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SOIL CARBON CYCLE AND SUSTAINABILITY OF ORGANIC FERTILIZATION IN A MEDITERRANEAN SHORT ROTATION WOODY CROPS

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ABSTRACT

Short Rotation Woody Crops (SRWC) is a method of cultivation of selected material of fast growing species (poplar, willow, eucalyptus, black locust) to produce in short time large quantity of biomass for energy purposes (calorific or/and electric energy). In a SRWC very high plant density per hectare is used (from 5-10,000 to 15,000 plants per hectare), the rotation age is 2-3 years. It is estimated that a SRWC *Eucalyptus* requires an average of at least 100 kg of N year, thus the use of irrigation and fertilization to meet the demand of this type of crop is not feasible because is a very uneconomic crop. Irrigation can be used as a rescue intervention and inorganic fertilizer at planting and after the cuts. For this reason, the fertilization with manure, where available, may be an attractive option because allows to properly dispose of the sewage and to give to the plant nutrients necessary for growth.

The European Union Directive 91/676/EEC, known as Nitrates Directive, has dictated basic agronomic principles regarding the use of animal manure source, zoo-technical and waste waters from small food companies.

The use of nitrification inhibitors together with animal effluents may be beneficial for nutrient recycling, soil quality, plant productivity and greenhouse gas emission, and offers economic advantages to make it an alternative to conventional fertilizers.

The aim of this research was to investigate the sustainability of organic fertilization combined with a nitrification inhibitor in relations to carbon dynamics.

Were compared the effectiveness of the nitrification inhibitor 3,4 dimethyl pyrazole phosphate (DMPP), together with the addition of fresh organic matter (bovine effluent, BE) to the soil of a short rotation forestry.

The results of microcosm experiments suggested that the combined use of DMPP and BE could be a good solution to limit the drastic effect of BE application on soil biological properties and microbial dynamics. The preliminary study of field application of DMPP and BE evidenced that this strategy of fertilization could be also a good solution to reduce the ammonia and carbon dioxide emissions and to improve the productivity in SRWC agro-ecosystems of Mediterranean environment and preserve the soil functions.

PREFACE

This research is the results of a collaboration between the University of Tuscia and the Agriculture Research Council, Research Center for the Soil-Plant System (CRA-RPS) with the economic support of K+S Nitrogen and Timac Italy to the doctoral fellowship.

This thesis is divided into 6 chapters, the first is an introductory one that explains the features of SRWC in the Mediterranean environment and the related environmental issues with particular attention to soil management. In the second section, all materials and methods used are described: site description, laboratory techniques and field analysis, and last the statistical methods data processing. The Chapters 3, 4, 5, 6 resumed the experimental works and have been written with the intent to submit three different publications. In the first part of the research (Chapters 3 to 5) a microcosm experiment was conducted to determine the biochemical and microbial response to the treatments. The analysis of biochemical indicators were carried out at Agricultural Research Council (CRA), Research Center for the soil-plant system, Roma (RPS) under the Supervision of Dr. Dell'Abate, while the microbial growths and structural analysis were carried at University of Lund under the Supervision of Prof. E. Bååth. The aim of second part of my research was to investigate significant differences of treatments with bovine manure with and without addiction of a nitrification inhibitor, 3,4 DMPP (Nib) on CO_2 and NH_3 emissions and plant productivity. The field experiment was conducted at the intensive Eucalyptus crop of CRA-PLF under the Supervision of Dr. Mughini and the supervision of Dr. Luca Salvati essential for the processing data (Chapter 6).

CHAPTER 1: INTRODUCTION

1.1 Short Rotation Woody Crops (SRWC) in Mediterranean Countries

In European Countries, trials on willows grown in short rotation for the purpose of biomass production initiated in the 1980s. Commercial willow plantations are grown in a short rotation system on 15,000 ha in Sweden, while in Poland the planted area is about 6,800 ha and in Germany less than 1,000 ha. In general, this kind of short rotation coppice consists of fast growing trees and is characterized by higher wood productivity in time and space than conventional cultivated forests, due to high juvenile growth rates of the trees. Short Rotation Woody Crops (SRWC) are mainly grown for producing wood fuel for heat and power production. In the last years, the cultivation of fast growing species was boosted thanks to the increasing interest in biomass aimed to energy production. These species are characterized by very short turns, 2-4 years, with high density spacing (from 5-10,000 to 15,000 plants/ha) *(De Franchi et al., 2010).* The most used species are poplar (*Populus nigra, Populus alba*), eucalyptus (*Eucalyptus camaldulensis*), willow (*Salix cinerea, Salix alba*), and false acacia (*Robinia pseudoacacia*) which all are characterized by fast juvenile growth, often with the capacity for asexual reproduction and an ability to re-grow from rootstocks or stools. The plantations are established at high densities on arable land in spring and harvested in winter during vegetation dormancy when the ground is frozen. In Mediterranean Countries where the climate is the limiting factor for the crop growth, it is particularly used the Eucalyptus as a species adaptable to these conditions.

