatic sites of Northern Europe. These field experiments have confirmed high carbon sequestration rates, especially under *Miscanthus*, showing high potential to increase SOC stocks under former arable lands. The potential zone for *Miscanthus* production has been extended in Northern European areas by the screening and breeding of more frost-tolerant genotypes. In Table 1, the main studies on *Miscanthus* to which this research refers, are classified in accordance to the environmental stratification of Europe, suggested by Metzger et al. (2005). This classification assumes similar environmental parameters where agricultural land could be suitable for non-food crops cultivation. Considering also Fig. 1, it appears that these experimental studies have been conducted in different bioclimatic areas, with well distinct meteorological parameters. They extend from the Atlantic Central zone, quite good for agriculture in spring/summer time but not during winter because of low temperatures, to Continental climate, less favourable for the relatively low annual rainfall.

Table 1 Location, meteorological parameters and environmental classification (Metzger et al., 2005) of the above mentioned studies on *Miscanthus* and of this thesis. Description of geographic allocation of environmental zones from EBONE (European Biodiversity Observation Network).

Authors	Experimental Site	Mean Temperature (°C)	Annual Rainfall (mm)	Months Temp < 0°C	Environmental zone
Clifton-Brown et al. (2007)	Southern Ireland	10	912.9	0.2	Atlantic Central
Dondini et al. (2009)	Souht-East of Ireland	10	912.9	0.2	Atlantic Central
Zatta et al. (2013)	West Wales	10	912.9	0.2	Atlantic Central
Schneckenberger et al. (2007)	Southern Germany	8.6	774.3	4.1	Continental
Hansen et al. (2004)	Denmark (North Jutland)	8.6	774.3	4.1	Continental
this research (2014)	Po Valley	13.2	695.9	0.4	Mediterranean-North

In contrast, the present research was located in the South-East of the Po Valley (Northern Italy), included in the Mediterranean-North zone, that, with its warm temperatures coupled with sufficient precipitations, may be considered better suited for growing a wide array of energy crops. The area of the Po Valley is characterised by average low SOC levels (<10 g C kg^{-1} up to 0.30 m; ARPA, 2009), largely due to anthropic practices as intensive tillage, a strong specialisation of cropping systems and a concurrent decline of the livestock farm activity. Perennial biomass crops are being introduced here, besides bioenergy aims, to foster C sequestration. In this light, we investigated a 9-year old conversion from two annual crop systems, continuous wheat and a maize/wheat rotation, to *Miscanthus* and giant reed, assessing their contribution to SOC storage and possible benefits on biological aspects of soil, in less favourable conditions than in Northern Europe.

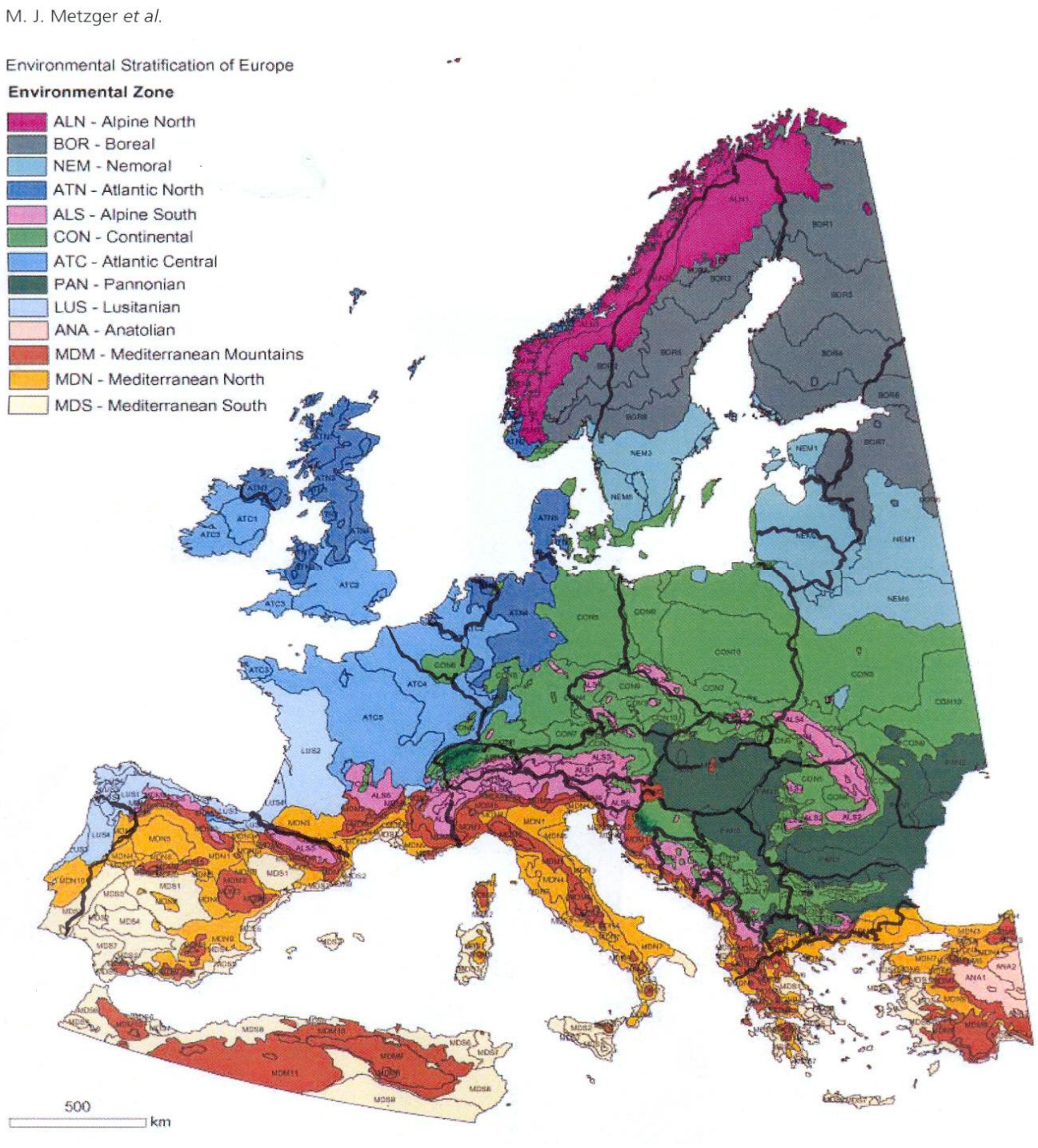

Figure 1 Environmental stratification of Europe, suggested by Metzger et al. (2005).

GENERAL AIMS

This research is carried out in the frame of the multidisciplinary BIOSEA Project ("Ottimizzazione delle filiere bioenergetiche esistenti per una sostenibilità economica e ambientale"; http://www.biosea.dista.unibo.it), funded by MIPAAF (Italian Ministry of Agricultural Policy). The aim of the project is to improve the bioenergy sector in a sustainable agricultural context, through the interaction of genetic, economic, agricultural and environmental analysis.

As shown in the General Introduction, perennial energy grasses exhibit different levels of adaptation and tolerance to a large range of climatic conditions, from Northern to Southern Europe. Results of long-term field experiments in Northern Europe, about the evaluation of SOC storage potential of perennial grasses, represent a promising starting point to estimate it under warm-temperate conditions, with increased temperatures, of Southern Europe. Comparing the literature mentioned above and considering the environmental characteristics of the experimental sites, the main objectives of this thesis are:

1) To assess the impact of 9 years of *Miscanthus* and giant reed on SOC stock. In particular, changes in overall SOC storage, after 9 years of perennial energy crops were measured, comparing *Miscanthus* plots with continuous wheat and giant reed plots with maize/wheat rotation. Through the ^{13}C natural abundance analysis, the amount of SOC sequestered by the two perennial crops, down to 0.60 m depth, was estimated.

2) To evaluate the impact of the conversion to perennial energy crops on soil aggregates and different carbon pools. To measure the stability of the newly sequestered carbon, derived from *Miscanthus* and giant reed, into SOM pools, a physical soil fractionation, combined with the ^{13}C natural abundance method, was carried out.

3) To investigate the changes occurred on soil enzyme activities and on microbial communities, after the land use change to perennial energy crops. The functional aspects of soil were investigated through the determination of three enzyme activities, involved in soil carbon, nitrogen and phosphorous cycles (β-glucosidase, urease and alkaline phosphatase, respectively). The structure of the microbial community under annual and perennial crops was studied by using the biomolecular approach of PCR-DGGE (Denaturing Gradient Gel Electrophoresis).

SECTION ONE

Soil carbon sequestration potential of *Miscanthus* and giant reed estimated by natural ^{13}C abundance

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Based on: Cattaneo, F., Barbanti, L., Gioacchini, P., Ciavatta, C., Marzadori, C., 2014. ^{13}C abundance shows effective soil carbon sequestration in *Miscanthus* and giant reed compared to arable crops under Mediterranean climate. Biology and Fertility of Soils (submitted)

ABSTRACT

Many studies on soil organic carbon (SOC) sequestration in perennial biomass crops are available under Atlantic and Continental environments of North-Central Europe, while there is insufficient information for Southern Europe. Therefore, we assessed SOC turnover under Mediterranean-temperate climate, after a 9-year old conversion from two annual crop systems, continuous wheat and maize/wheat rotation, to *Miscanthus* (*Miscanthus × giganteus* Greef and Deuter) and giant reed (*Arundo donax* L.), respectively. The ^{13}C natural abundance analysis, down to 0.60 m, was used to evaluate the total amount of SOC in annual *vs* perennial species, and determine the portion of SOC derived from perennial species.

SOC was significantly higher under perennial (average, 91 Mg C ha^{-1}) than annual species (average, 56 Mg C ha^{-1}), with a stronger accumulation in the topsoil (0-0.15 m). This difference was consistent with the reduced soil disturbance associated with perennial crop management. After 9 years of *Miscanthus* plantation, the amount of C_4-derived carbon was 18.7 Mg ha^{-1}, mostly stored at 0-0.15 m, whereas the amount of C_3-derived carbon under giant reed was 34.7 Mg ha^{-1}, more evenly distributed through soil depths. This difference is echoed in the deeper root apparatus evidenced for giant reed in literature, providing a stronger contribution to SOC in deeper layers.

Comparing our results with the available studies, only for *Miscanthus*, in North-Central Europe, we conclude that *Miscanthus* and giant reed own a remarkable potential for SOC sequestration also in Mediterranean conditions, exerting effective belowground carbon sink potential while supporting the growing bioenergy sector with aboveground biomass supply.

INTRODUCTION

Soil organic carbon (SOC) is the major C stock in terrestrial ecosystems (Batjies, 1996; Amundson, 2001) with the slowest turnover rate. SOC plays an important role in global C cycling exerting positive effects on soil quality as concerns nutrient availability, soil moisture and health (Sarkhot et al., 2012). Soil has a great potential of C sink, but intensive land management and land use change from (semi-) natural ecosystems to agriculture, as well as changes from grasslands to croplands, can cause a loss of SOC accumulated at a more rapid rate than it is stored. On a long-term scale, it has been estimated that, at a world level, these anthropic disturbances have added about 124 Pg of C to the atmosphere (Houghton, 1999; Smith, 2008). In Europe C loss has been evaluated in 300 Tg C per year (Janssens et al., 2003).

To promote C sequestration, agricultural management systems should provide increased organic C input into soils, as plant litter and root material, while, concurrently, reducing soil disturbance and preserving or increasing soil quality, structurally as well as biologically (Post and Kwon, 2000). Traditional practices to enhance SOC stocks include: afforestation, reducing tillage and land use change to permanent pasture or perennial energy crops. As identified by Smith et al. (2000), perennial grasses for energy uses have attracted significant research attention across Europe, because of their great potential for C mitigation. The increase in stored C pools in soil results from the relatively large quantities of root deposition belowground, as well as enhanced SOM content (Clifton-Brown, 2007). The low fertiliser and pesticide inputs and the reduction of soil disturbance, associated with these crops' low input management, contribute to their high C sequestration potential (Kahle et al., 2001; Freibauer et al., 2004). In Continental and Oceanic regions of Northern Europe, the perennial rhizomatous grass *Miscanthus* (*Miscanthus × giganteus*, Greef and Deuter) has been especially focused. In South European areas, another perennial grass, giant reed (*Arundo donax* L.) has been identified as promising biomass crop for energy uses (Lewandowski et al., 2000).

Miscanthus is a perennial rhizomatous C_4 grass, endemic of South-East Asia, with a considerable biomass production potential even under cool-temperate conditions (Lewandowski et al., 2000). The genotype widely used in Europe for bioenergy production is *Miscanthus × giganteus*, introduced from Japan to Denmark in 1930 (Lewandowski et al., 2003). Yields above 30 t dry matter ha^{-1}, only with irrigation, are reported for locations in Southern Europe (e.g. Southern Portugal), with high annual incident global radiation and high average temperatures. In Central and Northern Europe (from Austria to Denmark), where global radiation and average temperatures are lower, yields without irrigation are more typically 10-25 t dry matter ha^{-1} (Lewandowski et al., 2000).

Moreover, Qin et al. (2011) reported that *Miscanthus* cultivation can be feasible in marginal lands or recently abandoned cropland, reducing possible competition with food crops as well as possible C emissions due to land use change (Clifton-Brown et al., 2007; Heaton et al., 2008; Qin et al., 2011).

The perennial rhizomatous C_3 plant giant reed (*Arundo donax* L.), native from East-Asia, has been diffused in riparian habitats of Mediterranean environment. Giant reed is a warm-temperate or subtropical species, but it is able to survive frost. Serious damage can occur in case of late season frosts after sprouting (Perdue, 1958). Recently, in Europe, this species has been indicated like the one of the most promising for energy production for the Southern European areas. Yields reported in Spain showed 45.9 t dry matter ha^{-1} on average, ranging from 29.6 to 63.1 t (Hidalgo et al., 2001). A high biomass productivity has been observed also reducing crop inputs, such as fertiliser and plant density (Angelini et al., 2009).

Many studies on SOC sequestration potential of *Miscanthus* have been carried out in areas featuring Atlantic (Clifton-Brown et al., 2007; Dondini et al., 2009; Zimmermann et al., 2012; Schneckenberger et al., 2007), as well as Continental climate (Hansen et al., 2004), according to a recent classification of European environments (Metzger et al., 2005). In contrast, no study to our knowledge has so far addressed SOC sequestration in perennial rhizomatous grasses under Mediterranean climate, where higher temperatures favour SOC mineralisation, hampering C storage.

A cost efficient technique, used to study how land use change can act on SOC sequestration and to understand the fate of SOM within and between ecosystems, is the analysis of stable isotopes (^{13}C isotope) (Balabane and Balesdent, 1992; Balesdent and Balabane, 1992; Flessa et al., 2000; Garten and Wullschleger, 2000; Foereid et al., 2004; Pelz et al., 2005). The stable carbon isotope signature is described using the δ notation. During the photosynthesis, plants generally discriminate against the heavier carbon isotope ^{13}C (Farquhar et al., 1989), leading to a depletion of ^{13}C levels in plant organic material and therefore a lower $\delta^{13}C$ value compared to the atmosphere. This depletion is due to the fact that the heavier ^{13}C forms slightly more stable chemical bonds and diffuses more slowly, through stomata (O'Leary, 1988). The level of discrimination depends on the different photosynthetic pathways (C_3- and C_4 plants) and, for this reason, the analysis of stable isotopes is appreciable in the case of a land use change, involving a vegetation change.

As plants with different photosynthetic pathways (C_3- and C_4-plants) have different ^{13}C-signature, when the dominant vegetation, at a particular location, is converted from C_3- to C_4-plants, or viceversa, there is a shift in the isotopic signature of the C sequestered to the soil, which can be used to determine the proportion of SOC derived from the new vegetative sources (Balesdent et al., 1987).

With these premises, the present research was carried out in the South-East of the Po Valley (Northern Italy), identified as part of the Mediterranean North environmental zone (Metzger et al., 2005). This agro-ecosystem is characterised by low average SOC levels (<10 g C kg^{-1} up to 0.30 m; ARPA, 2009), largely due to anthropic practices as intensive tillage, a strong specialization of cropping systems and a concurrent decline of the livestock farm activity.

Perennial crops have been introduced here, besides bioenergy aims, to restore soil fertility and productivity, by enhancing SOC levels.

In this light, we investigated a 9-year old conversion from two annual crop systems, continuous wheat and a maize/wheat rotation, to *Miscanthus* and giant reed, assessing their belowground biomass contribution (as roots) in SOC storage, changing climatic and environmental conditions.

Through the analysis of stable ^{13}C isotope of soil, down to a 0.60 m depth, we compared the C_4 (*Miscanthus*) and C_3 (giant reed) perennial species with their respective C_3 (wheat) and the C_4/C_3 (maize/wheat rotation) annual references. This allowed us to precisely determine the portion of SOC derived from the two perennial species in 9 years of cropping, as well as to compare the total amount of SOC between annual and perennial species at the end of this period (Hansen et al. 2004).

MATERIALS AND METHODS

EXPERIMENTAL SITE

The field experiment was located at the experimental farm of the University of Bologna (Italy), in Cadriano (44° 33’ N, 11° 21’ E, 32 m a.s.l.), with mean annual precipitation and temperature of 700 mm and 13.3 °C, respectively. With these data, the area precisely falls in the Mediterranean North environmental zone, implying mild winter and long growing season, in exchange for precipitation mostly concentrated in the cold semester (Metzger et al., 2005). A chemical and physical characterisation of the studied soils under the two perennial (*Miscanthus* and giant reed) and annual crops (continuous wheat and maize/wheat rotation) was carried out, according to the current Italian methods of soil analysis (D.M. 13/09, 1999), as shown in Table 2.

Table 2 Physical and chemical characterisation of the studied soils, under perennial (*Miscanthus* and giant reed) and annual crop (wheat and maize/wheat rotation) systems. Soil analysis according to the Italian method D.M. 13/09, 1999.

	Site (soil)			
	Miscanthus	**Giant reed**	**Wheat**	**Maize/Wheat**
pH_{H2O}	7.6	7.5	6.1	6.1
Texture (%)				
Sand	25	27	24	25
Silt	45	46	49	45
Clay	30	27	27	26
Limestone (%)				
total	1.7	1.7	< 0.5	< 0.5
active	1.6	1.6	< 0.1	< 0.1
Vegetation type	C_4	C_3	C_3	C_4/C_3
C_{ORG} (g kg^{-1})	16	11.5	7.4	6.3
N_{TOT} (g kg^{-1})	1.5	1.5	0.9	0.8
C:N	10.7	7.7	7.4	7.9
Available P (mg kg^{-1})	32.7	12.4	31.9	20.9

CROP MANAGEMENT AND EXPERIMENTAL LAYOUT

Continuous wheat (*Triticum aestivum* L.) and maize/wheat rotation (*Zea mays* L./*Triticum aestivum* L.) are the two annual systems that were established in experimental plots in the late 1960's. *Miscanthus* and giant reed plots were established in 2002 in soils previously cultivated with annual C_3 and C_4/C_3 species, respectively.

In this thesis, the expression *land use system* will be used to indicate perennial *vs* annual crops, while the term *plant group* will distinguish between *Miscanthus vs* continuous wheat and giant reed *vs* maize/wheat rotation.

In both annual and perennial crops 0 (N_0) and 120 (N_{120}) kg ha^{-1} of mineral N, supplied as urea, were applied annually during the spring time. Moreover, 50 kg P_2O_5 ha^{-1}, as triple superphosphate, were applied every year in annual crops. In *Miscanthus* and giant reed, 200 kg P_2O_5 ha^{-1} were added prior to planting in spring 2002. No K fertiliser was applied, given the sufficient level of this nutrient in the soil.

Tillage at 0.30 m was carried out every year in annual crops; only prior to planting (autumn 2001) in perennial ones. In both annual and perennial species, the rest of crop husbandry reflected the normal practice followed in the experimental area. In particular, perennial crops were always harvested every year in the early autumn (September 25 – October 15); annual crops were harvested at maturity (wheat, early summer; maize, late summer) and the recoverable fraction of their residues (wheat straw and maize stover) was always removed from the field.

Soil sampling took place in March 2011, at the beginning of the 10th growing season, using a 85 mm diameter soil corer up to a depth of 0.60 m, divided in three soil layers (0-0.15, 0.15-0.30, 0.30-0.60 m). For each crop, three replicates were taken according to a completely randomised experimental design. Each replicate was composed of three subsamples, which were put together and transported to the laboratory on the same day. Then soil samples were sieved (2 mm), plant organic material as leaves, stubbles and litter were removed and then soils were air-dried.

An aliquot was used for total C, total N and $\delta^{13}C$ signature analysis and another aliquot was stored for further analysis. In the same date, separate samples (50 mm diameter) of undisturbed soil were monitored for bulk soil.

TOTAL C, TOTAL N AND ^{13}C ANALYSIS

Total soil organic carbon (SOC) and nitrogen (TN) contents were directly measured in soil samples with an elemental analyser (CHNS - O mod. EA 1110, Thermo Scientific GmbH, Dreieich, Germany), using acetanilide as a standard for C and N concentration. As traces of carbonates were detected in the studied soils, samples were pre-treated with acid. An aliquot of soil (10 - 13 mg) was weighed into silver capsules and, after adding a few drops of 6 M HCl, was heated to 80 °C on a heating plate until dry. Then the capsules were closed and analysed in the elemental analyser. The isotopic composition ($\delta^{13}C$ signature) of soil was measured by Continuous Flow - Isotope Ratio Mass Spectrometry (CF - IRMS), by introducing the combustion gas (CO_2) from the elemental analyser into the Isotope Ratio Mass Spectrometer (IRMS, Delta Plus, Thermo Scientific, Germany).

DATA ANALYSIS

The natural abundance $\delta^{13}C$ was measured to determine the proportion of C derived from the C_4 *Miscanthus* and how much C remained from wheat (C_3), in all soil samples (Lisboa et al., 2009).

Generally, photosynthesis leads to a discrimination against the heavier ^{13}C isotope in the plant organic matter compared to atmospheric CO_2. The degree of the discrimination is dependent on the photosynthetic pathway: C_4-plants show distinctly higher ^{13}C abundance than C_3-plants. In an environment with only one source of C_4-derived SOC (i.e. *Miscanthus*), the isotopic signal can be used to quantify the amount of carbon derived by that given source (Balabane and Balesdent, 1992; Balesdent and Balabane, 1992) using the isotope mass balance.

The ^{13}C abundance was expressed in delta - units ($\delta^{13}C$ ‰) and calculated according to the equation:

$$\delta^{13}C\ ‰ = [(R_{\mathrm{sample}}/R_{\mathrm{standard}}) - 1] \times 1000$$

where R_{sample} is the isotope ratio $^{13}C/^{12}C$ of the sample and R_{standard} is the $^{13}C/^{12}C$ ratio of the international Pee Dee formation belemnite carbonate standard (PDB).

The proportion of C derived from *Miscanthus* (F_M) was calculated by using the isotope mass balance equation (Balesdent et al., 1987):

$$F_M = [(\delta^{13}C_{\mathrm{new}} - \delta^{13}C_{\mathrm{old}})/(\delta^{13}C_{\mathrm{new\ crop}} - \delta^{13}C_{\mathrm{old\ crop}})] \times 100 \qquad [1]$$

where $\delta^{13}C_{new}$ is the $\delta\ ^{13}C$ of the soil under *Miscanthus*, $\delta\ ^{13}C_{old}$ is the $\delta\ ^{13}C$ of the soil under wheat, $\delta^{13}C_{new\ crop}$ is the $\delta^{13}C$ of *Miscanthus* material entering the soil (-12.66 ‰) and $\delta^{13}C_{old\ crop}$ is the $\delta^{13}C$ of wheat (-27 ‰) (Dondini et al., 2009). The proportion of C derived from wheat ($C_{old\ \%}$) was obtained by the equation:

$$C_{old} = 100 - F_M$$

The amount of C derived from giant reed (F_G), a C_3 species, was calculated according to the following equation, proposed by Hansen et al. (2004):

$$F_G = [(\delta^{13}C_{\mathrm{new}} - \delta^{13}C_{\mathrm{old}})/(\delta^{13}C_{\mathrm{new\ crop}} - \delta^{13}C_{\mathrm{old}})] \times 100 \qquad [2]$$

where the $\delta\ ^{13}C$ of soil under maize/wheat was used as $\delta\ ^{13}C_{old}$, since it was not known the $\delta\ ^{13}C$ of the rotation before the introduction of giant reed. Soil organic C was expressed as the amount of C per unit crop surface (Mg C ha^{-1}) considering the bulk density of the three soil layers.

STATISTICAL ANALYSIS

Normal distribution and equal variance of data were controlled through the Kolmogorov-Smirnov and Bartlett test, respectively. Data were then submitted to the analysis of variance (ANOVA) through the CoStat 6.3 software (CoHort Software, Monterey, California, USA), according to a completely randomised factorial scheme: in each trait the significance of the investigated sources (crop, N fertilisation, soil depth and their interactions) was determined. The Student - Newman-Keuls (SNK) test at $P \leq 0.05$ was adopted to separate means of statistically significant sources.

RESULTS

SOIL C AND N CONTENT

Mineral N fertilisation did not significantly influence SOC and TN contents under perennial and annual crops (Table 3.a, b), except for a modest (6%; $P = 0.043$*) TN increase under fertilisation, in the combination of giant reed and maize/wheat (Table 3.b). No significant interaction was observed between nitrogen, on one side, and the other two investigated factors (crop and soil depth), on the other side (Table 3.a, b).

Table 3 Soil organic carbon (SOC) and nitrogen (TN) in *Miscanthus vs* continuous wheat (**A**) and in giant reed *vs* maize/wheat rotation (**B**), depending on crop, N fertilisation, soil depth and their interactions.

A

Sources	**SOC (g kg^{-1})**	**TN (g kg^{-1})**
Crop (C)		
Miscanthus	12.2 a	1.3 a
Wheat	7.4 b	0.9 b
P	< 0.001***	< 0.001***
Fertilization (N; kg ha^{-1})		
0	10.0	1.1
1	9.6	1.1
P	**0.290 ns**	**0.7 ns**
Depth (D; m)		
0-0.15	11.7 a	1.2 a
0.15-0.30	9.2 b	1.0 b
0.30-0.60	8.5 b	1.0 b
P	< 0.001***	< 0.001***
P (C x D)	< 0.001***	< 0.001***
P (C x N)	**0.867 ns**	**0.640 ns**
P (D x N)	**0.685 ns**	**0.238 ns**
P (C x D x N)	**0.879 ns**	**0.915 ns**

B

Sources	**SOC (g kg^{-1})**	**TN (g kg^{-1})**
Crop (C)		
Giant reed	10.0 a	1.3 a
Maize/Wheat	6.2 b	0.7 b
P	< 0.001***	< 0.001***
Fertilization (N; kg ha^{-1})		
0	8.0	1.0 b
1	8.3	1.1 a
P	**0.116 ns**	0.043*
Depth (D; m)		
0-0.15	8.9 a	1.1
0.15-0.30	7.8 b	1.0
0.30-0.60	7.8 b	1.0
P	< 0.001***	0.056 ns
P (C x D)	< 0.001***	0.011*
P (C x N)	**0.631 ns**	**0.185 ns**
P (D x N)	**0.277 ns**	**0.239 ns**
P (C x D x N)	**0.60 ns**	**0.065 ns**

ns, *, ** and *** mean non-significant and significant at $P \leq 0.05$, ≤ 0.01 and ≤ 0.001, respectively.
Different letters indicate significantly different means (SNK test; $P \leq 0.05$).

Integrated through the soil profile, SOC under perennial energy crops was remarkably higher than under annual arable crops (ca. +60%) (Table 3). Within the same land use system, small differences can be observed between the two perennial crops (12.2 *vs* 10.0 g C kg^{-1} in *Miscanthus* and giant reed, respectively), as well as the two annual references (7.4 and 6.2 g C kg^{-1} in continuous wheat and the maize/wheat rotation, respectively). In soil profile, the top layer (0-0.15 m) showed higher SOC content than the two deeper ones (0.15-0.30 and 0.30-0.60 m). However, the significant crop × depth interaction indicates a different behaviour between the two land use systems (Fig. 2.a and b): the topsoil under *Miscanthus* and giant reed contained significantly more C (16.0 and 11.5 g C kg^{-1} in the two respective crops) than the two lower soil layers (on average, 10.3 and 9.4 g C kg^{-1} in the same two crops), while continuous wheat and maize/wheat rotation showed a consistent SOC distribution along depth.

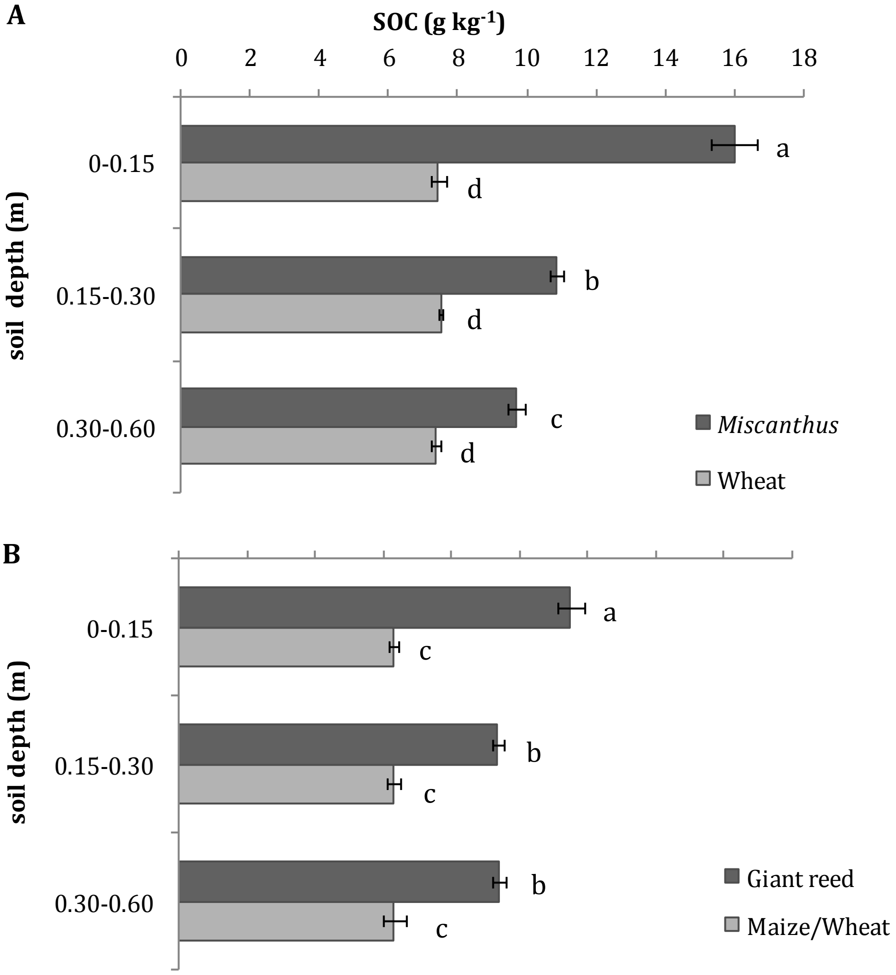

Figure 2 Crop × depth (C × D) interaction of soil total organic carbon (SOC) in *Miscanthus vs* continuous wheat (**A**) and in giant reed *vs* maize/wheat rotation (**B**). Different letters indicate significantly different means (SNK test; $P \leq 0.05$). Horizontal bars represent ± standard errors (n= 6).

Overall, the total quantity of C per unit crop surface amounted to 98.6 Mg C ha^{-1} under *Miscanthus* and to 84.3 Mg C ha^{-1} under giant reed.

A similar trend can be observed for TN content, which was significantly higher under perennial crops than annual ones (1.3 *vs* 0.8 g N kg^{-1}) (Table 2). Likewise, under *Miscanthus* and giant reed, the topsoil (0-0.15 m) contained significantly more N (1.48 and 1.47 g N kg^{-1}, respectively) than the two deeper layers (on average, 1.22 and 1.30 g N kg^{-1} in the two respective species), while, under wheat and the maize/wheat rotation, TN was consistent among soil depths (Fig. 3.a and b).

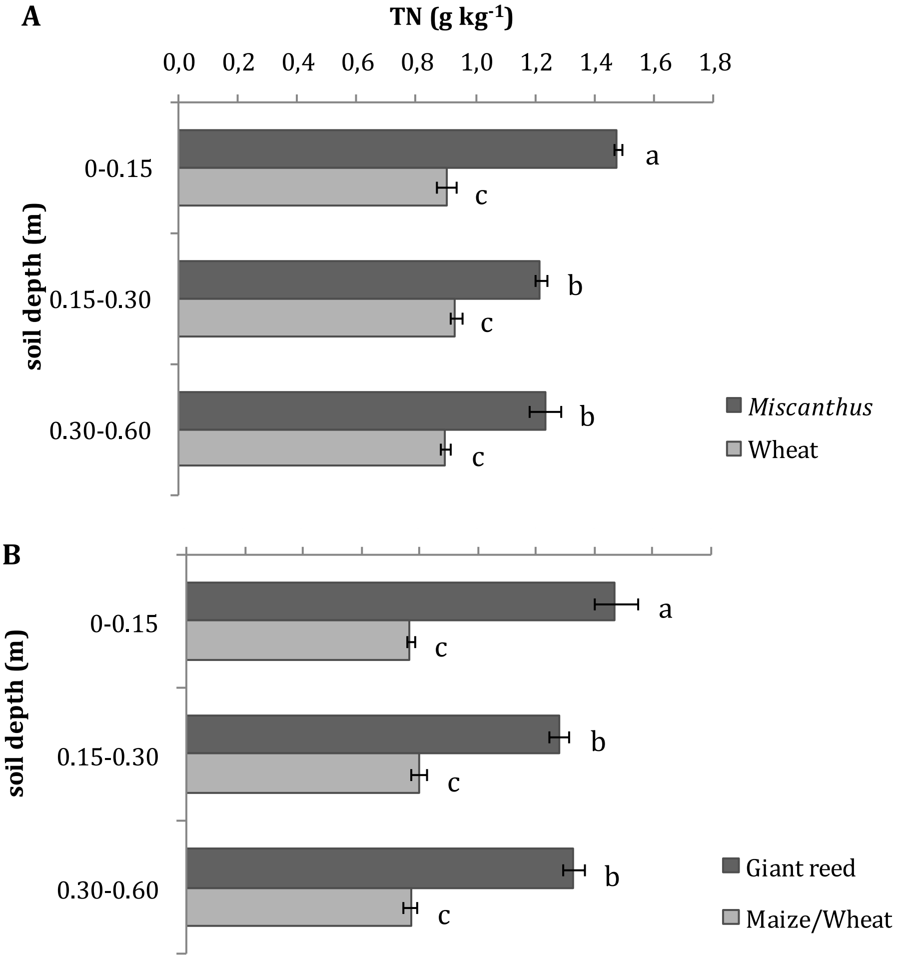

Figure 3 Crop × depth (C × D) interaction of soil total nitrogen (TN) in *Miscanthus vs* continuous wheat (**A**) and giant reed *vs* maize/wheat rotation (**B**). Different letters indicate significantly different means (SNK test; $P \leq 0.05$). Horizontal bars represent ± standard errors (n= 6).

ISOTOPE MASS BALANCE AND CONTRIBUTION OF PERENNIAL SPECIES TO SOC

$\delta^{13}C$ values and distribution in perennial and annual crops appear to be strongly influenced by their C_3 or C_4 photosynthetic pathways (Fig. 4).

While in annual crops $\delta^{13}C$ values of SOM remained consistent through soil depths, a major difference appears when comparing the two perennial crops: giant reed showed a $\delta^{13}C$ value that kept consistently lower (i.e., more negative) than maize/wheat through soil profile (Fig. 4.a), whereas *Miscanthus* exhibited quite higher (i.e., less negative) $\delta^{13}C$ values in the shallow layer, then converged towards $\delta^{13}C$ values of continuous wheat in the two deeper layers (Fig. 4.b).

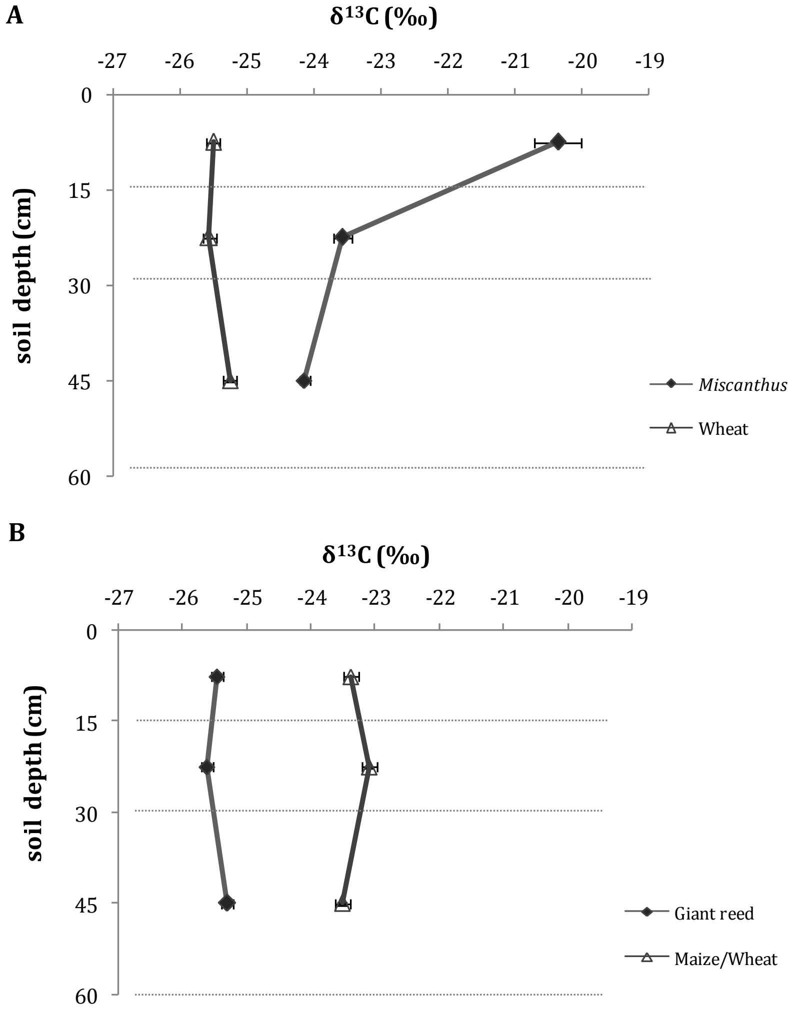

Figure 4 $\delta^{13}C$ values at three soil depths, in *Miscanthus* *vs* continuous wheat (**A**) and giant reed *vs* maize/wheat rotation (**B**). Horizontal bars represent ± standard errors (n= 6).

The effects of the substitution of arable crops with perennial grasses, in terms of SOC storage and relative share of C_3- and C_4-derived C, indicate a different behaviour between *Miscanthus* and giant reed.

The input of C_4-derived C from *Miscanthus* was principally found in the 0-0.15 m soil layer, with a contribution of 35.8% to the total C pool in that layer (Fig. 5). This value declined to 13.8% at 0.15-0.30 m and to only 7.7% in the 0.30-0.60 m soil depth (Fig. 5). Based on these data, after 9 years of *Miscanthus* plantation the amount of C_4-derived C in the whole profile was 18.7 Mg C ha^{-1}, with a variable distribution among the three layers: 65% was stored in the top 0-0.15 m, 17% was accumulated at 0.15-0.30 m and another 17% in the 0.30-0.60 m depth.

In contrast to this, the input of C_3-derived C from giant reed was more evenly distributed among the three soil layers (Fig. 6): 42.5% of the total C pool was C_3-derived in the 0-0.15 m soil layer, 48.6% at 0.15-0.30 m and 36.8% in the 0.30-0.60 m layer. Based on this, after 9 years of giant reed plantation the amount of C_3-derived C in the whole profile was 34.7 Mg C ha^{-1}, distributed in the following way: 30% was stored in the shallow layer (0-0.15 m), 28% at 0.15-0.30 m and 42% in the deep layer (0.30-0.60 m). Thereby, based on $\delta^{13}C$ data, giant reed fostered a much higher accumulation of newly derived-C in the whole profile (+86%), and this difference was mainly due to a stronger contribution from the two deeper layers.

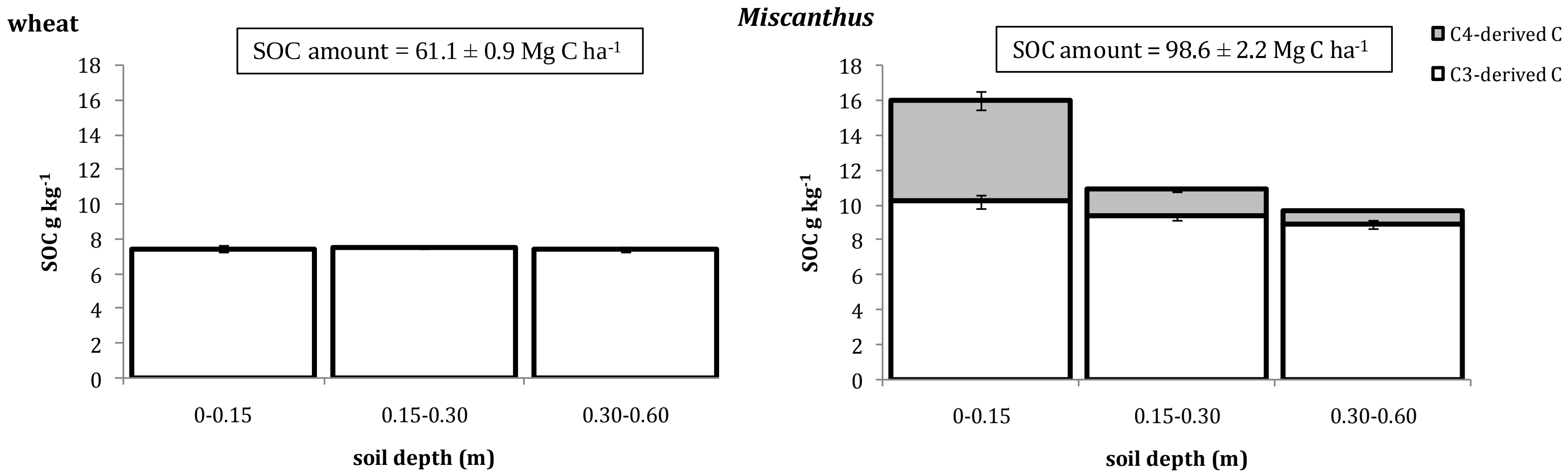

Figure 5 C_3 and C_4-derived carbon content at three soil depths in continuous wheat and *Miscanthus*. The total amounts of C (0-0.60 m) are also indicated as means ± standard errors. Vertical bars represent ± standard errors (n= 6).