Prior to the plantation establishment, chemical or mechanical weed control is needed to minimize competition for resources and thereby allows for vigorous growth of the planted crop *(Larsson et al., 1991).* In many sites, especially in Central Europe, fertilization is not needed if the plantation is established on former arable land. When plantations are fertilized with sewage sludge, which is common in Sweden, the plantations act also as vegetation filters. The demand for wood as a renewable resource for energetic use is currently

increasing due to increasing energy use, the decline of fossil fuels and increasing energy prices. Thus, short rotation forestry for energy purposes is rapidly expanding in Europe in the last years because of the reduced dependence on foreign sources of energy, the availability of large areas of set aside land, and because of the environmental benefits, being CO_2-neutral and giving low sulfur emissions compared to fossils fuels.

If plants are grown in unfit locations and under the difficult Mediterranean climate, carrying out adequate trials in order to avoid dangerous expectations by farmers is particularly important and the *Eucalyptus* is a species adaptable to the Mediterranean climates.

Lately, especially in hilly and mountain environments, there is large extension of soils which are semi-abandoned, arid, consumed by intensive cropping, salty, strongly sloping, hardly accessible, etc.. In these cases, the limiting factors can be chemical, physical, topographic or climatic and for these soils, no more used for agriculture or pasture, biomass production can be a good way to avoid their abandonment. However, only a few of these soils are able to support intensive SRWC practices.

To increase production in quantity and quality, in the mid and long terms, profitability and environmental sustainability are research strategies in Mediterranean environment. In Central Italy precipitation during the growth season is variable with an average annual rainfall of about 755 mm *(Bergante et al., 2010)* which implies that water availability may be a crucial growth factor at many willow sites and for the sustainability of the system.

Moreover, irrigation is not believed to be economically and environmental viable, although it is well documented that SRWC has a high water demand *(Lindroth et al., 1996; Hunt et al., 1997).* Instead, SRWC should be planted on soils that can adequately supply the trees with water.

Much of the current concerns about environmental effects of short rotation forestry relate to nutrient removals in harvest. In a conventional forestry context, this concern is entirely appropriate. The whole tree harvesting together with the short harvest cycles, often with leaves intact, result in a nutrient depletion rate that is far greater than for conventional forest harvests.

Research studies show that quantities of nutrients removed from SRWC harvest vary with yield, species of tree or clone and time of harvest *(Ciancio et*

al., 2000). However, SRWC trees are usually harvested throughout the year, including when leaves are present. If leaves are removed from the site, nutrient removals will be significantly greater than these numbers. The quantity of nutrients removed with leaves will vary depending on time in the growing season and degree of removal of leaves from the site.

Nutrient removal in the above study was related to yield, but clones varied substantially in their efficiency of nutrient use in terms of unit of woody biomass produced per unit of nutrient consumed. Few data have been reported on nutrient losses in SRWC plantations other than for removals in harvest. Such losses can occur from leaching, erosion and in the case of nitrogen, from de-nitrification processes. In SRWC, most of these losses, except for de-nitrification, are likely to occur in early years of establishment and following harvest because of lack of soil protection during these periods and the absence of nutrient uptake by the crop *(Hardcastle, 2006).*

However, inappropriate fertilization with N fertilizer can result in high nitrate levels below the rooting zone of young, shallow-rooted SRWC species. The nitrification potential of the soil is a critical factor governing the quantity of nitrogen in water following application of urea and ammonium sources of N.

In SRWC, soil availability and quality play a crucial role because biomass production clearly clashes with the classical agricultural productions *(Bisoffi et al., 2000; De Franchi et al., 2010).*

To satisfy the need for agro ecosystem sustainability and the demands of society, most cropping system must evolve toward more environmentally friendly practices.

It can be concluded that soil management in SRWC is likely to be a problem and must be considered in planning all field activities to assure high production, to minimize production costs and to limit off-site effects of fertilizers application, so it needs to study strategies appropriate to improve soil fertility and preserve water for irrigation..