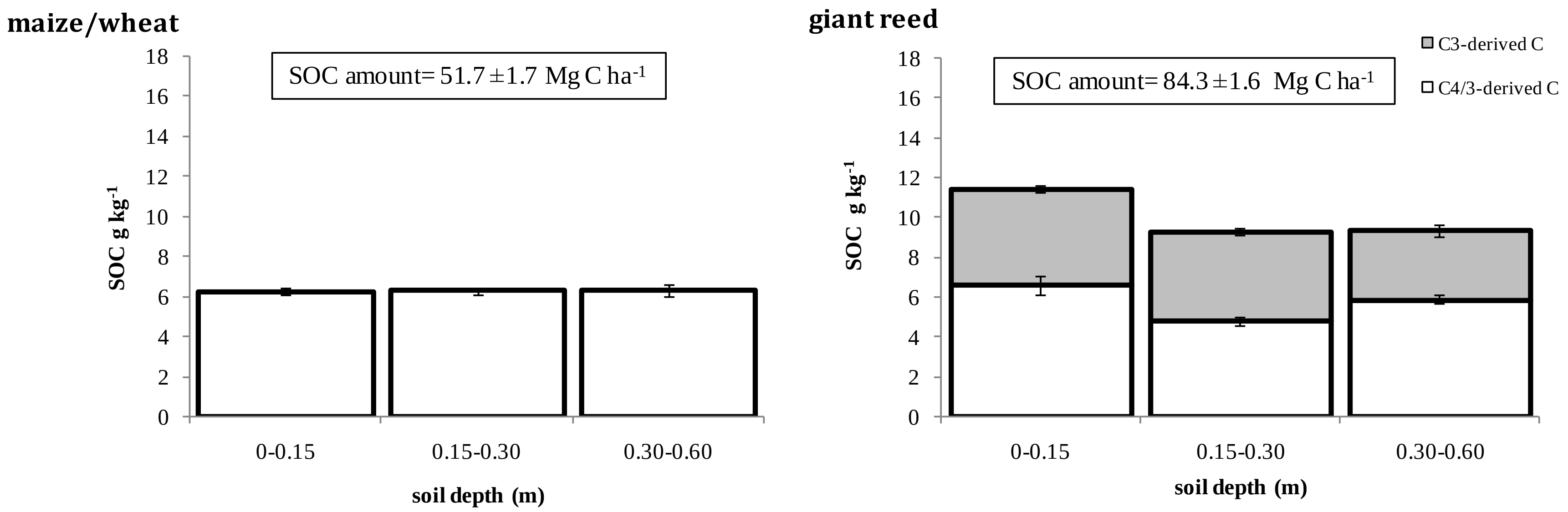

Figure 6 C_3 and C_4-derived carbon content at three soil depths in the maize/wheat rotation and giant reed. The total amounts of C (0-0.60 m) are also indicated as means ± standard errors. Vertical bars represent ± standard errors (n= 6).

DISCUSSION

SOIL C AND N CONTENT

Nitrogen fertilisation did not enhance the SOC pool in both land use systems. Moreover, the supply of mineral fertiliser did not influence the other two investigated factors (crop and soil depth) in any trait, apart from TN in giant reed-maize/wheat (Table 3). This preliminary and unexpected finding ruled out N fertilisation from the effects on SOC, that is the principal focus of our study.

In exchange for that, the combination of N_0 and N_{120} data in the factorial ANOVA, addressing the effects of crops, depths and their interaction, contributed to higher experimental precision.

The effects of N fertilisation were more noticeable in terms of aboveground biomass, at least in giant reed (data not shown), although this falls beyond the scope of this thesis. It is therefore evinced that N fertilisation in perennial energy crops may contribute to biomass production but does not benefit C sequestration. In agreement with this point, Lewandowski et al. (2003) affirmed that, since *Miscanthus* did not respond to N fertilisation in several sites across Europe, N fertilisation may be necessary only on soils with low N status.

As assumed by Dondini et al. (2009) and Hansen et al. (2004), we supposed that initial SOC levels were the same under annual and perennial species. Based on this, after 9 years of perennial energy crops, the amount of SOC (Mg ha^{-1}), stored in the whole profile, was increased to a similar extent in the two perennial species: +61% in *Miscanthus* and +63% in giant reed over their respective references (Figs. 4 and 5, respectively). This strong increase is consistent with the crop management: unlike annual crops, perennial grasses only need tillage in the year of establishment. The ecological advantages of the long period without tillage are reduced risk of soil erosion and a foreseeable increase in soil carbon content (Kahle 2001; Ma et al. 1999), thanks to reduced mineralisation under conditions of lower aeration (King et al. 2004).

ISOTOPE MASS BALANCE AND CONTRIBUTION OF PERENNIAL SPECIES TO SOC

Carbon distribution observed in the three soil layers is consistent with the root dry weight (RDW) of *Miscanthus* and giant reed, analysed by Monti and Zatta (2009) in the same experimental site: the shallow layer (0-0.15 m) accounted for 63% and 54% of the total RDW amount over the 0-0.60 m soil profile in the two respective species. It appears therefore that a greater root biomass in the shallow layer provided a stronger contribution to SOC than in deeper layers (below 0.15 m). In these layers, a major difference in root biomass between the two perennial crops was observed (Monti and Zatta 2009), reflecting in the $\delta^{13}C$ trend evidenced in this research (Fig. 3): under the top 0.15 m, *Miscanthus* root biomass rapidly decreased, in contrast to giant reed. Compared to this, soil C distribution under the two annual reference systems (continuous wheat and maize/wheat rotation) did not show any particular trend along soil profile (Fig. 3).

The different $\delta^{13}C$ trend in soil profile between *Miscanthus* and giant reed indicates that other sources of C accumulation, as the burrowing activity of earthworms or the leaching of dissolved organic C, play a minor role in C turnover below the top layer (0- 0.15 m).

The shallow C deposition, shown by *Miscanthus* in this experiment, is consistent with other sources. Dondini et al. (2009), comparing a 14-year *Miscanthus* substituting an arable cropping system in the Atlantic Central environmental zone, according to the proposed classification (Metzger et al. 2005), observed significant differences along soil depths, with 82% of the total C stock stored in the upper 0.30 m under *Miscanthus*, compared to 62% in the arable soil. In another study in the Continental zone, Schneckenberger et al. (2007) compared a loamy and a sandy soil with similar cultivation periods of *Miscanthus*. They noticed that more SOC was found in the upper 0.30 m of the sandy than the loamy soil (4.6 kg C m^{-2} *vs* 4 kg C m^{-2}). This is explained by slower decomposition of plant residues in the loamy soil, due to less aeration, associated with higher protection of SOC by clay particles. These two studies and ours suggest that the potential of soil C storage under perennial energy crops depends on several factors as the past land management and the amount of aboveground biomass remaining after harvest and being recycled to the soil. As supposed by Ceotto et al. (2011), we should also consider that the prolonged canopy cover of *Miscanthus* and giant reed could determine suitable micro-climatic conditions to increase SOC storage. In fact, as stated by Grigal and Berguson (1998), SOC tends to increase with a decrease in mean temperature and with an increase in precipitation.

The conversion from a C_3 to a C_4 perennial plant rapidly increased $\delta^{13}C$ signature in 0-0.15 m (Fig. 3.b), making soil organic matter under *Miscanthus* more ^{13}C-enriched in the shallow layer but depleted in the deeper ones, in contrast to giant reed and the two annual reference systems, maintaining steady isotopic values with soil depth. Nevertheless, ^{13}C abundance derived from giant reed and *Miscanthus* is consistent with their root biomass distribution, as previously discussed (Monti and Zatta 2009).

Since these results are based on changes in ^{13}C abundance, it may be stated, in agreement with Hansen et al. (2004), that the fraction of topsoil SOC (0-0.15 m) involved in short- to medium-term turnover is substantial. In our experiment the annual SOC gain, originated by *Miscanthus* (2.07 Mg C ha^{-1} y^{-1}), ranks at intermediate level between that observed by Dondini et al. (2009) (3.2 Mg C ha^{-1} y^{-1}) and those evidenced by Hansen et al. (2004) (approximately 0.6 and 0.9 Mg C ha^{-1} y^{-1} after 9 and 16 years of *Miscanthus* plantation, respectively) and Schneckenberger (2007) (ca. 0.6 and 0.7 Mg C ha^{-1} y^{-1} after 9 and 12 years of *Miscanthus* plantation, respectively). The variety in these data is coupled with a large range of climates, soil types, sampling depths and, especially, initial SOC levels (from 7.4 g SOC kg^{-1} in our experiment to ca. 23 g SOC kg^{-1} in Dondini et al. 2009). Therefore, it appears that no simple explanation can be provided for the gain in SOC level recorded after a period varying from 9 to 16 years of *Miscanthus* plantation.

As concerns giant reed, to our knowledge, no data on ^{13}C abundance is available so far in the literature; hence our results represent a new source of information about the role of this species in improving SOC levels, while being a promising plant for biomass production in Southern Europe (Angelini et al. 2009; Lewandoski et al. 2003).

CONCLUSION

After 9 years of *Miscanthus* and giant reed cropping, soil organic carbon content was increased along soil profile, compared to the respective soils under annual crops.

Through the ^{13}C abundance assessment, *Miscanthus* appears to have a greater potential of carbon accumulation in soil but giant reed can store more carbon to deeper soil layers (0.15-0.60 m), thanks to its deeper rooting.

Based on our data, the first registered in a Mediterranean-temperate climate, these two perennial energy crops can actually contribute to carbon sequestration potential of soils depleted by intensive agricultural management. Since both *Miscanthus* and giant reed are suited to environmental conditions of warm-temperate areas of Southern Europe, cultivations with these two plants can represent a promising and sustainable solution, not only for energy production, but also for the restoration of soil fertility and the enhancement of carbon sink potential.

SECTION TWO

Physical fractionation and natural ^{13}C abundance: Combined analysis to trace soil organic carbon distribution in soil aggregates under *Miscanthus* and giant reed

ABSTRACT

The introduction of perennial energy crops as feedstock for bioenergy production has been a focus in recent research. Their use may be also extended to other purposes, as, for instance, a viable alternative to overcome some of the negative aspects of annual crops, such as maize or wheat. Indeed, perennial energy crops can recover the loss of soil organic carbon due to ongoing soil disturbance in annual cropping systems, thanks to their high carbon sequestration potential.

In this thesis, we studied a 9-year old conversion from two annual crop systems, continuous wheat and maize/wheat rotation, to the perennial species, *Miscanthus* (*Miscanthus × giganteus* Greef and Deuter) and giant reed (*Arundo donax* L.), respectively, under Mediterranean-temperate climate.

Considering the significant differences in carbon sequestration, we investigated more in detail the impact of land use change from annual crops to *Miscanthus* and giant reed, on different soil fractions, as well as newly derived-carbon stocks.

Combining the ^{13}C natural abundance analysis with physical fractionation, we observed that the sequestered carbon, derived from the two perennial crops, was mainly found as particulate organic matter (68.1% and 71% in *Miscanthus* and giant reed, respectively) and therefore it is in labile state with short turnover rates. Considering the newly sequestered carbon, derived from perennial crops, it was observed that, under giant reed, microaggregates and mineral fraction played a relevant role, storing more newly derived carbon than in *Miscanthus*. This latter appears to be a preservative system of old derived carbon, because of the amount of C_3-derived carbon relative to continuous wheat observed in all fractions, at all soil depths.

In conclusion, after 9 years of perennial energy crops, the resulting increase in soil organic carbon storage contributes to recover the previous loss of organic carbon and the limited soil tillage, as management practice of these crops,

causes a relevant carbon storage also in physically protected fractions, where carbon turnover is slower.

INTRODUCTION

In the previous Section, it was shown that perennial energy crops, in particular *Miscanthus* and giant reed, have a high soil carbon sequestration potential, compared to annual crops or grassland systems (Hansen et al., 2004). Our results evidenced that, after 9 years, SOC content was significantly enhanced under the two perennial crops, with a different distribution of stored C in soil profile. However, it is important to measure the stability of perennial crops-derived C and how its distribution may change, to better understand the impact of *Miscanthus* and giant reed plantations. In fact, elucidating and accurately quantifying the capacity and longevity of C pools in agricultural lands is essential, given that soil has a great potential of C sink and that approximately 10% of the earth's total SOC (1500 Pg) (Post et al., 1990; Eswaran et al., 1993) is stored within agricultural soils (Schlesinger, 1984; Paustian et al., 1997a).

Agricultural systems relying on sustainable management practices (reduced or no-tillage, crop residue retention at soil surface, etc.) support soil C sequestration, also reducing negative environmental impacts and attenuating anthropic CO_2 emissions (Kong et al., 2005). Moreover, as different studies suggest, conservation practices as those cited can also protect SOM from decomposition (Tisdall and Oades, 1982; Jastrow and Miller, 1998), in contrast to intensive tillage (Paustian et al., 2000; West and Post, 2002; Lal, 2004; Six et al., 2004). This latter has been identified to be a major driver of SOC loss (Paustian et al., 2000), due to increased aeration and a reduction in the physical protection of SOM, leading to increased decomposition rates (Oades, 1984; Roberts and Chan, 1990).

Preserving the organic matter content in agricultural soils is of great importance, because of SOM central role in determining soil properties that strongly affect crop production and the environmental quality in a wider perspective (Lal, 2009; Powlson et al., 2012). SOM stabilisation depends on biochemical processes, including formation and preservation of molecules, structural rearrangements and molecular associations more resistant to decomposition (Piccolo, 2001; Six et al., 2002a; Schnitzer and Monreal, 2011).

However, its persistence in soil is also linked to the combined action of physical and chemical protection mechanisms (Six et al., 2002a; Von Lützow et al., 2006; Schmidt et al., 2011). Physical protection mechanisms refer to the occlusion of SOM within aggregates, which form a physical barrier limiting O_2 diffusion and the accessibility of decomposers and enzymes to the organic substrates (Plaza et al., 2013). Physical protection depends on the level of aggregation and has been shown to be much greater within microaggregates than within macroaggregates (Pulleman and Marinissen, 2004).

Chemical stabilisation mechanisms refer to the intimate association of organic matter with mineral particles, which reduce the degrading capacity of decomposers and enzymes (Six et al., 2002a). As Golchin et al. (1994) described in their study, fresh plant material entering the soil is colonised by microorganisms and encrusted by primary particles through the binding action of microbial agents (e.g., mucilage and polysaccharides), thus forming stable macroaggregates. With time, the fresh plant material within macroaggregates is selectively decomposed, leaving more chemically recalcitrant plant structural materials, which are coated with microbial metabolites and mineral particles to form stable microaggregates (Plaza et al., 2013). According to the model developed by Six et al. (1998, 1999, 2000), soil tillage increases macroaggregates turnover and thereby inhibits the formation of microaggregates within macroaggregates in which particulate organic matter is stabilised in the long term (Plaza et al., 2013).

Because of the strong influence of agricultural management practices on SOM stabilisation, physical fractionation (by density, size or aggregation) proves one of the most used techniques supplying relevant information on the location and dynamics of SOM, after a land use change. As shown by Tisdall and Oades (1982), three different physical soil fractions are obtained through physical fractionation, in association with three classes of organic matter (persistent, transient and temporary): macroaggregates (>250 μm), microaggregates (53-250 μm), silt and clay (<53 μm).

In this frame, the cultivation of perennial energy crops may be considered a viable option to improve SOC storage and enhance SOM concentration and stabilisation, because of these crops' low need for soil tillage. This typical crop management, together with a high biomass production and deep rooting systems, supports their high C sequestration potential (Lewandowski et al., 2003).

In this research, we investigated a 9-year old conversion from two annual crop systems, continuous wheat and a maize/wheat rotation, to the perennial energy crops, *Miscanthus* and giant reed. Focusing the results in the portion of SOC derived from the two perennial species in 9 years of cropping (see Section One), we analysed the C distribution within soil aggregates to (i) understand if C distribution changed, depending on the different agricultural management of annual and perennial crops; (ii) ascertain if the amount of stored SOC, in soil fractions and increasing layers, diverged in the two land use systems; (iii) quantify the proportion of newly derived, as well as old, C associated with the three soil fractions.

MATERIALS AND METHODS

EXPERIMENTAL SITE

The study site was located at the experimental farm of the University of Bologna (Italy), in the South-East of the Po Valley (44° 33' N, 11° 21' E, 32 m a.s.l.). Mean annual precipitation and temperature are of 700 mm and 13.3 °C, respectively. Thus, the area is included in the Mediterranean North environmental zone, implying mild winter and long growing season, in exchange for precipitation mostly concentrated in the cold semester (Metzger et al. 2005).

A chemical and physical characterisation of the studied soils, under the two perennial (*Miscanthus* and giant reed) and annual crops (continuous wheat and maize/wheat crop rotation), was carried out according to the current Italian methods of soil analysis (D.M. 13/09, 1999), as shown in Table 4.

Table 4 Physical and chemical characterisation of the studied soils under perennial (*Miscanthus* and giant reed) and annual crop (continuous wheat and maize/wheat rotation) systems. Soil analysis according to the Italian method D.M. 13/09, 1999.

	Site (soil)			
	Miscanthus	**Giant reed**	**Wheat**	**Maize/Wheat**
pH_{H2O}	7.6	7.5	6.1	6.1
Texture (%)				
Sand	25	27	24	25
Silt	45	46	49	45
Clay	30	27	27	26
Limestone (%)				
total	1.7	1.7	< 0.5	< 0.5
active	1.6	1.6	< 0.1	< 0.1
Vegetation type	C_4	C_3	C_3	C_4/C_3
C_{ORG} (g kg^{-1})	16	11.5	7.4	6.3
N_{TOT} (g kg^{-1})	1.5	1.5	0.9	0.8
C:N	10.7	7.7	7.4	7.9
Available P (mg kg^{-1})	32.7	12.4	31.9	20.9

CROP MANAGEMENT AND EXPERIMENTAL LAYOUT

Continuous wheat (*Triticum aestivum* L.) and maize/wheat rotation (*Zea mays* L./*Triticum aestivum* L.) are the two annual systems that were established in experimental plots in the late 1960's. *Miscanthus* and giant reed plots were established in 2002 in soils previously cultivated with annual C_3 and C_4/C_3 species, respectively.

Each plot of both annual and perennial crops was split to receive 0 (N_0) and 120 (N_{120}) kg ha^{-1} of mineral N, supplied as urea, were applied annually during the spring time. Moreover, 50 kg P_2O_5 ha^{-1}, as triple superphosphate, were applied every year in annual crops. In *Miscanthus* and giant reed, 200 kg P_2O_5 ha^{-1} were added prior to planting in spring 2002. No K fertiliser was applied, given the sufficient level of this nutrient in the soil.

Tillage at 0.30 m was carried out every year in annual crops; only prior to planting (autumn 2001) in perennial ones. In both annual and perennial species, the rest of crop husbandry reflected the normal practice followed in the experimental area. In particular, perennial crops were always harvested every year in the early autumn (September 25 – October 15); annual crops were harvested at maturity (wheat, early summer; maize, late summer) and the recoverable fraction of their residues (wheat straw and maize stover) was always removed from the field.

Soil sampling took place in March 2011 at the beginning of the 10th growing season, using a 85 mm diameter soil corer up to a depth of 0.60 m, divided in three layers (0-0.15, 0.15-0.30, 0.30-0.60 m). For each crop three replicates were taken according to a completely randomised experimental design. Each replicate was composed of three subsamples, which were put together and transported to the laboratory on the same day. Soil samples were subsequently sieved (2 mm); visible plant debris were removed and then soils were air-dried.

While an aliquot was used for the analysis described in Section One, the other aliquot was subjected to physical fractionation and then to total C and $\delta^{13}C$

signature analysis of soil aggregates. In the same date, separate samples (50 mm diameter) of undisturbed soil were monitored for bulk soil.

SOIL PHYSICAL FRACTIONATION

Soil fractions were mechanically isolated from the whole soil using the method developed by Elliott (1986), partially modified (Fig. 7). An aliquot of 5 g of soil samples was used to separate aggregates by wet sieving on two sieves, with a slacking time of 10 min and a further "up and down" movement of sieves of 20 min. The fraction retained on the 250 µm mesh consisted of particulate organic matter (POM, >250 µm) and 250-2000 µm sand, setting up the *macroaggregates*. Materials passing the 250 µm sieve were flushed into the underlying sieve (53 µm mesh sieve) by a continuous water flow. These materials retained on the 53 µm sieve constituted the *microaggregate* fraction, called intraggregate light fraction (IALF, 53-250 µm). The suspension passing the 53 µm sieve was flocculated using 0.5 mM $CaCl_2$ and let to stand all day long to settle. The supernatant was composed by *silt and clay*, representing the mineral fraction (MF, <53 µm). Consequently, three aggregate size fractions were separated: (i) small macroaggregates (>250 µm), (ii) microaggregates (53-250 µm), (iii) silt and clay (>53 µm) fractions. Once dried at 60 °C, all the soil fractions were weighed and analysed for total C and $\delta^{13}C$ signature by CF - IRMS (Delta Plus, Thermo Scientific).

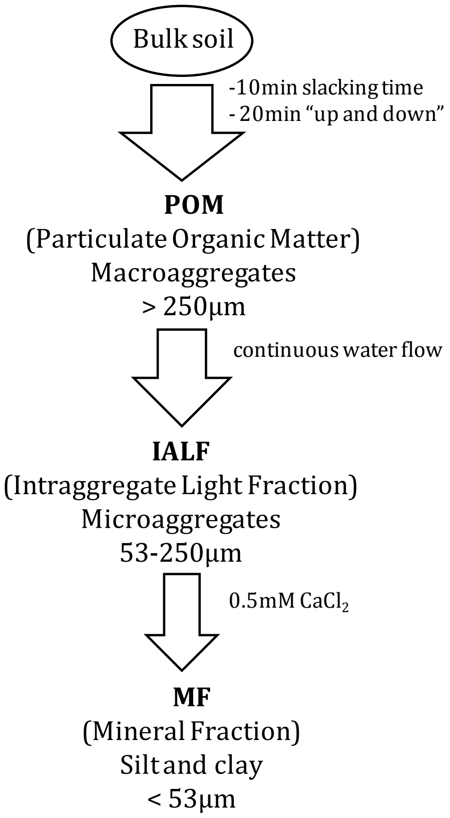

Figure 7 Scheme of physical fractionation of soil adopted in this research, modified after Elliott (1986).

TOTAL C AND ^{13}C ANALYSIS OF SOIL AGGREGATES

Total soil organic C (SOC), N (TN) and δ^{13}C values were determined for each soil fraction by using an elemental analyser (CHNS - O mod. EA 1110, Thermo Scientific GmbH, Dreieich - Germany), with acetanilide as a standard for C concentration. As traces of carbonates were detected in the studied soils, samples were pre-treated with acid. An aliquot of soil (10 - 13 mg) was weighed into silver capsules, and, after adding a few drops of 6 M HCl, was heated to 80 °C on a heating plate until dry. Then the capsules were closed and analysed in the elemental analyser. The isotopic composition (δ^{13}C signature) of soil aggregates was measured by Continuous Flow - Isotope Ratio Mass Spectrometry (CF - IRMS), by introducing the combustion gas (CO_2) from the elemental analyser into the Isotope Ratio Mass Spectrometer (IRMS, Delta Plus, Thermo Scientific).

DATA ANALYSIS

The natural abundance δ^{13}C was measured to determine the proportion of C derived from the C_4 *Miscanthus* and how much C remained from wheat (C_3), in soil fractions (Lisboa et al., 2009).

Generally, photosynthesis leads to a discrimination against the heavier ^{13}C isotope in the plant organic matter compared to atmospheric CO_2. The degree of the discrimination is dependent on the photosynthetic pathway: C_4-plants show distinctly higher ^{13}C abundance than C_3-plants. In an environment with only one source of C_4-derived SOC (i.e. *Miscanthus*), the isotopic signal can be used to quantify the amount of carbon derived by that given source (Balabane and Balesdent, 1992; Balesdent and Balabane, 1992) using the isotope mass balance. The ^{13}C abundance was expressed in delta - units (δ^{13}C ‰) and calculated according to the equation:

$$\delta^{13}\text{C ‰} = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 1000$$

where R_{sample} is the isotope ratio $^{13}C/^{12}C$ of the sample and R_{standard} is the $^{13}C/^{12}C$ ratio of the international Pee Dee formation belemnite carbonate standard (PDB).

The percentage of C derived from *Miscanthus* (F_M) and stored in soil aggregates was calculated by using the isotope mass balance equation (Balesdent et al., 1987):

$$F_M = [(\delta^{13}C_{\mathrm{new}} - \delta^{13}C_{\mathrm{old}})/(\delta^{13}C_{\mathrm{new\ crop}} - \delta^{13}C_{\mathrm{old\ crop}})] \times 100 \qquad [1]$$

where $\delta^{13}C_{new}$ is the $\delta\ ^{13}C$ of the soil under *Miscanthus*, $\delta\ ^{13}C_{old}$ is the $\delta\ ^{13}C$ of the soil under wheat, $\delta^{13}C_{new\ crop}$ is the $\delta^{13}C$ of *Miscanthus* material entering the soil (-12.66 ‰) and $\delta^{13}C_{old\ crop}$ is the $\delta^{13}C$ of wheat (-27 ‰) (Dondini et al., 2009). The proportion of C derived from wheat ($C_{old\ \%}$) was obtained by the equation:

$$C_{old} = 100 - F_M$$

The amount of C derived from giant reed (F_G), a C_3 species, and stored in soil aggregates, was calculated according to the following equation, proposed by Hansen et al. (2004):

$$F_G = [(\delta^{13}C_{\mathrm{new}} - \delta^{13}C_{\mathrm{old}})/(\delta^{13}C_{\mathrm{new\ crop}} - \delta^{13}C_{\mathrm{old}})] \times 100 \qquad [2]$$

where the $\delta\ ^{13}C$ of soil under maize/wheat was used as $\delta\ ^{13}C_{old}$, since it was not known the $\delta\ ^{13}C$ of the rotation before the introduction of giant reed.

SOC was also expressed as the amount of C per unit crop surface (Mg C ha^{-1}) considering the bulk density of the three soil layers.

STATISTICAL ANALYSIS

Normal distribution and equal variance of data were controlled through the Kolmogorov-Smirnov and Bartlett test, respectively. Data were then submitted to the analysis of variance (ANOVA) through the CoStat 6.3 software (CoHort Software, Monterey, California, USA), according to a completely randomised factorial scheme: the four-way ANOVA involved 4 sources (crop and N fertilisation, at two levels; soil depth and soil fractions, at three levels) and their resulting interactions (six interactions of 1st order, four of 2nd and one of 3rd order). In this complex dataset, special attention will be paid to ANOVA sources involving the effects of soil depth.

The Student - Newman-Keuls (SNK) test at $P \leq 0.05$ was adopted to separate means of statistically significant sources. The *LSD* test at P= 0.05 has been used to graphically separate data of significant traits (SOC, TN, SOC distribution in aggregate size fractions, distribution of soil aggregates).

RESULTS

ANOVA RESULTS

The four-way ANOVA involved 4 simple sources (crop, C; N fertilisation, N; soil depth, D; soil fraction, F) and the numerous 1st, 2nd and 3rd order interactions, in relation with 4 main traits (SOC, TN, SOC distribution in aggregate size fractions and distribution of soil aggregates) (Table 5).

For each investigated trait, every single source and the derived interactions exerted a variable influence, as shown by ANOVA statistical significance. Through the simple sources, a special attention must be paid to N fertilisation, always resulting statistically insignificant in *Miscanthus vs* continuous wheat, apart from two 2nd order interactions (C × D × N; C × N × F) referring to TN, SOC distribution in fractions and distribution of soil aggregates (Tab. 5.a). On the other hand, N fertilisation was often significant in giant reed *vs* maize/wheat rotation from both 1st and 2nd order interactions (Tab. 5.b). Focusing on the two perennial species, giant reed also showed a larger responsiveness to added N than *Miscanthus* in aboveground biomass during the 9 years of cultivation (data not shown). These results are in accordance with those observed in Section One for the two plant groups, as only a modest TN increase under fertilisation was shown under giant reed *vs* maize/wheat rotation, compared to none under *Miscanthus vs* continuous wheat.

Considering the complex dataset, in the following paragraphs of Results, a particular focus will be assigned to the interactions involving the soil depth factor.

Table 5 ANOVA sources (simple factors, 1st, 2nd and 3rd order interactions) and statistical significance on soil organic carbon (SOC), total nitrogen (TN), soil organic carbon distribution in fractions ($SOC_{\text{in fractions}}$) and distribution of soil aggregates, in *Miscanthus vs* continuous wheat (**A**) and giant reed *vs* maize/wheat rotation (**B**).

ANOVA sources	P_{SOC}	P_{TN}	$P_{\text{SOC in fractions}}$	$P_{\text{distribution}}$
A				
Crop (C)	< 0.001***	< 0.001***	< 0.001***	0.329 ns
Fertilisation (N)	0.463 ns	0.833 ns	0.589 ns	0.658 ns
Depth (D)	< 0.001***	< 0.001***	< 0.001***	0.618 ns
Soil fraction (F)	< 0.001***	< 0.001***	< 0.001***	< 0.001***
C × D	< 0.001***	0.001**	0.010*	0.863 ns
C × N	0.918 ns	0.199 ns	0.981 ns	0.372 ns
D × N	0.226 ns	0.381 ns	0.883 ns	0.846 ns
C × F	< 0.001***	0.022*	< 0.001***	< 0.001***
D × F	< 0.001***	0.357 ns	0.001**	0.101 ns
F × N	0.598 ns	0.474 ns	0.983 ns	0.639 ns
C × D × N	0.208 ns	0.032*	0.763 ns	0.681 ns
C × D × F	0.005**	0.307 ns	< 0.001***	0.002**
C × N × F	0.556 ns	0.245 ns	0.002**	< 0.001***
D × N × F	0.724 ns	0.397 ns	0.309 ns	0.350 ns
C × D × N × F	0.660 ns	0.839 ns	0.252 ns	0.164 ns
Mean trait value	9.543 g kg^{-1}	1.274 g kg^{-1}	3.107 g fraction^{-1}	0.333 kg kg^{-1}soil
B				
Crop (C)	< 0.001***	< 0.001***	< 0.001***	0.003**
Fertilisation (N)	0.006**	0.090 ns	0.096 ns	0.392 ns
Depth (D)	< 0.001***	0.145 ns	0.001**	0.214 ns
Soil fraction (F)	< 0.001***	< 0.001***	< 0.001***	< 0.001***
C × D	< 0.001***	< 0.001***	0.126 ns	0.612 ns
C × N	0.384 ns	0.684 ns	0.934 ns	0.541 ns
D × N	0.007**	0.133 ns	0.111 ns	0.608 ns
C × F	< 0.001***	< 0.001***	< 0.001***	< 0.001***
D× F	0.127 ns	0.233 ns	0.005**	< 0.001***
F× N	0.911 ns	0.587 ns	0.580 ns	0.034*
C × D × N	0.017*	< 0.001***	0.418 ns	0.631 ns
C × D × F	0.858 ns	0.660 ns	0.065 ns	< 0.001***
C × N × F	0.955 ns	0.872 ns	0.041*	< 0.001***
D × N × F	0.632 ns	0.604 ns	0.066 ns	< 0.001***
C × D × N × F	0.720 ns	0.866 ns	0.046*	0.014*
Mean trait value	8.215 g kg^{-1}	1.091 g kg^{-1}	2.598 g fraction^{-1}	0.333 kg kg^{-1}soil

ns, *, ** and *** mean non-significant and significant at $P \leq 0.05$, ≤ 0.01 and ≤ 0.001, respectively.

AGGREGATE SIZE FRACTIONS

The three classes of weight distribution of aggregate size (macro-, micro-aggregates, silt and clay) followed different patterns across both land use systems (perennial and annual) and through soil layers (0-0.15, 0.15-0.30, 0.30-0.60 m). In general, the most significant differences were observed within the soil aggregate class than among crop types.

Particulate organic matter (POM) significantly contained the highest proportion of soil, compared to microaggregates (IALF) and mineral fraction (MF), but some differences were observed between *Miscanthus* and giant reed.

Comparing *Miscanthus* and continuous wheat, about the same C content was observed at 0-0.15 m (19.2% and 18.9% in *Miscanthus* and continuous wheat, respectively), although this difference was not statistically significant (Fig. 8.a).

On the contrary, soil distribution in microaggregates (IALF) and mineral fraction (MF) increased under the perennial crop, compared to continuous wheat, through soil depth.

In giant reed, 22.4% of POM was incorporated in the topsoil (0-0.15 m), compared to 20.8% under maize/wheat rotation (Fig. 8.b). Integrated through the whole profile, giant reed soil had a higher percentage of POM fraction compared to the crop rotation (67.2% *vs* 61.3% in giant reed and maize/wheat rotation, respectively), whereas soil distribution slightly decreased in the IALF and MF fractions, with respect to maize/wheat rotation. Differences in these soil fractions were not significant, apart from soil contained in MF at 0.15-0.30 m (1.3% and 3.2% under giant reed and maize/wheat rotation, respectively) (Fig. 8.b).

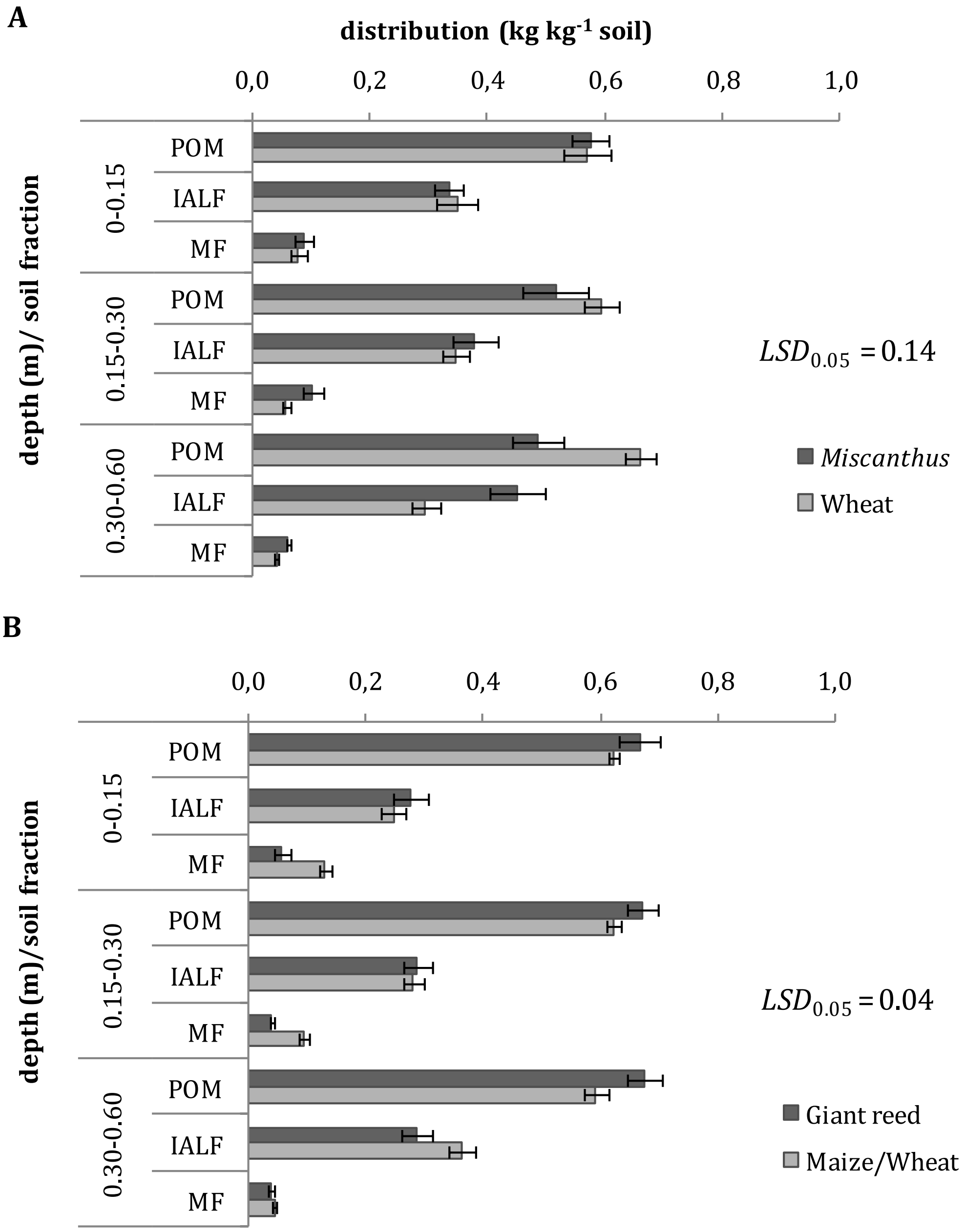

Figure 8 Soil weight distribution among aggregate size classes (POM-IALF-MF), at three soil depths, in *Miscanthus vs* continuous wheat (**A**) and in giant reed *vs* maize/wheat rotation (**B**). Horizontal bars represent ± standard errors (n= 6). $LSD_{0.05}$, least significant difference at $P \leq 0.05$.

DISTRIBUTION OF TOTAL SOC STOCKS WITHIN SOIL FRACTIONS

Both *Miscanthus* as well as giant reed soils contained significantly higher total SOC than the annual systems, continuous wheat and the maize/wheat rotation (97.8 Mg C ha^{-1} and 77.9 Mg C ha^{-1} *vs* 59.6 Mg C ha^{-1} and 58.1 Mg C ha^{-1}, respectively). SOC distribution through the three soil depths (0-0.15, 0.15-0.30 and 0.30-0.60 m) was significant for both land use systems and, as shown in Section One, C distribution was especially restricted in the topsoil, with a major evidence for *Miscanthus* (29.2 Mg C ha^{-1}) than giant reed (21.3 Mg C ha^{-1}), compared to the deeper layers.

SOC distribution within soil aggregates in the three layers (C × D × F interaction) resulted in a significant interaction in both the plant groups (Tab. 5). In general, the higher amount of organic C was found in the POM fraction under both perennial crops (average 17.9 and 18.1 Mg C ha^{-1} in *Miscanthus* and giant reed, respectively), while under annual crops a lower C storage was observed (12.6 and 11.1 Mg C ha^{-1} in continuous wheat and maize/wheat rotation, respectively) (Fig.9). In the POM fraction, 58.1% and 72% of total SOC were stored in *Miscanthus* and giant reed soils, respectively. Under *Miscanthus*, total SOC was stored in all three soil aggregate classes, in all soil layers, whereas, under giant reed, the POM fraction evenly stored a greater C content through soil depths, compared to IALF and MF.

Shares of the total SOC stocks decreased in IALF and MF fractions, with a similar trend between the two perennial grasses: in *Miscanthus*, C stored in microaggregates (IALF), silt and clay (MF), was higher than C stored in the same aggregate classes under continuous wheat (7.9 Mg C ha^{-1} and 2.4 Mg C ha^{-1} *vs* 4.2 Mg C ha^{-1} and 1 Mg C ha^{-1}, respectively) (Fig. 9.a). Under giant reed, the share of total SOC in microaggregates was higher than under the maize/wheat rotation (average 5.8 and 4.4 Mg C ha^{-1}, respectively) but lower in the mineral fraction (average 1.3 Mg C ha^{-1} and 2 Mg C ha^{-1}, respectively) (Fig. 9.b).

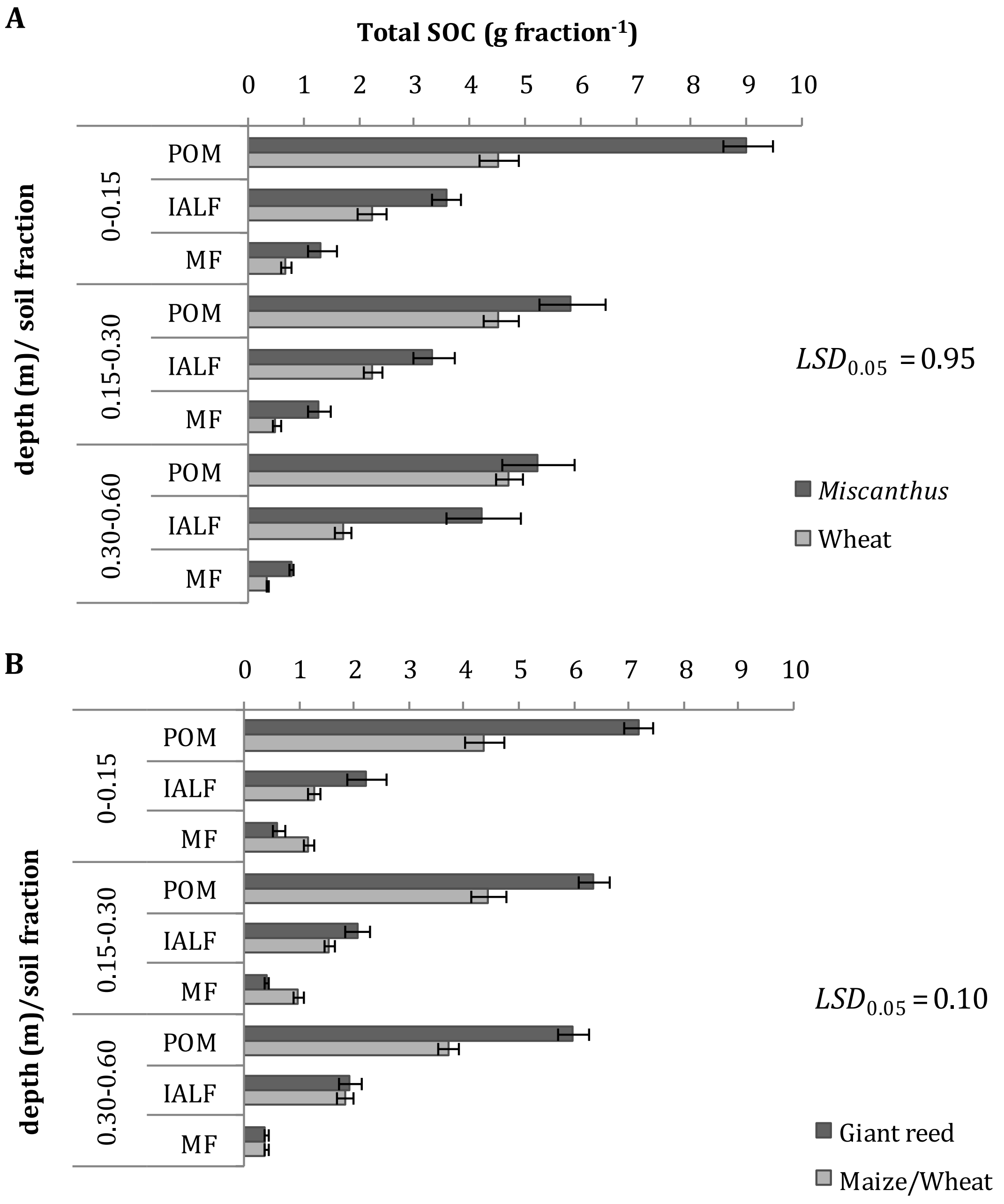

Figure 9 Total soil organic carbon (SOC) within the soil fractions, in the three soil depths, in *Miscanthus vs* wheat (**A**) and in giant reed *vs* maize/wheat rotation (**B**). Horizontal bars represent standard errors (n= 6). $LSD_{0.05}$, least significant difference at $P \leq 0.05$.

ISOTOPE MASS BALANCE AND CONTRIBUTION OF PERENNIAL SPECIES TO SOC IN SOIL FRACTIONS

As seen in Section One, $\delta^{13}C$ values and distribution in soil aggregates, both in perennial and annual crops, appear to be strongly influenced by their C_3 or C_4 photosynthetic pathways. Within soil layers, the $\delta^{13}C$ values of all aggregates size fractions remained consistent in annual crops, while a great difference appeared when comparing the two perennial plants. $\delta^{13}C$ values were significantly higher under *Miscanthus* (i.e., less negative) (Table 6) and significantly lower under giant reed (i.e., more negative) (Table 7).

Table 6 $\delta^{13}C$ values (‰) for soil aggregate fractions in the two land use systems, *Miscanthus* (C_4) and continuous wheat (C_3). Standard errors are in brackets.

$\delta^{13}C$				
Depth (m)	**Land use**	**POM**	**IALF**	**MF**
0-0.15	*Miscanthus*	-20,72	-22,12	-22,32
		(0.35)	(0.21)	(0.20)
	Wheat	-25,35	-25,47	-25,25
		(0.08)	(0.07)	(0.05)
0.15-0.30	*Miscanthus*	-23,78	-24,43	-23,96
		(0.10)	(0.15)	(0.08)
	Wheat	-25,48	-25,58	-25,29
		(0.06)	(0.05)	(0.06)
0.30-0.60	*Miscanthus*	-24,18	-24,33	-24,15
		(0.08)	(0.06)	(0.02)
	Wheat	-25,38	-25,45	-25,23
		(0.15)	(0.08)	(0.08)

Table 7 $\delta^{13}C$ values (‰) for soil aggregate fractions in the two land use systems, giant reed (C_3) and maize/wheat rotation ($C_{4/3}$). Standard errors are in brackets.

$\delta^{13}C$				
Depth (m)	**Land use**	**POM**	**IALF**	**MF**
0-0.15	Giant reed	-25,30	-25,62	-25,22
		(0.13)	(0.08)	(0.09)
	Maize/Wheat	-23,32	-23,65	-23,24
		(0.14)	(0.10)	(0.06)
0.15-0.30	Giant reed	-24,85	-25,20	-24,85
		(0.04)	(0.06)	(0.08)
	Maize/Wheat	-23,21	-23,28	-22,44
		(0.18)	(0.08)	(0.14)
0.30-0.60	Giant reed	-25,15	-25,33	-24,84
		(0.07)	(0.05)	(0.12)
	Maize/Wheat	-23,17	-23,63	-23,19
		(0.18)	(0.06)	(0.09)

The effects of the substitution of arable crops with perennial grasses, in terms of SOC storage and distribution in soil aggregates and relative share of C_3- and C_4-derived C, indicates a different behaviour between *Miscanthus* and giant reed.

Under *Miscanthus,* in the 0-0.15 m soil layer, 48.8% of all C_4-derived C was located in macroaggregates (POM); in the two deeper layers, 12% and 7.3% were concentrated in POM, respectively (Fig. 10). Moreover, with reference to the IALF and MF fractions, the input of C_4-derived C from *Miscanthus* was principally found in the shallow layer, where 14.3% was located in microaggregates and 4.7% in silt and clay. Based on these data, after 9 years of *Miscanthus* plantation, the amount of C_4-derived C in the whole soil profile was 12.6 Mg C ha^{-1}, with the most part of total SOC localised in the topsoil, according to the distribution in soil aggregates described above.

Furthermore, under *Miscanthus,* in all soil aggregate classes, it was observed an average amount of C_3-derived C, higher than in wheat soil (Fig. 10).

On the other hand, under giant reed, 28.1%, 20.2% and 22.6% of all C_3-derived C was located in the POM fraction in the shallow, intermediate and deep layers, respectively (Fig. 11). The topsoil (0-0.15 m) stored 6 Mg C ha^{-1} of C_3-derived C, while in the other soil fractions (IALF and MF), a lower input of C_3-derived C was observed. On the whole, 71%, 23.7% and 5.4% of the C_3-derived C was located in the POM, IALF and MF soil fractions, respectively.

Based on these data, after 9 years of giant reed plantation, the amount of C_3-derived C in the whole soil profile was 27.9 Mg C ha^{-1}, according to the distribution through the soil layers and within soil aggregates, described above (Fig. 11).

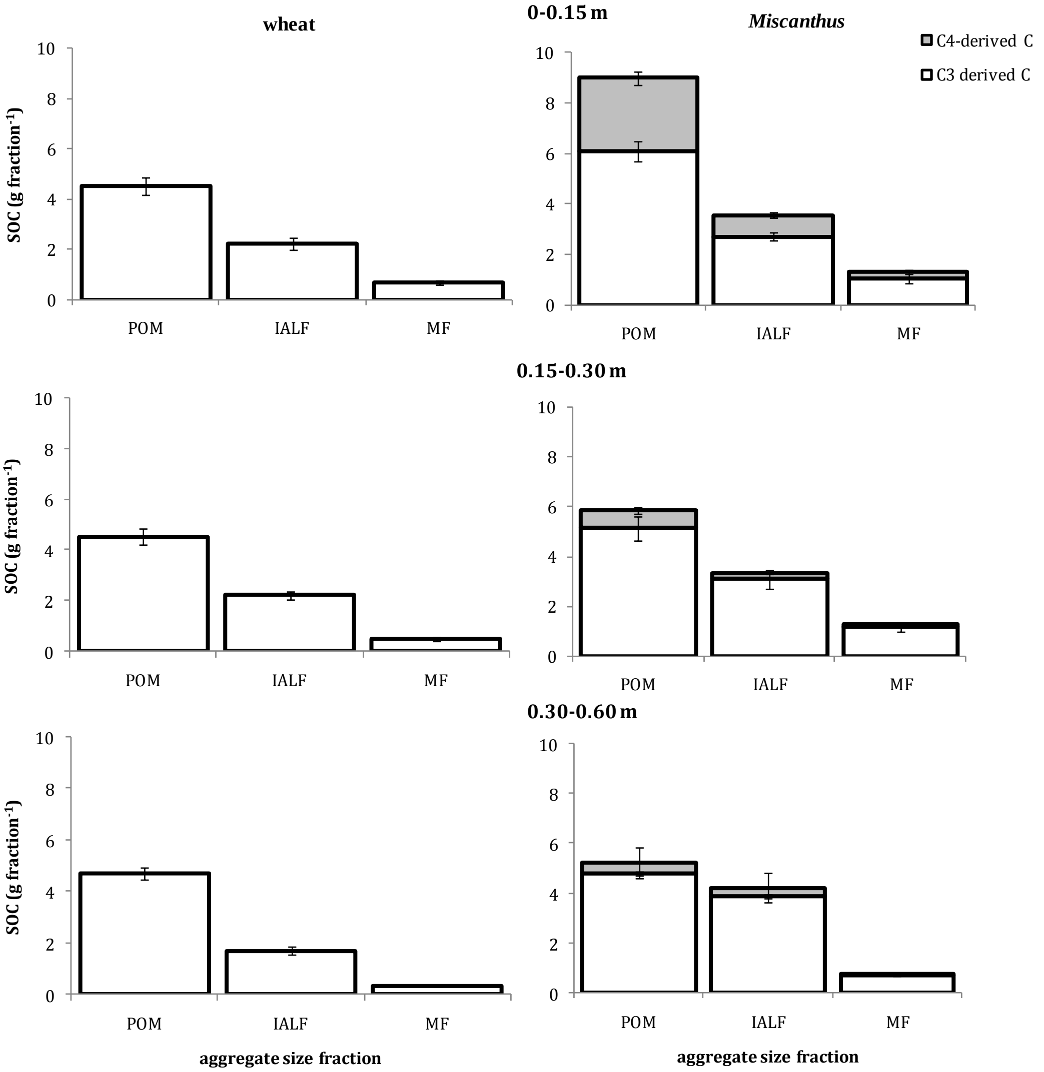

Figure 10 Soil organic carbon (SOC) content in the aggregate soil fractions (POM-IALF-MF), in the three soil layers, for *Miscanthus* and wheat. Vertical bars represent ± standard errors (n= 6).

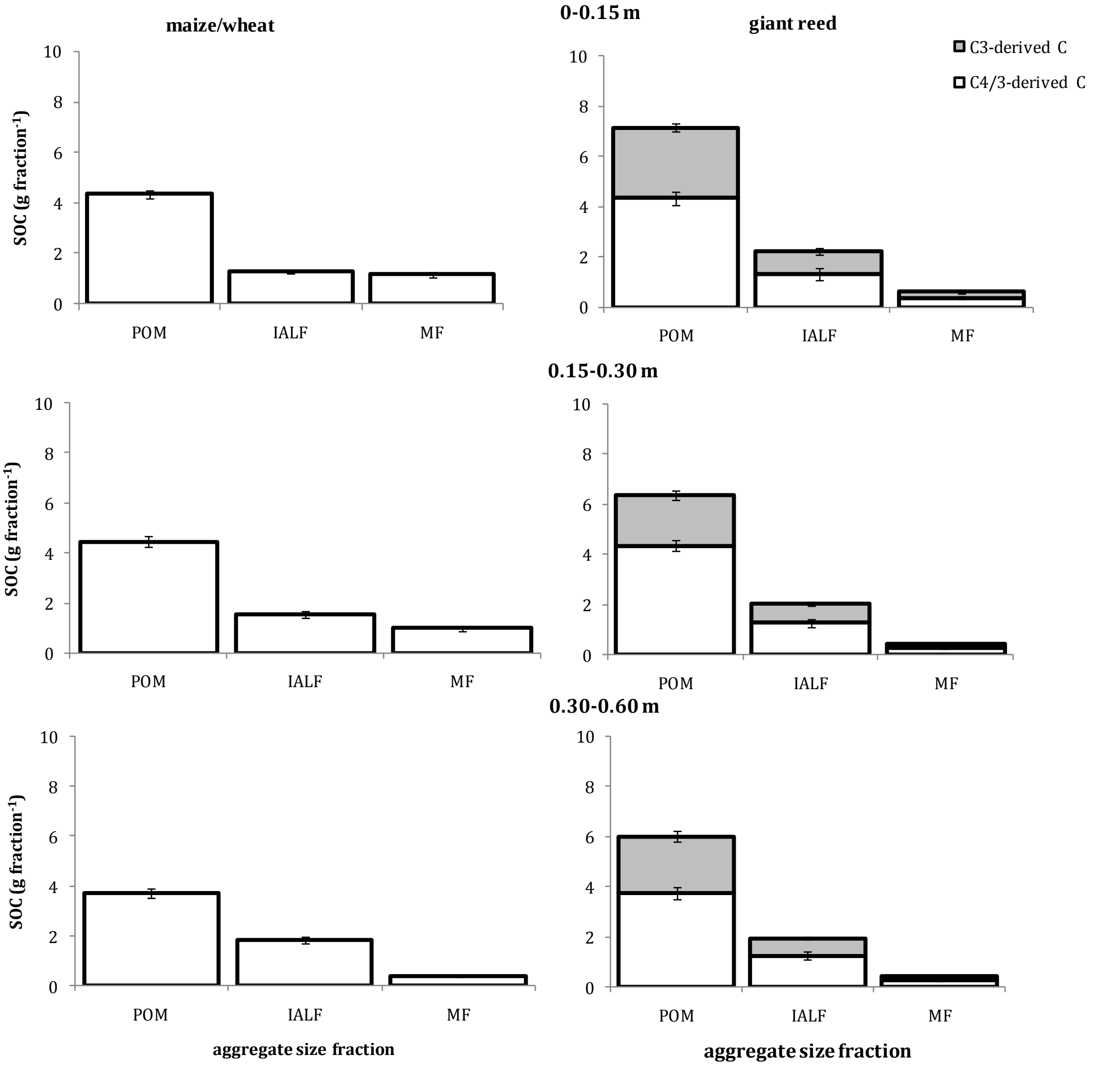

Figure 11 Soil organic carbon (SOC) content in the aggregate soil fractions (POM-IALF-MF), in the three soil layers, for giant reed and maize/wheat rotation. Vertical bars represent ± standard errors (n= 6).

DISCUSSION

Combining SOM physical fractionation with ^{13}C natural abundance analysis enabled us to further understand the SOC dynamics after the land conversion from annual crops to the two perennial species, *Miscanthus* and giant reed. In fact, as reported by many authors, physical fractionation has an important influence on sequestration of C in soils following land use change (Edwards and Bremner, 1967; Elliott, 1986; Six et al., 2002; Denef et al., 2004).

The comprehensive results show differences, not only between the land use systems (annual *vs* perennial), but also between the two perennial energy crops. Soil C content and distribution across the physical fractions and the soil layers mainly increased into macroaggregates (POM), with a significant amount in perennial crops. Under *Miscanthus,* SOC is evenly stored also into physical protected (microaggregates) and chemical protected SOM (silt and clay), while, under giant reed, SOC is preferentially accumulated into particulate organic matter (POM). These results correspond well with Kahlon et al. (2013), who compared, in a long-term experiment, the effects of no-till, ridge till and plow till on soil physical properties and SOC concentrations. The authors reported that macroaggregates contained more C than microaggregates (Dorodnikov et al., 2009), particularly in no-till fields. The amount of C content may be attributed to an increase in soil aggregation, especially macroaggregates, and to a relatively higher increase in labile C pools as a result of less disturbance and more residue retention (Havlin et al., 1990). As perennial energy crops need soil tillage only in the year of the establishment, we can consider soils under *Miscanthus* and giant reed as undisturbed crop systems. Thus, the assumptions of Kahlon et al. (2013) and other authors (Six et al., 1999, 2004, Paustian et al., 2000; West and Post, 2002; Lal et al., 2004; Plaza et al., 2013) may be efficient also in our case.

It is reasonable to suppose that the different SOC distribution between the two perennial species is due also to their different root biomass, as previously discussed in Section One. The greatest root biomass in *Miscanthus* and the resulting rhizodeposition activity occur in the topsoil and the shallow rooting growth results in a higher C storage in the macroaggregates, at 0-0.15 m. As reported in other studies (Plaza et al., 2013; Dondini et al., 2009), we may suppose that the high C content observed in POM at 0-0.15 m is sequestered in microaggregates within macroaggregates, considered by many authors the primary site for long-term soil C sequestration.

The role of microaggregates is important for the contribution to the long-term stabilisation of SOM in the perennial systems, by providing physical protection not only to mineral-free SOM but also to chemically-protected SOM adsorbed on mineral surfaces (Plaza et al., 2013). This main difference between the two perennial energy crops may also be due to the microbial activity that probably differs, as a possible consequence of their different root biomass, as discussed above. Recent work has found the contribution of microbial biomass to soil SOM to be much more important than previously thought (Miltner et al., 2012; Simpson et al., 2007b). In their study, Plaza et al. (2013) observed that a reduced aggregate turnover due to less soil disturbance enhances the formation of stable organo-mineral complexes between mineral particles and microbial materials. It is likely that, at least in part, microbes play an active role in anchoring themselves to clay surfaces, through the secretion of biofilms that mix with various inorganics to produce "hutches" (Lünsdorf et al., 2000). One anchored to clay, it is likely that the microbes can only access readily available components close to the mineral surfaces. Microbes and microbial by-products, adsorbed on mineral surfaces and physically protected by entrapment within very small microaggregates, appear to constitute an important pool of SOM stabilisation and C sequestration in soils under no-till.

Perennial energy crops show a distinct trend also in newly derived C storage within soil aggregates.

Most of the C_4-derived C under *Miscanthus* was found in the POM fraction in topsoil, while its content decreased with soil depth. On the other hand, microaggregates and mineral fraction appear to have a secondary role in newly derived C storage. Moreover, under *Miscanthus,* all soil fractions showed a greater amount of C_3-derived C relative to continuous wheat. This result is in agreement with Dondini et al. (2009), indicating that *Miscanthus* may be a preservative system in old derived C storage. Dondini et al. explained this fact by assuming that, at the start of their field experiment, the soils of the future *Miscanthus* plantation and the reference site were not fully in equilibrium with their use as arable land. Continued soil disturbance through ploughing would then further decrease soil C_3 stocks in the reference site. To explain the C_3-derived C under *Miscanthus,* we hypothesise also a slower C turnover associated with the preference of soil microbial biomass to use the newly derived C as substrate for degradation, because of better chemical and structural quality.

Under giant reed, most of the C_3-derived C was stored in the macroaggregates, but a considerable share of newly derived C was stored also in the microaggregates and in the mineral fraction, through all soil layers. From this evidence, giant reed appears to be a less preservative system of old derived C, compared to *Miscanthus*.

In agreement with Von Lützow et al. (2008), we can confirm that the potential for SOM stabilisation and C sequestration in agricultural soils is site- and depth-specific, but our results demonstrate that it is also crop-specific. Indeed, we observed that between the same land use system of perennial crops, both *Miscanthus* and giant reed can differently act in the enhancement of soil as a C sink.

CONCLUSION

After 9 years of *Miscanthus* and giant reed plantations, the majority of newly sequestered carbon was found in the relatively labile particulate organic matter (POM).

Differences in the distribution of newly sequestered carbon in soil fractions have been evidenced between the two perennial crops: *Miscanthus* appears to be a more preservative system of old derived carbon in all fractions, whereas giant reed can store a higher content of new carbon in the microaggregates (IALF) and the mineral fraction (MF).

Under giant reed, along with its root biomass distribution, the carbon storage is evenly distributed in all soil layers.

The conversion from annual arable crops to perennial energy species reflected in a land management conversion, from tillage to a reduced soil disturbance. Continuous disturbance, such as long-term arable farming of annual crops, shows to have a significant effect on carbon associated with stable aggregates. Indeed, while intensive tillage disturbs the labile and stable carbon pools by leading to a reduction in associated carbon stocks, a no-till practice, like perennial energy crops management, may help to maintain and preserve soil organic matter.

The experimental approach used in this Section allowed to investigate more in detail the fate of the newly sequestered carbon in soil physical aggregates. Results support the idea that both *Miscanthus* and giant reed can represent a sustainable solution for the restoration and enhancement of soil carbon sink potential, also in warm-temperate environments.

SECTION THREE

Soil enzyme activities and microbial community structure after 9 years of perennial energy crops: Impact of *Miscanthus* and giant reed

Based on: Cattaneo, F., Di Gennaro, P., Barbanti, L., Giovannini, C., Labra, M., Moreno, B., Benitez, E., Marzadori, C., 2014. Ten-year perennial energy cropping systems affect soil enzyme activities and bacterial community structure in a South European agricultural area. Applied Soil Ecology (submitted)

ABSTRACT

In the recent decades perennial rhizomatous grasses have been introduced in the Po Valley (Northern Italy), not only for their energetic potential, but also to face the loss of soil organic carbon, largely due to anthropic practices. As literature data especially concern Northern Europe and continents as North America and Africa, we investigated an experimental site in Southern Europe, under a Mediterranean temperate climate, to improve the level of information on these crops' potential in different agro-climatic conditions. Also, there is a need for sustainable production of bioenergy services research in the Mediterranean area, especially considering their fast expansion in the last years. The aim of this research was to evaluate changes induced by the introduction of perennial energy crops on soil microbial community structure and on some key aspects of soil functionality. We compared two 9-year old perennial energy crop systems, *Miscanthus* and giant reed, with two annual crop systems, established in the late 1960's.

The structure of the bacterial community was studied by denaturing gradient gel electrophoresis (PCR-DGGE), a profiling-sequence analysis of PCR-amplified 16S rDNA fragments. The functional aspects were investigated through the determination of three soil enzyme activities, involved in soil carbon, nitrogen and phosphorous cycles (β-glucosidase, urease and alkaline phosphatase, respectively). Introduction of perennial energy crops positively stimulated soil enzymes, especially in the shallow layer (0-0.15 m), where storage of carbon and nitrogen was stronger. Enzyme activities were also positively correlated to organic carbon, especially in soils under perennial species. A significant but weaker correlation was also observed with total nitrogen. The DGGE profiles revealed the relationship between land use systems (annual *vs* perennial) and soil microbial communities. Community richness was higher in perennial than in annual crops but no effect of soil depth was observed. In contrast, Shannon

index of diversity was not influenced by land use system, but only by soil depth, with a 32% increase in the top layer.

Results highlight that the introduction of perennial energy crops in a South European agricultural area increases both soil biochemical activity and microbial diversity, thanks to the absence of soil disturbance and to the ability of these crops to stabilise SOM in soil. This is relevant mainly because this geographical area is notoriously characterised by a rapid turnover of organic carbon and nitrogen. Moreover, perennial energy crops could represent a sustainable choice for the recovery of soils exhausted by intensive agricultural management.

INTRODUCTION

Perennial energy crops, such as giant reed (*Arundo donax* L.) and *Miscanthus (Miscanthus × giganteus* Greef and Deuter), have been proposed as potential biofuel feedstocks in alternative to fossil fuels, because of their annual biomass production. The interest in these crops as a component in a portfolio of climate mitigation measures is promoting their diffusion in many agricultural areas of the world (Sims et al., 2006; Rechberger and Lötjönen, 2009; IEA, 2013).

The introduction of perennial species is expected to take place at the expense of the traditional annual crops with consequent changes in applied agricultural techniques. Unlike annual species, the need for soil tillage in perennial crops is limited to the year of the establishment. The ecological advantages of the long periods without tilling are reduced risk of soil erosion and a likely increase in soil carbon content (Kahle et al., 2000; Ma et al., 1999). Furthermore, due to the recycling of nutrients by their rhizome systems, perennial grasses have a low demand for nutrient inputs (Christian et al., 1997; Barbanti, 2014) and N fertilisation (Fazio and Monti, 2011). Since they have few natural pests, they may also be produced with little or no pesticide use (Lewandowski et al., 2000).

As shown by previous studies, these aspects could have a direct impact on SOC accumulation with subsequent influence on microbial structure community and functioning and soil biogeochemical cycles of the elements, particularly C and N (Hakey et al., 2010; Mao et al., 2011; Piotrowska and Wilczewski, 2012). As reported by Paul and Clark (1996), the deep rooting system and the higher root density of perennial species allow to allocate more resources to belowground organs (rhizomes) and to maintain more complex food webs and, in turn, may accommodate a larger population of beneficial microorganisms.

Perennial crops are typically harvested after aboveground biomass has senesced, leaving stems and leaves accumulating on soil surface as pre-harvest losses, hence causing high SOC input (Beuch, 1999). The incorporation of plant litter by the soil fauna is a major source of SOC, and the perennial nature of these species not only allows for senescence leading to higher litter input, but it has also been shown that the reduced disturbance has a positive impact on the soil fauna hence enhancing litter incorporation further (Chan, 2001; Van Eekeren et al., 2008; Ernst et al., 2009).

Compared to this, fewer information, concerning the impact of perennial energy crops on soil microbial functionality, is available. There is previous evidence that perennial crop residues, including plant root exudates, may either stimulate or inhibit the growth and activity of different fractions of soil microbial community; thus the planting of different crops may result in distinct microbial communities (Mao et al., 2011).

Identifying land use effects on soil microbiota, together with the knowledge of community-specific functions, can improve the understanding of fundamental controls on communities and their processes (Liang et al., 2012). This knowledge is crucial for the development of sustainable agriculture, as soil microorganisms represent an integrative component of soil quality because of their involvement in many ecosystems processes (i.e. SOM decomposition and nutrient cycling, N_2 fixation, aggregate formation and stabilisation) (Schutter et al., 2001).

Due to this reason and to its quick adaptability to changes of environmental conditions, soil microbiota represents an early and sensitive indicator of soil quality changes (Schloter et al., 2003, Dick, 1992; Bending et al., 2004; Winding et al., 2005). In both the US and in Europe, there are various candidate perennial grasses available which differ considerably in their potential productivity, chemical and physical properties of their biomass, environmental demands and crop management requirements.

As the first research on perennial grasses began in the humid-continental environments of North America and then in the Atlantic and Continental regions of Northern Europe, fewer information is available for Mediterranean-temperate areas of Southern Europe where average temperatures, annual precipitation, moisture and global radiation are different.

As affirmed by Lewandowski et al. (2003), the wide range of climatic conditions from Northern to Southern Europe influences yields of these perennial crops but also the soil C sink potential.

On these bases, the present research was carried out in the South-East of the Po Valley (Northern Italy), identified as part of the Mediterranean North environmental zone (Metzger et al., 2005). This agro-ecosystem is characterised by low SOC stocks (<10 g C kg^{-1} up to 0.30 m; ARPA, 2009), largely due to anthropic practices as intensive tillage, a strong specialisation of cropping systems and a concurrent decline of the livestock farm activity. We investigated the changes occurred on soil microbiota and its biological activity after 9 years of perennial energy crops, *Miscanthus* (*Miscanthus × giganteus* Greef and Deuter) and giant reed (*Arundo donax* L), replacing two 40-years annual arable systems.

The microbial community was studied in terms of structure and richness, while the functional aspects were investigated through the determination of three soil enzymes, involved in soil carbon, nitrogen and phosphorous cycles (β-glucosidase, urease and alkaline phosphatase, respectively).

MATERIALS AND METHODS

EXPERIMENTAL SITE

The field experiment was located at the experimental farm of the University of Bologna (Italy), in Cadriano (44° 33’ N, 11° 21’ E, 32 m a.s.l.), with mean annual precipitation and temperature of 700 mm and 13.3 °C, respectively. The area is identified as part of the Mediterranean North environmental zone, implying mild winter and long growing season, in exchange for precipitation mostly concentrated in the cold semester (Metzger et al. 2005). The soil at this site has a clayey-loamy texture. Continuous wheat (*Triticum aestivum* L.) and a maize/wheat crop rotation (*Zea mays* / *Triticum aestivum* L.) are the two annual systems which have been replaced, after 40-years, with the perennial energy crops, *Miscanthus* (*Miscanthus × giganteus* Greef and Deuter) and giant reed (*Arundo donax* L.), respectively.

Table 8 Physical and chemical characterisation of the studied soils under perennial (*Miscanthus* and giant reed) and annual crop (continuous wheat and maize/wheat rotation) systems. Soil analysis according to the Italian method D.M. 13/09, 1999.

	Soil	
	Perennial energy crops	**Annual arable crops**
pH $_{H2O}$	7.6	6.1
Texture (%)		
Sand	26.7	34.5
Silt	45.3	42
Clay	28	23.5
Limestone (%)		
total	1.7	< 0.5
active	1.6	< 0.1
C_{ORG} (g kg^{-1})	12.5	6.9
N_{TOT} (g kg^{-1})	1.5	0.9
C:N	8.3	8
Available P (mg kg^{-1})	19.4	26.4

A chemical and physical characterisation of the studied soils under perennial and annual crops was carried out, according to the current Italian methods of soil analysis (D.M. 13/09 1999). Results were averaged for crop type because very similar; they are reported in Table 8.

CROP MANAGEMENT AND EXPERIMENTAL LAYOUT

Two replicates from a field experiment, arranged in a completely randomised design, were used in the present research.

Continuous wheat and maize/wheat rotation are the two annual systems that were established in experimental plots in the late 1960's. *Miscanthus* and giant reed plots were established in 2002 in soils previously cultivated with annual C_3 and C_4/C_3 species, respectively.

Endemic to East Asia, *Miscanthus* is adapted to warmer climates but it has shown a good adaptability to the climatic conditions of Central and Southern Europe. European research has focused on the genotype *Miscanthus × giganteus*, which is a sterile, triploid interspecific hybrid. Its sterility necessitates vegetative propagation by rhizome division or in vitro cultures (Angelini et al., 2009).

Native from East Asia, giant reed is widely diffused in Mediterranean environment where it is frequently found in riparian habitats. It is reported that giant reed is an asexual reproductive species, due to seed sterility (Bhanwra et al., 1982). Usually this crop does not set fruit because the pollen results unfruitful; consequently, the better propagation method is the use of rhizomes (Angelini et al., 2009).

In both annual and perennial crops 0 (N_0) and 120 (N_{120}) kg ha^{-1} of mineral N, supplied as urea, were applied annually during the spring time. Moreover, 50 kg P_2O_5 ha^{-1}, as triple superphosphate, were applied every year in annual crops. In *Miscanthus* and giant reed, 200 kg P_2O_5 ha^{-1} were added prior to planting in spring 2002. No K fertiliser was applied, given the sufficient level of this nutrient in the soil.

Tillage at 0.30 m was carried out every year in annual crops; only prior to planting (autumn 2001) in perennial ones. In both annual and perennial species, the rest of crop husbandry reflected the normal practice followed in the experimental area. In particular, perennial crops were always harvested every year in the early autumn (September 25 – October 15); annual crops were harvested at maturity (wheat, early summer; maize, late summer) and the recoverable fraction of their residues (wheat straw and maize stover) was always removed from the field.

Soil sampling took place in March 2011 at the beginning of the 10th growing season, using a 85 mm diameter soil corer up to a depth of 0.60 m, divided in three soil layers (0-0.15, 0.15-0.30, 0.30-0.60 m). For each crop two replicates were taken. Each replicate was composed by three subsamples, which were put together and transported back to the laboratory the same day.

Soil samples for microbiological analysis were subsequently sieved (2 mm mesh sieve), plant organic material as leaves, stubbles and litter were removed and samples were stored at -20 °C. Soil samples for chemical analysis (SOC; TN; P_{AVAIL} contents; microbial biomass carbon, C_{mic}) were also sieved as described above but then air-dried outdoors.

TOTAL C, TOTAL N AND AVAILABLE P

Soil samples were air-dried and finely ground before analysis for total soil organic C (SOC) and N (TN) The C and N contents of soils were directly measured with an elemental analyser (CHNS-O mod. EA 1110, Thermo Fischer, Germany) using acetanilide as a standard for C and N concentration. As traces of carbonates were detected in the studied soils, samples were pre-treated with acid. An aliquot of soil (10 - 13 mg) was weighed into silver capsules and, after adding a few drops of 6 M HCl, heated to 80°C on a heating plate until dry. Then the capsules were closed and analysed in the elemental analyser. Soil available P (P_{AVAIL}) was measured by the Olsen method (Olsen and Sommers, 1982). For each sample 2 g of soil were dispersed in 0.5 M $NaHCO_3$ (pH 8.5), extracted on a horizontal shaker for 30 min and finally filtered through Whatman no.42 filter paper and analysed for the available P content with an inductively coupled plasma mass spectrometry (ICP-OES, Spectro Arcos, Ametek, Germany).

MICROBIAL BIOMASS CARBON

Microbial biomass carbon (C_{mic}) was determined using the chloroform fumigation extraction method (Brookes et al., 1985; Vance et al., 1987). The equivalent of 10 g of oven-dried soil was fumigated with ethanol-free chloroform for 24 h at 25 °C, in a desiccators, in the dark. Fumigated and unfumigated samples were dispersed in 40 ml of 0.5 M K_2SO_4 and extracted on a horizontal shaker at 250 rev min^{-1} for 30 min. Extracts were filtered through Whatman no. 42 filter paper and analysed 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.

SOIL ENZYME ACTIVITIES

β-glucosidase (GLU) activity was determined using 1 g of soil (dry weight, d.w.), according to Eivazi and Tabatabai (1988). Absorbance was measured at 400 nm and activity expressed as μg PNG g^{-1} h^{-1}.

Urease activity (UR) was determined using 3 g of soil (d.w.), according to Kandeler and Gerber (1972). The absorbance was measured in the supernatant at 600 nm and the enzyme activity was expressed as mg $N\text{-}NH_4^+$ g^{-1} h^{-1}.

Alkaline phosphatase (P_{AL}) activity was performed according to Eivazi and Tabatabai (1977), using 1 g of soil (d.w.) and measuring the absorbance at 400 nm. Phosphatase activity was expressed as mg PNP g^{-1} h^{-1}.

SOIL DNA EXTRACTION

Based on the results of soil enzyme activities, the DNA was extracted only from soil samples taken at the 0-0.15 and 0.30-0.60 m depths. Before DNA extraction, soil samples were homogenized with pestle and mortar. Four 1 g subsamples (dry weight, d.w.) of each replicate were used for DNA extraction by the bead-beating method, following the manufacturers' instructions of the MoBio

UltraClean Soil DNA Isolation Kit (MoBio Laboratories Inc., Solana Beach, California, USA) with a few modifications, including the repetition of the second step (Inhibitor Removal Solution) to remove trace concentrations of PCR inhibitors. Then the DNA samples were checked for concentration and quality using the NanoDrop ND-1000 Spectrophotometer (NanoDrop® Technologies, Wilmington, Delaware, USA).

PCR-DGGE ANALYSIS

PCR on the DNA samples was performed with the 16S rDNA universal bacterial denaturing gradient gel electrophoresis (DGGE), with primers 907R (5'-CCGTCAATTCCTTTGAGTTT-3') and GC-341F (P3) (5'-CCTACGGGAGGCAGCAG-3') (TIB®MOLBIOL, Berlin, Germany) to amplify the V3–V5 hypervariable regions of 16S rDNA genes. Primer P3 contains the same sequence as 341F but with an additional 40-nucleotide GC-rich sequence (GC clamp) at its 5' end (Yu and Morrison, 2004).

The PCR programme was performed as described by Vivas et al. (2009) with some modifications. It was initiated by a hot start of 9 min at 95 °C; after 1 min of initial denaturation at 94 °C, a touchdown thermal profile protocol was used, and the annealing temperature was decreased by 1 °C per cycle from 65 °C to 55 °C. Amplification was carried out with 1 min of denaturation at 94 °C, 1 min of primer annealing at 55 °C and 1.5 min of primer extension at 72 °C, followed by 10 min of final primer extension. The total reaction mixture of the first PCR consisted of 25 µl containing: a range of extracted DNA concentrations approximately between 4.5 ng and 5.5 ng; 2.5 µl of 10X PCR Buffer; 0.75 µl $MgCl_2$ 50 mM; 2.5 µl dNTPs 10 mM; 1 µl primer P3 10 µM and 1 µl primer 907R 10 µM; 0.15 µl 0.025 U/µl BIOTAQ DNA and sterile Milli-Q water to a final volume.

PCR products were analysed by electrophoresis in 2% agarose gel to select the best amplified DNA concentrations (ng).

The second amplification was performed using the selected DNA concentrations tested in the first PCR as template to a final volume of 50 µl. In this amplification, primers P3 and 907R were used under the same conditions as described above.

Then PCR products were purified by "DNA Clean & Concentrator™-5 Kit" (Zymo Research Corp., California, USA) and checked for concentration and quality using the NanoDrop ND-1000 Spectrophotometer (NanoDrop® Technologies, Wilmington, Delaware; USA).

DGGE analysis was conducted using 550 ng of concentrated PCR products and loaded into a 30%-55% urea-formamide-polyacrilamide gel. Electrophoresis was performed at 200 V for 10 min and then at 75 V for 18 h at 58 °C, using an INGENYphorU System (Ingeny International BV, The Netherlands). Gel was silver stained with the Bio-Rad Silver Stain, according to the standard DNA-staining protocol and then photographed.

STATISTICAL ANALYSIS

Normal distribution and equal variance of data were controlled through the Kolmogorov-Smirnov and Bartlett test, respectively. Data were then submitted to the analysis of variance (ANOVA) through the CoStat 6.3 software (CoHort Software, Monterey, California - USA), according to a completely randomised factorial scheme: in each trait the significance of the investigated sources (crops, soil depths and their interaction) was determined. The Student - Newman-Keuls (SNK) test at $P \leq 0.05$ was adopted to separate means of statistically significant sources.

Data were also analysed by the orthogonal contrasts method (or single degree of freedom procedure) (Snedecor and Cochran, 1980), to test selected effects addressing specific combinations of crop groups (perennial *vs* annual) and soil depths (shallow *vs* deep).

The structural diversity of microbial community was examined by the Shannon diversity index (H') and by the community richness (R). The intensity of bands was reflected as peak heights in the densitometric curve. Community richness was evaluated by the presence or absence of the band in the DGGE analysis. The

H' value was calculated from the number of bands and their relative intensities in each lane, with the following equation:

$$H' = -\Sigma(\mathrm{P}i \times \log \mathrm{P}i)$$

where $\mathrm{P}i = ni/N$; ni is the height of peak and N is the sum of all peak heights in the curve.

To test if the similarities observed within and between samples were larger or smaller than those expected by the different treatments, band matching data were stored as a binary matrix and analysed using Raup and Crick's probability-based index of similarity S_{RC} (Raup and Crick, 1979). S_{RC} indicates the probability that the randomised similarity would be larger than or equal to the observed similarity, and S_{RC} values above 0.95 or below 0.05 signify differences, which are not random assortments of the same species (bands or OTUs) (Rowan et al., 2003).

Pearson's correlation (r) was used to study the relationships among soil enzyme activities, biochemical properties and biodiversity indices.

RESULTS

SOIL ENZYME ACTIVITIES

Soil enzyme activities were significantly influenced by crops, soil depths and their interaction (Table 9). Giant reed particularly stimulated GLU, achieving a 65% higher activity than continuous wheat. Likewise, *Miscanthus* boosted UR and P_{AL} activities, attaining +250% and +130% than maize/wheat rotation and wheat, respectively.

The shallow layer (0-0.15 m) consistently showed a higher enzyme activity: +106%, +22% and +52% in GLU, UR and P_{AL}, respectively, than the deep layer (0.30-0.60 m).

Table 9 Soil enzyme activities depending on crops, soil depths and their interaction. GLU, β-glucosidase; UR, urease; P_{AL}, alkaline phosphatase.

Sources	**GLU (μg PNG g^{-1} h^{-1})**	**UR (mg N-NH^{4+} g^{-1} h^{-1})**	**P_{AL} (mg PNP g^{-1} h^{-1})**
Crop (C)			
Miscanthus	30.04 ab	50.88 a	79.03 a
Giant reed	39.34 ab	36.16 b	56.79 b
Wheat	24.64 b	31.52 b	34.01 c
Maize/Wheat	30.49 ab	14.45 c	62.13 b
P	0.024*	< 0.001***	< 0.001***
Depth (D; m)			
0-0.15	52.14 a	37.49 a	72.01 a
0.15-0.30	23.21 b	32.25 b	52.82 b
0.30-0.60	25.27 b	30.79 b	47.25 b
P	< 0.001***	0.006**	< 0.001***
P (C × D)	< 0.001***	0.145 ns	0.001***

ns, *, ** and *** mean non-significant and significant at $P \leq 0.05$, ≤ 0.01 and ≤ 0.001, respectively. Different letters indicate significantly different means (SNK test; $P \leq 0.05$).

GLU and P_{AL} also exhibited a significant interaction between the two factors considered, indicating a positive effect of perennial species in the topsoil (Fig. 12), especially in the case of *Miscanthus* with P_{AL}, and giant reed with GLU activity.

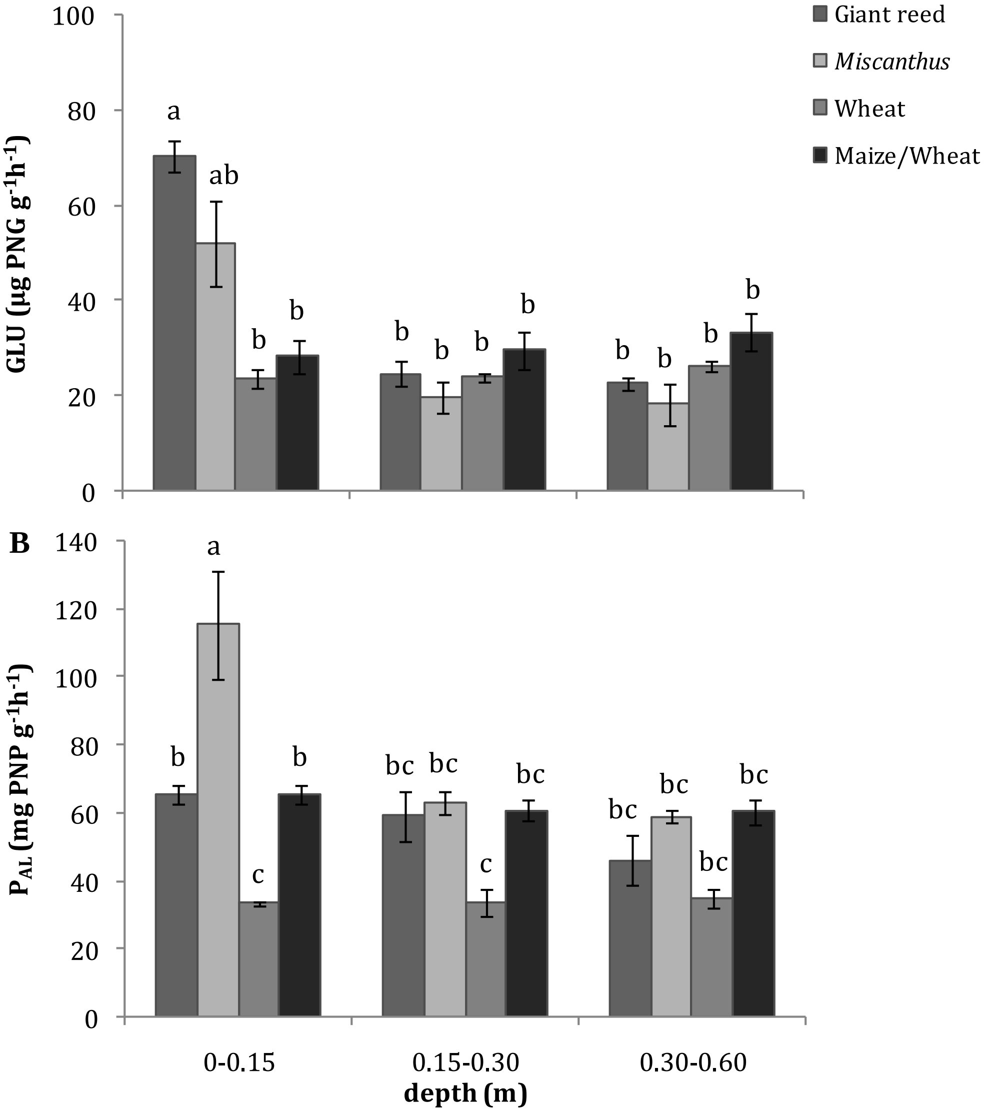

Fig. 12 Significant crop × depth (C × D) interaction of β-glucosidase (GLU) (**A**) and alkaline phosphatase (P_{AL}) (**B**). Different letters indicate significantly different means (SNK test; $P \leq 0.05$). Vertical bars represent ± standard errors (n= 3).

The effect of land use systems (perennial *vs* annual) and soil depths (shallow *vs* deep layer) was highlighted by the linear contrasts test (Table 10).

The group composed by the two perennial species displayed a 36%, 77% and 32% increase in GLU, UR and P_{AL}, respectively, over the group represented by the two annual crops. Regardless of the crop system, in the shallow layer a 115%, 19% and 44% increase in the three respective enzymes was observed over the two combined deep layers (0.15-0.60 m). As a result, the effect of perennial over annual species appeared further enhanced in the shallow layer (+167%, +101% and +76% in the three respective enzymes).

Table 10 Most relevant orthogonal contrasts of soil enzyme activities between crop types, soil layers and their combinations. A, annual crop systems (continuous wheat and maize/wheat rotation); P, perennial energy species (*Miscanthus* and giant reed); GLU, β-glucosidase; UR, urease; P_{AL}, alkaline phosphatase.

Orthogonal contrast	**Enzyme activity**		
	GLU (μg PNG g^{-1} h^{-1})	**UR (mg N-NH^{4+} g^{-1} h^{-1})**	**P_{AL} (mg PNP g^{-1} h^{-1})**
A *vs* P	27.5 *vs* 37.5*	22.9 *vs* 40.5**	48.1 *vs* 63.5**
0-0.15 *vs* 0.15-0.60	52.1 *vs* 24.2**	37.4 *vs* 31.5**	72.0 *vs* 50.0**
(A *vs* P) $_{0\text{-}0.15}$	26.0 *vs* 69.5**	23.3 *vs* 46.9**	49.4 *vs* 87.1**

* and ** mean significant at $P \le 0.05$ and ≤ 0.01, respectively.

Significant correlations were evinced between enzyme activities and chemical properties. In fact, GLU, UR and P_{AL} were positively correlated to SOC (r= 0.56**, 0.82*** and 0.75***, respectively) (Fig. 13), because of the role of perennial crops and the shallow soil layer in enhancing both SOC and enzyme activities.

There was also a positive correlation between GLU, UR and P_{AL} activities and TN (r= 0.50*, 0.72*** and 0.42*, respectively) (Fig. 14), although weaker than those with SOC (Fig. 13). However, it is still possible to observe the combined effect of perennial crops and the shallow layer (0-0.15 m) in enhancing TN and enzyme activities. UR was the enzyme showing the best correlations with both SOC and TN.

In contrast, no correlation was observed between enzyme activities and P_{AVAIL} (data not shown).

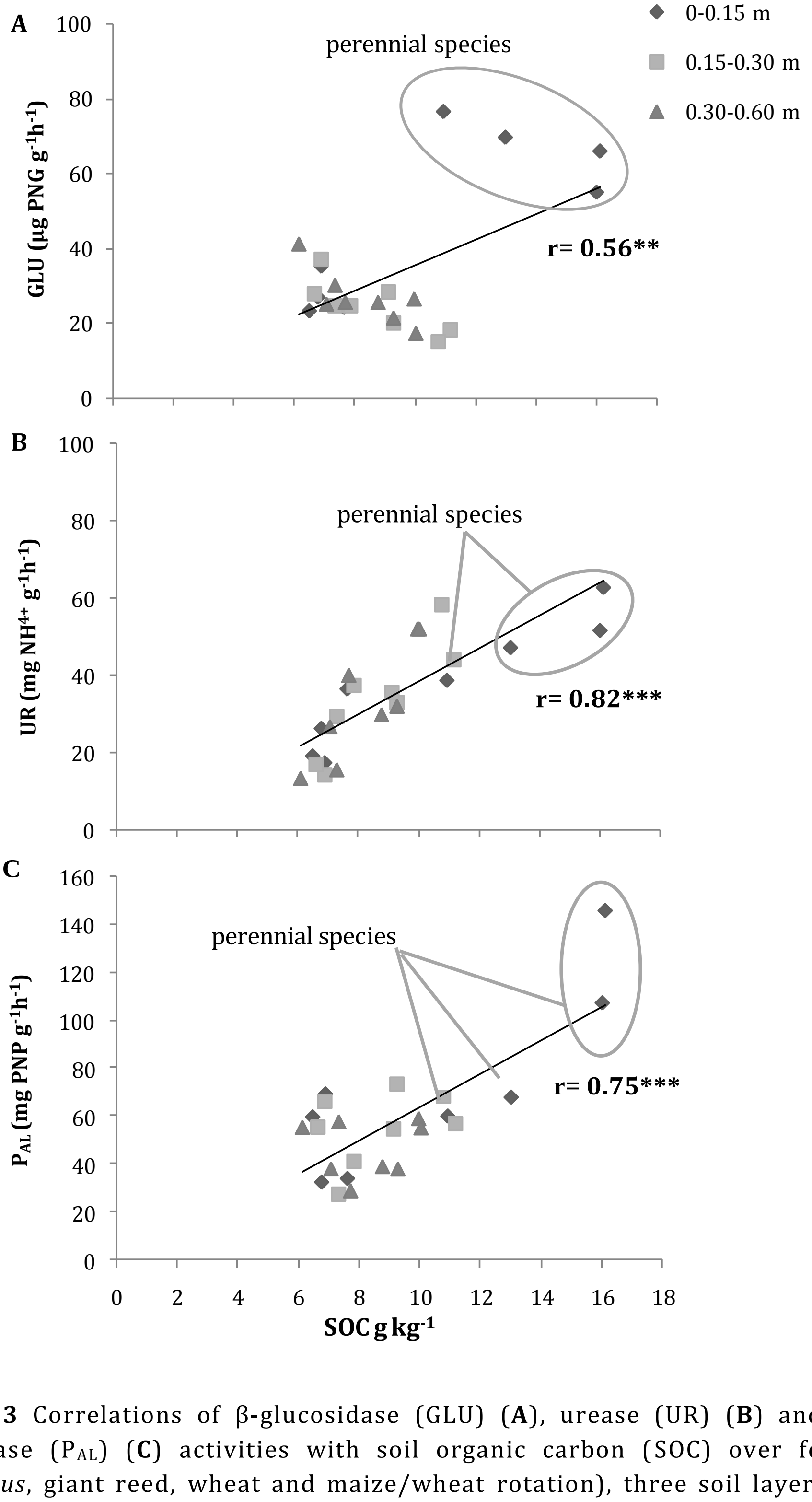

Figure. 13 Correlations of β-glucosidase (GLU) (**A**), urease (UR) (**B**) and alkaline phosphatase (P_{AL}) (**C**) activities with soil organic carbon (SOC) over four crops (*Miscanthus*, giant reed, wheat and maize/wheat rotation), three soil layers (0-0.15, 0.15-0.30 and 0.30-0.60 m) and two replicates (**= *P* ≤0.01; ***= *P* ≤0.001) (n= 24).

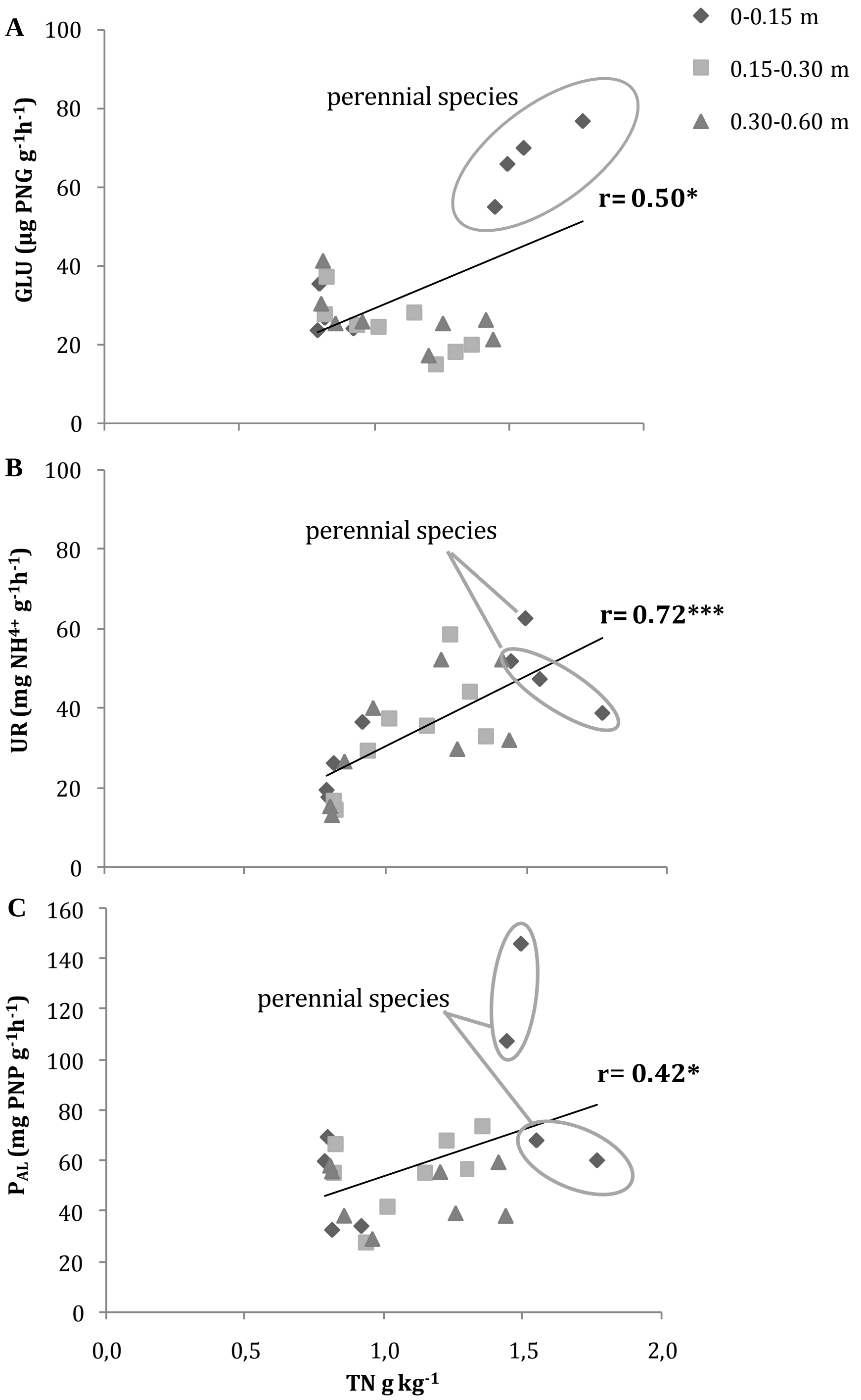

Figure 14 Correlations of β-glucosidase (GLU) (**A**), urease (UR) (**B**) and alkaline phosphatase (P_{AL}) (**C**) activities with soil total nitrogen (TN) over four crops (giant reed, *Miscanthus*, wheat and maize/wheat rotation), three soil layers (0-0.15, 0.15-0.30 and 0.30-0.60 m) and two replicates (*= $P \leq 0.05$; ***= $P \leq 0.001$) (n= 24).

The activity of soil enzymes was also analysed in connection with microbial biomass carbon (C_{mic}). C_{mic} content was significantly correlated with UR and P_{AL} activities, exhibiting respective correlation coefficients (r) of 0.59** and 0.75***, and weakly correlated with GLU (r= 0.47*), as shown in Table 11. In all correlations between C_{mic} and enzyme activities, the strong effect of *Miscanthus* on microbial biomass C in the topsoil (0-0.15 m) is particularly evident (Fig. 15).

Table 11 Matrix of correlations (r) between soil enzyme activities, chemical properties and microbial biomass carbon in the three soil depths (0-0.15; 0.15-0.30 and 0.30-0.60 m) (n = 24). GLU, β-glucosidase; UR, urease; P_{AL}, alkaline phosphatase; SOC, soil organic carbon; TN, total nitrogen; P_{AVAIL}, available phosphorus; C_{mic}, microbial biomass carbon.

	GLU	***UR***	***P_{AL}***	***SOC***	***TN***	***P_{AVAIL}***	***C_{mic}***
GLU	1						
UR	0.20 ns	1					
P_{AL}	0.56**	0.44*	1				
SOC	0.56**	0.81***	0.75***	1			
TN	0.50*	0.72***	0.42*	0.81***	1		
P_{AVAIL}	-0.50*	-0.41 ns	0.21 ns	-0.29 ns	-0.62**	1	
C_{mic}	0.47*	0.59**	0.75***	0.79***	0.52*	0.01 ns	1

ns, *, ** and *** mean non-significant and significant at $P \leq 0.05$, ≤ 0.01 and ≤ 0.001, respectively.

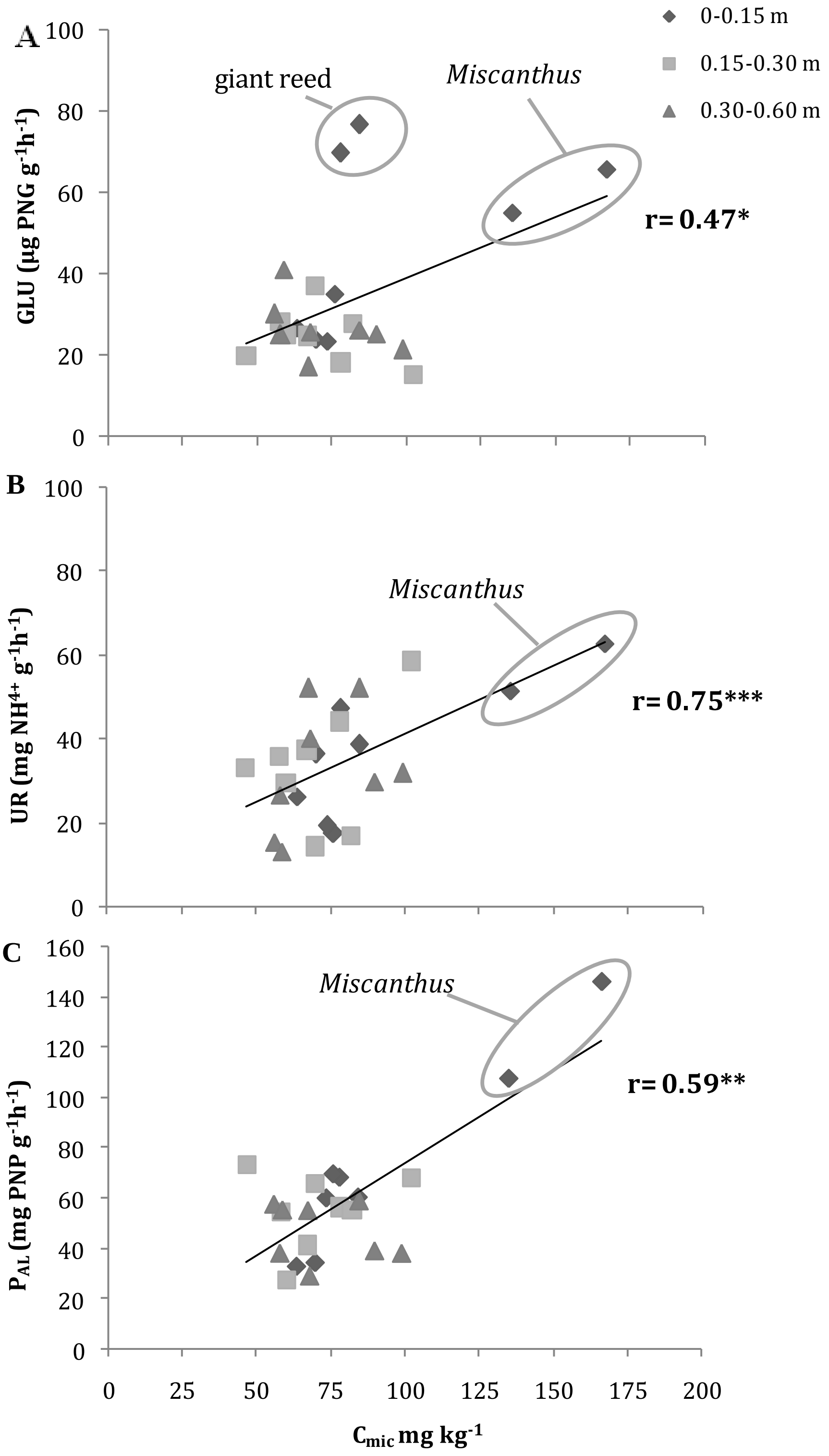

Figure 15 Correlations of β-glucosidase (GLU) (**A**), urease (UR) (**B**) and alkaline phosphatase (P_{AL}) (**C**) activities with microbial biomass carbon (C_{mic}) over four crops (giant reed, *Miscanthus*, wheat and maize/wheat rotation), three soil layers (0-0.15, 0.15-0.30 and 0.30-0.60 m) and two replicates (*= $P \leq 0.05$; ***= $P \leq 0.001$) (n= 24).

PCR-DGGE ANALYSIS

Comparison of bacterial communities between soils under perennial and annual crops showed quite markedly different profiles across the 16S rDNA-DGGE gel (Fig. 16). The resulting genetic profile, representing the community structure, is characterised by rare intensity and relatively few DGGE bands. Nevertheless, it is possible to observe the main differences between perennial and annual crop soils in the high part of the DGGE gel.

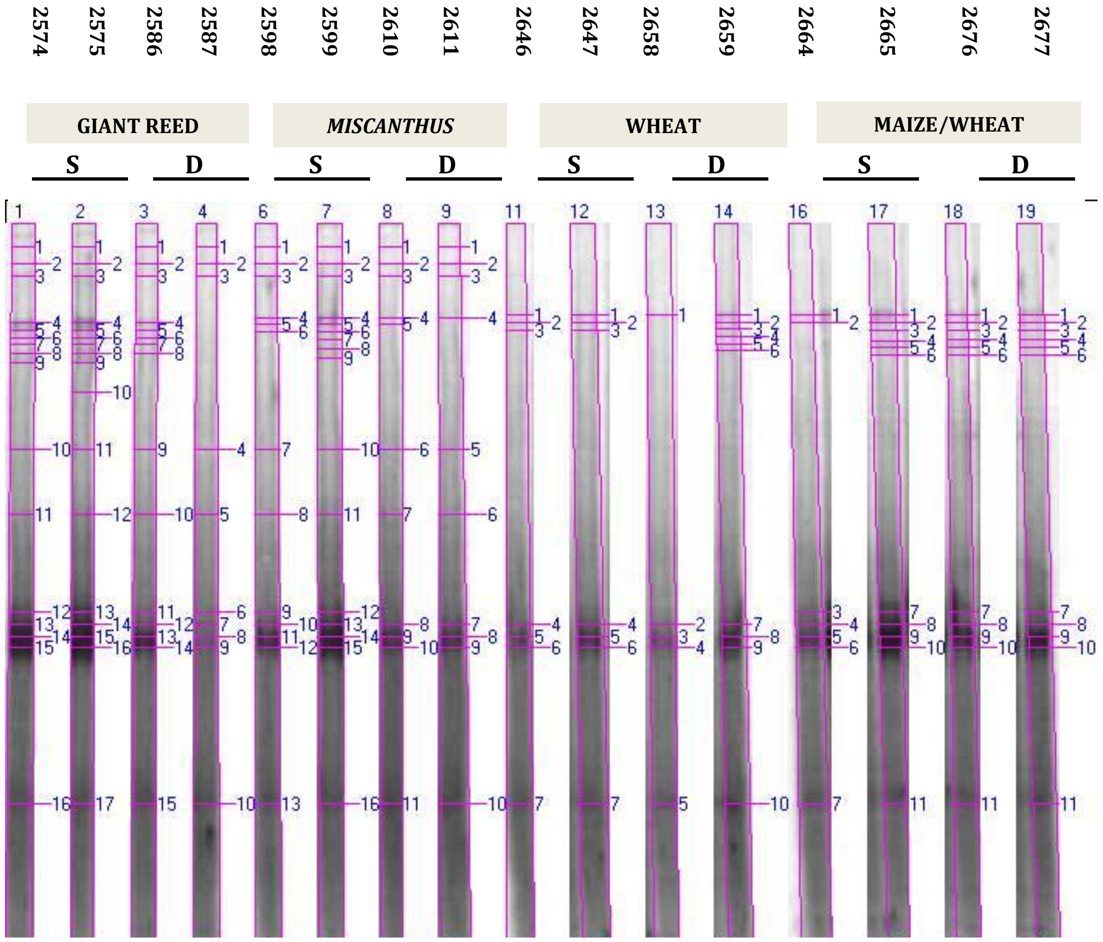

Figure 16 PCR-DGGE patterns of V3-V5 hypervariable regions of 16S rDNA of soil microbial community in giant reed, *Miscanthus*, wheat and maize/wheat rotation over two soil layers (S-shallow layer, 0-0.15; D-deep layer, 0.30-0.60 m) and two replicates (samples 2574-2575, 2586-2587 for giant reed; 2598-2599, 2610-2611 for *Miscanthus*; 2646-2647, 2658-2659 for wheat; 2664-2665, 2676-2677 for maize/wheat rotation).

The structure of bacterial community was influenced by the investigated factors to a variable extent (Table 12). Community richness (*R*) showed a significant crop effect, evidencing higher values in perennial than in annual crops. Conversely, the effect of soil depth was negligible. By contrast to this, *H'* was not significantly influenced by crop species, whereas the shallow soil layer exhibited a 32% higher value over the deep layer. In both *R* and *H'*, the crop × depth interaction was not significant.

Table 12 Community richness (*R*) and Shannon index of general diversity (*H'*) for DGGE (Denaturing Gradient Gel Electrophoresis) profiles, depending on crops, soil depths and their interaction.

Sources	*R*	*H'*
Crop (C)		
Miscanthus	10.00 a	1.95
Giant reed	12.25 a	2.16
Wheat	5.50 b	2.11
Maize/Wheat	7.00 b	2.07
P	0.016*	0.853 ns
Depth (D; m)		
0-0.15	9.37	2.38 a
0.30-0.60	8.00	1.80 b
P	0.282 ns	0.009**
P (C × D)	0.500 ns	0.462 ns

ns, * and ** mean non-significant and significant at $P \leq 0.05$, ≤ 0.01 and ≤ 0.001, respectively.

Figure 17 shows the dendrogram generated by the Raup and Crick cluster analysis. Similarities within replicates were significant in all cases (S_{RC}=1, data not shown). Soils managed with the same crop system (perennial *vs* annual) proved significantly similar (S_{RC}>0.95). By contrast, in the pair-compared DGGE profiles, a significant dissimilarity (S_{RC}<0.05) was observed between *Miscanthus* and giant reed profiles in contrast with wheat and maize/wheat rotation.

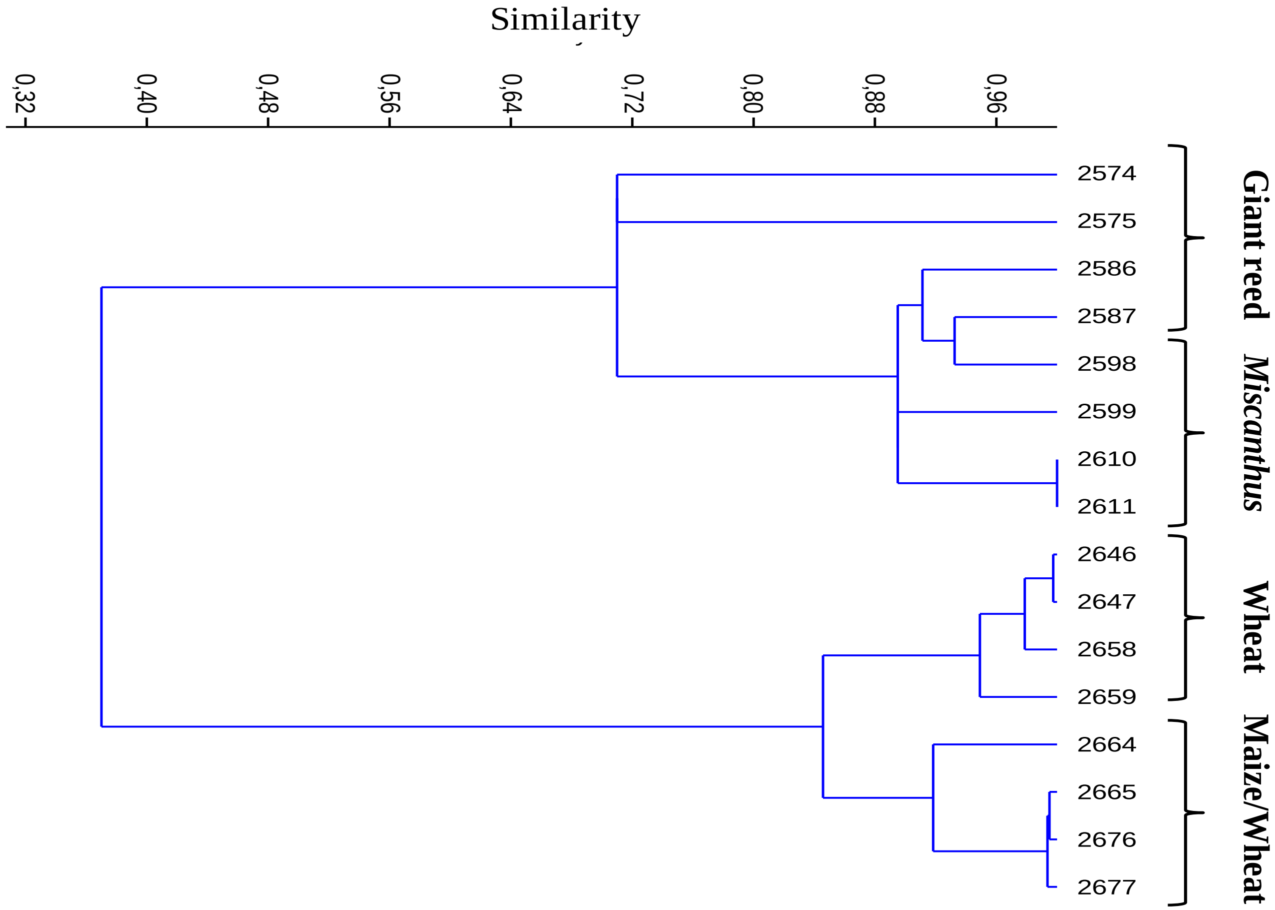

Figure 17 Raup and Crick probability-based index of similarity cluster analyses for DGGE profiles.

Richness of microbial species, deriving from DGGE profiles, was then correlated with the enzyme activities and the chemical properties of soil determined in this research. The intention was to detect the effect of perennial energy crops on the potential relation between soil bacteria diversity and biochemical functions. This approach is based on the relationships between microbial diversity and soil functionality, considering that most of processes in soil are mediated by bacteria. Correlations are summarised in Table 13.

As shown in Figure 18, community richness (*R*) was directly correlated with GLU activity (r= 0.62**) and also with SOC and TN (r= 0.65** and 0.73**, respectively).

In contrast, community richness was not well correlated with UR and P_{AL}. Figure 18 also illustrates how richness was distributed through soil layers (0-0.15 and 0.30-0.60 m), showing an evident gap established by perennial crops in the topsoil (0-0.15 m), relative to the deeper layers.

Table 13 Matrix of correlations (r) between soil enzyme activities, chemical properties and biodiversity indices through the topsoil (0-0.15 m) and the deeper layer (0.30-0.60 m). GLU, β-glucosidase; UR, urease; P_{AL}, alkaline phosphatase; SOC, soil organic carbon; TN, total nitrogen; P_{AVAIL}, available phosphorus; *R*, community richness; *H'*, Shannon index of diversity.

	GLU	*P_{AL}*	*UR*	*SOC*	*TN*	*P_{AVAIL}*	*R*	*H'*
GLU	1							
P_{AL}	0.64*	1						
UR	0.38 ns	0.52*	1					
SOC	0.68**	0.80***	0.83***	1				
TN	0.64**	0.42 ns	0.73**	0.79***	1			
P_{AVAIL}	-0.19 ns	0.21 ns	-0.39 ns	-0.30 ns	-0.65**	1		
R	0.62**	0.40 ns	0.39 ns	0.65**	0.73**	-0.49 ns	1	
H'	0.57*	0.33 ns	0.01 ns	0.25 ns	0.08 ns	-0.01 ns	0.29 ns	1

ns, *, ** and *** mean non-significant and significant at $P \leq 0.05$, ≤ 0.01 and ≤ 0.001, respectively.

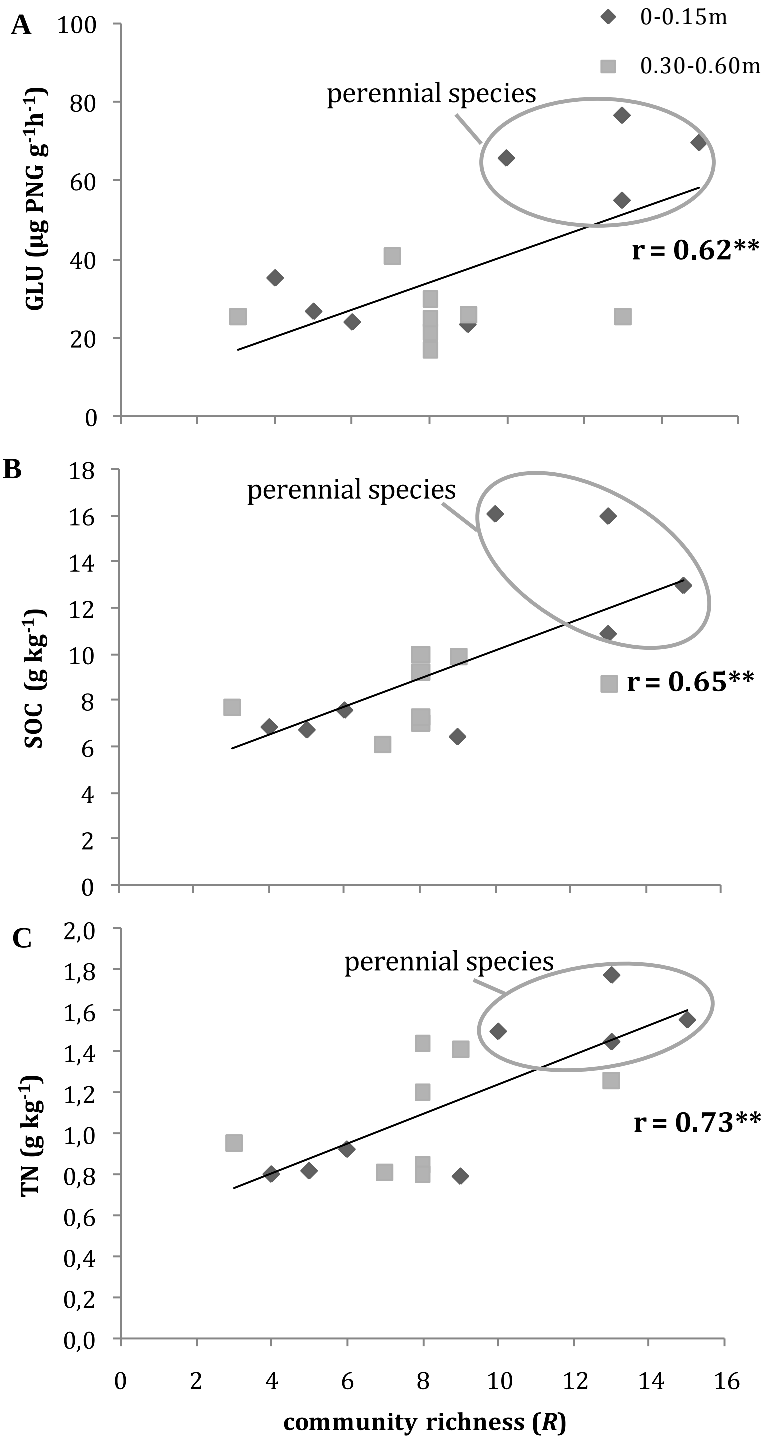

Figure 18 Correlations of β-glucosidase activity (GLU) (**A**), soil organic carbon (SOC) (**B**) and total nitrogen (TN) (**C**), with community richness over four crops (giant reed, *Miscanthus*, wheat and maize/wheat rotation), two soil layers (0-0.15 and 0.30-0.60 m) and two replicates (*= $P \leq 0.05$; **= $P \leq 0.01$; ns= non significant) (n= 16).

DISCUSSION

The land conversion from annual arable crops to perennial energy grasses positively influenced the three soil enzymes surveyed in this research. The effect was particularly evident in the shallow layer where soil C input is mostly determined by turnover of large roots and harvest losses, and where microorganisms are most active. The positive effect is likely due to the different agricultural management of perennial species: unlike annual crops, the need for soil tillage in perennial grasses is limited to the year in which these crops are established. The reduced soil disturbance by tillage could reflect in increased soil C storage thanks to reduced microbial oxidation under conditions of lower aeration (King et al., 2004).

Soil organic carbon and nitrogen are among the most important factors that may considerably influence the activity of soil enzymes (Gianfreda and Bollag, 1996). It is well known that soil enzymes are early indicators of changes in soil physical and chemical properties (Amador et al., 1997), induced by tillage (Carter, 1986; Powlson et al., 1987; Friedel et al., 1996; Salinas-Garcia et al., 1997), fertilisation regimes (Giacometti et al., 2013), vegetation changes (Waldrop et al., 2000; Sinsabaugh et al., 2002), disturbance (Bolton et al., 1993; Eivazi and Bayan, 1996; Garcia and Hernández, 1997; Boerner et al., 2000) and plant succession (Tscherko et al., 2003).

Results confirm this assumption, showing that, limited to the upper soil layer, a remarkable stimulation of GLU, UR and P_{AL} activities was linked to the introduction of plants involving low or no tillage as perennial crops. A high activity of soil enzymes may indicate insufficient nutrient supply for microorganisms, but the significant correlation of GLU, UR and P_{AL} activities with microbial biomass C and SOC allows to suppose that this is not the reason. On the contrary, the amount of SOC and microbial biomass C, observed in soils under *Miscanthus* and giant reed, may have caused the high activity of GLU, UR and P_{AL}.

A similar result, comparing perennial and annual cropping systems, has been previously reported by Dick et al. (1996). We observed this occurrence in a South European site, notoriously characterised by a rapid turnover of organic carbon and nitrogen, thus hampering their stabilisation in soil.

Based on soil enzyme activity data, we addressed soil microbial community structure and distribution considering the top (0-0.15 m) and the deep (0.30-0.60 m) layers as the two opposite cases in a gradient of richness distribution. In this frame, the microbial community structure was examined by using the bacterial DNA profiles, produced by 16S rDNA-DGGE. DGGE banding data were used to estimate the diversity indices (R and H'), by treating each band as an individual operational taxonomic unit (OTU). Moreover, because it is not possible to be sure whether the different H' index reflects differences in species richness, species evenness or simply sampling differences, the H' and the Raup and Crick's probability-based index of similarity (S_{RC}) were combined.

Previous works (De Deyn et al, 2010; Liang et al., 2012) indicated that soil microbial communities are differentially influenced by host plant species and by other microorganism communities of varying diversity. Soils of different types, harbouring specific microbial communities, are also assumed to exert a selection as shown in a continental-scale study by Fierer and Jackson (2006). In agreement with these authors, data of R and H' provide evidence that the characteristics of the bacterial community have changed after 9 years of *Miscanthus* and giant reed, introduced in the agricultural management of the study site. Dendrogram generated by Raup and Crick's probability-based index of similarity cluster analysis from PCR-DGGE profile reinforced this assumption (Fig. 17).

Community richness is positively correlated with SOC and TN contents, suggesting possible effects of the host plants and of the reduced disturbance characterising perennial crops management on soil microbiota. One mechanism by which plants and plant communities may exert selection on soil microbial community is by modifying resource availability. Richness is consistent with a mechanism in which plants impact soil microbial communities through the quantity of resources provided. In fact, provision of C substrates via rhizodeposition is commonly assumed to be a primary mechanism for plant-driven impacts on soil microbial communities (De Deyn et al., 2010).

In this research, it is necessary to consider the significant contribution in terms of aboveground biomass, that typically characterises perennial crops, in the form of stem and leaves accumulated on soil surface as pre-harvest losses (Anderson-Teixeira et al., 2009). In spite of all evidence, supporting a picture of general advantage from enhanced soil microbial community, data of this research reveal a lack of relationship between bacterial diversity (R and H' indices) and two of the three enzyme assayed (UR and P_{AL}). The approach is based on the potential relationships between microbial diversity and soil functionality, considering that 80 - 90% of the processes in soil are reactions mediated by microbes (Nannipieri et al., 2003; Caldwell, 2005).

However, it is perceived as soil enzymes are a key point in the evaluation of biological soil quality, as described by Burns et al. (2013): extracellular enzymes represent the pre-requisite for the success of microorganisms that rely on the degradation of polymeric substrates and must prioritise carbon and nutrient allocation to enzyme production, in order to prevent starvation. The weak correlations may be explained by the fact that a considerable part of soil enzymes is present in protected extracellular form and therefore their activity is not always directly correlated with soil microbial populations (Burns et al., 2013; Boyd and Mortland, 1986; Burns, 1986; Dick and Tabatabai, 1987).

At the same time, a good correlation between enzyme activities and SOC was found in this research and this is consistent with the assumption that soil organic matter is one of the main factors involved in soil protection of extracellular enzymes (Burns et al., 2013).

CONCLUSION

In their work Liang et al. (2012) showed that microbial communities were considerably impacted by cropping system, with a significantly lower microbial biomass in conventional maize than in mixed prairie.

In this research we can confirm how, in a land use change from arable cropping to perennial species, the choice of crop types, and their consequently agricultural management, is crucial in determining structural and functional aspects of soil microbial community. Integrating soil ecosystem with perennial rhizomatous grasses increases the biochemical activity of soil thanks to their ability to sequester and stabilise organic carbon in soil, along with the reduced disturbance of tillage. This results in less aeration and lower decomposition rates of organic matter.

Along with other studies demonstrating the potential of perennial energy crops in terms of enhanced chemical (carbon and nitrogen) (Dondini et al., 2009; Mao et al., 2011) and biological properties (soil enzymatic activity and microbial community structure), this research supports the role of these crops as a sustainable option for the recovery of soils depleted by long periods of intensive management also in Southern Europe.

GENERAL CONCLUSIONS

This long-term field experiment involved a change in land use system from 40 years of annual arable crops (continuous wheat and a maize/wheat rotation) to 9 years of perennial biomass crops (*Miscanthus* and giant reed). To date, these two species have mainly been studied in view of their conversion into various forms of bioenergy (heat and power, biogas and 2nd generation biofuels). Hence, the net energy yield and environmental impact associated with their cultivation have especially been focused in previous research dealing with *Miscanthus* and giant reed. Conversely, a large uncertainty lies in the side effects determined by their cropping on soil properties in warm-temperate environments.

In fact, it has been frequently remarked in literature that perennial energy crops have a high carbon sequestration potential, due to high below-ground allocation to storage organs (rhizomes)and dense rooting, in association with undisturbed soil after tillage in the year of establishment. There are many studies on soil organic carbon potential of perennial crops, especially *Miscanthus*, carried out in Atlantic and Continental zones of North-Central Europe. In contrast, this research has been performed in a South European site, the Po Valley, classified as part of the Mediterranean North environmental zone (Metzger et al., 2005). This agro-ecosystem is generally characterised by low soil organic carbon, due to intensive anthropic practices and higher temperatures hampering carbon storage. Responding to these constraints, this thesis addresses the carbon sequestration potential of *Miscanthus* and giant reed under these climatic conditions and, what is more, investigates in detail the newly sequestered carbon distribution in soil aggregates and the changes occurred in soil microbial communities, after perennial energy crops introduction.

Applying the ^{13}C natural abundance analysis, combined with soil physical fractionation, has made it possible to precisely determine the portion of total soil organic carbon derived from *Miscanthus* and giant reed in 9 years of cropping , as well as its allocation trend, by quantifying organic carbon in the three soil fractions. Results show that soil organic carbon is significantly higher in the whole investigated profile (0-0.60 m) under perennial crops, compared to the respective soils under annual species. Besides the two land use systems (annual *vs* perennial species), differences have been observed between the two perennial crops, reflecting their fibrous root biomass: *Miscanthus* appears to have a greater carbon accumulation potential, but limited to the topsoil (0-0.15 m), while giant reed can store more soil carbon in deeper soil layers (0.15-0.60 m).

Soil physical fractionation shows that, 9 years after planting *Miscanthus* and giant reed, the majority of newly sequestered carbon is found in the relatively labile particulate organic matter (POM). Even in this case, differences in the distribution of total organic carbon have been evidenced between *Miscanthus* and giant reed: the former represents a more preservative system of old derived carbon in all fractions, while the latter can store a higher content of new carbon in the stable and protected pools (IALF and MF). The amount in the macroaggregates under perennial species is likely a result of both lower soil disturbance, carried out only in the year of the establishment, and their developed root biomass that entails a greater C storage potential. Less disturbance leads to a reduced aggregate turnover of soil organic matter and to a consequent formation of stable organo-mineral complexes between mineral particles and microbial materials. This explains why, in opposition to long-term conventional tillage, long-term no-till as in perennial energy crops can maintain more carbon in soil by having positive effects also on soil microbial communities.

Considering that different management practices may result in variable soil organic carbon contents and also in different soil microbial communities, a biomolecular approach, based on soil DNA extraction and PCR-DGGE, was used for soil community analysis, in terms of species richness estimate. However, this approach only informs about bacteria presence in soil; it cannot give information about gene expression, which is important to understand bacterial activities in soil, such as their growth, degradation of various compounds, and responses to environmental factors. Further insight could be provided by the study of the small subunit ribosomal RNA gene through real-time PCR (RT-PCR), which is considered able to improve the knowledge on bacterial responses to treatments or natural changes in the soil environment.

In conclusion, in the frame of a sustainable agriculture with an increasing commitment to environment protection, the use of *Miscanthus* and giant reed to counter soil organic carbon loss and restore soil fertility, appears a suitable solution in the warm-temperate areas of Southern Europe. As it was previously stated, these perennial energy crops can, not only recover the carbon loss due to previous soil disturbances, but enhance the soil carbon sink potential, storing significant newly derived carbon portions, with a considerably smaller environmental impact than annual crops.

Nevertheless, the "future of soil organic carbon research" requires collaboration and communication between the scientific community and the "practice sector", facilitated by individuals with hard knowledge and social intelligence. In order to be applicable to the practice sector, new findings in soil organic carbon dynamics need to be addressed in a conceptual framework to communicate the necessity for change as being "compatible", "observable", "achievable" and foremost "economical" (Robertson et al., 2012).

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RINGRAZIAMENTI

Grazie al Prof. Marzadori per avermi dato l'opportunità di realizzare questo progetto, permettendomi di svilupparlo, non solo seguendo ambiti per me del tutto nuovi, ma anche secondo i miei personali interessi.

Grazie ai miei correlatori.
Grazie alla Dott.ssa Di Gennaro per l'entusiasmo e la disponibilità mostrati fin dagli inizi di questa collaborazione, e soprattutto durante i momenti più difficoltosi.
Grazie al Dr. Barbanti per avermi supportata e sopportata in questi tre anni. Grazie per le consulenze statistiche e metodologiche che mi hanno aiutata a proseguire il mio progetto sempre meglio e con maggior competenza.

Grazie al Prof. Emilio Benítez per avermi accolta nel proprio laboratorio presso l'Estación Experimental del Zaidin, durante i miei 6 mesi trascorsi a Granada, mettendo a disposizione materiale e attrezzature.
Grazie alla Dott.ssa Beatriz Moreno per avermi aiutata nello svolgimento delle analisi biomolecolari, insegnandomi nuove tecniche e dimostrandosi un valido esempio da seguire per perfezionare il mio metodo di lavoro.

Grazie ai colleghi del gruppo dell'Area di Chimica Agraria.
In particolare, grazie a Paola per le analisi alla massa, per l'aiuto nel frazionamento fisico e per la preziosa consulenza durante la stesura degli articoli.
Grazie a Luigi , soprattutto per l'amicizia.

Grazie di cuore ai miei genitori per essere sempre in prima fila a fare il tifo per me. Grazie per gli scossoni arrivati sempre al momento giusto, quando lasciarmi andare allo sconforto sarebbe stata la via più facile; grazie per credere in me così smisuratamente.

Grazie ad Andrea per non avermi mai fatto sentire in colpa per le mie scelte, spesso difficili e faticose da condividere...grazie per aver invece scelto di viverle insieme a me, sostenendomi con forza e amore.

Grazie agli amici di una vita per aver pazientemente aspettato il mio ritorno e salutato ogni mia partenza, standomi sempre accanto: Valentina, Fabio, Sabrina, Daniele.

Grazie a Laura e Danila per la semplicità e la forza con cui non smettete mai di esserci, seppur la distanza spesso sia davvero fastidiosa.

Gracias à Teresa, Teresita, Viviana, Arnaud y Maykel, mi segunda familia que conoció viviendo en Granada. Los seis meses que pasé con vosotros han sido entre los màs hermosos de mi vida.

Grazie agli amici che Bologna mi ha regalato, per le esperienze condivise, per la leggerezza e per aver reso questa città ancor più bella di quanto già non sia: Stefy, Caterina, Elisabetta, Michela, Denise, Danilo...grazie.