1.2 The EU Normative

When high quantities of nutrients from sewage or fertilizers contaminate water bodies, they can cause eutrophisation. This describes the excessive growth of

weeds and algae that choke and discolour the waters, disrupting normal ecosystems and depriving fish of oxygen *(Hunt et al., 1997).*

The "Nitrates Directive" was promulgate in the 1991 and is one of the earliest pieces of EU legislation aimed at controlling pollution and improving water quality. While nitrogen is a vital nutrient that helps plants and crops to grow, high concentrations are harmful to people and nature. The agricultural use of nitrates in organic and chemical fertilizers has been a major source of water pollution in Europe. For the first time mineral fertilizer consumption registered a progressive reduction in the early 1990s and stabilized during the last four years in the EU-15, but across all 27 Member States nitrogen consumption has increased by 6%. Generally, farming remains responsible for over 50% of the total nitrogen discharge into surface waters *(EU Nitrate Directive* 91/676/EEC*).*

The European Union Directive 91/676/EEC knows as Nitrates Directive, has dictated basic agronomic principles regarding the use of animal manure source, zoo-technical and waste waters from small food companies. The Directive allows for Member States to get derogations to go beyond the 170 kg N ha^{-1} limit, under strict conditions. They have to demonstrate that they can meet the Directive's objectives by improving other measures and reducing nutrient losses in other ways. They must offer objective justifications for using higher quantities of manure than correspond to 170 kg nitrogen per hectare per year, which are allowed under the Directive: for example, long growing seasons, crops with high nitrogen uptake, high net precipitation or exceptional soil conditions. The derogation is granted through a Commission decision, following a positive opinion from the Nitrates Committee.

The recycling of animal manures from intensive zoo-technical industries in the agro-ecosystem could increase soil organic matter and nutrient contents, and help to solve the environmental and economic problems related to the disposal of these waste materials *(Plaza et al., 2004; Melero et al., 2006),* especially in Mediterranean areas, characterized by a low organic matter contents.

The application of zoo-technical waste needs appropriate treatments before it is applied to soil, to preserve the soil and the ground water from pollution, such as nitrate leaching. In fact, uncontrolled application of slurry to soil can generate an excess of nitrates in the soil and consequently in the ground water,

salts, trace metal, undesirable xenobiotic organic compounds from agricultural source and green house emissions.

The application of fresh animal manure may produce diverse effects on soil properties including the modification of the composition, size and activity of soil *microflora* and in the extracellular enzymes activities.

As a matter, ammonia present in, or mineralized from manure, can rapidly be nitrified. This process continues during all seasons. The nitrification leads to production of nitrous oxide, that has a detrimental effect on the ozone layer and contributes to the greenhouse effect, and to nitrate formation which can be leached eventually leading to nitrate concentrations in groundwater exceeding the European Community standard for drinking water (*Schoder et al., 1993).*

1.3 The soil C cycle

The carbon cycle summarizes the fluxes of carbon among four main reservoirs: fossil carbon, the atmosphere, the oceans, and the terrestrial biosphere. Soil contains the largest near-surface reservoir of terrestrial carbon and so knowledge of the factors controlling soil carbon storage and turnover is essential for understanding the carbon global cycle *(Neff et al., 2002).* Seventy-four percent of the Earth is covered with water, but only three percent is fresh and 80 to 90 percent of that fresh water is used for irrigation *(Gleick et al., 1997).* Organic matter in the topsoil helps it hold a greater amount of water which can lessens the need for irrigation. Tests have shown up to 70 percent less in some cases. In the 1994 Intergovernmental Panel Assessment on Climate Change (IPCC), an effort was made to improve the quantification of terrestrial exchanges and potential feedbacks from climate, changing CO_2, and other factors.

The release of CO_2 through respiration is not unique to plants, but is something all organisms do, including microscopic organisms living in soil. When dead organic matter is broken down or decomposed (consumed by bacteria and fungi), CO_2 is released into the atmosphere at an average rate of about 60 PgC/year globally. Because it can take years for a plant to decompose (or decades in the case of large trees), carbon is temporarily stored in the organic matter of soil.

When viewing the Earth as a system, these components can be referred to as carbon *pools* (sometimes also called *stocks* or *reservoirs*) because they act as storage houses for large amounts of carbon. Any movement of carbon between these reservoirs is called a *flux*. In any integrated system, fluxes connect reservoirs together to create cycles and feedbacks. An example of such a cycle is seen in Figure 1.1, where carbon in the atmosphere is used in photosynthesis to create new plant material. On a global basis, this process transfers large amounts of carbon from one pool (the atmosphere) to another one (plants). Soil organic matter is a major terrestrial pool of C, and the cycle and the availability of this element is constantly being altered by microbial mineralization and immobilization processes. Inorganic constituents of soil play a major role in retaining cations and non polar organic compounds and anions. The original sources of soil organic matter are plant tissues, whereas animals are secondary sources of organic matter. In a mature natural ecosystem or in a stable agro-ecosystem, the release of carbon as carbon dioxide by oxidation of soil organic matter (mostly by microbial respiration) is balanced by the input of carbon into the soil as plant residues (and, to a far smaller degree animal residues) *(Wick et al., 1998).* Organic matter in the soil reduces the need for fertilizers by holding the nutrients in a non-leachable form, making fertilizers less polluting and more efficient. Plants grow healthier with more production, and less need for pesticides *(Rengel et al., 1995).*

Over time, these plants die and decay, are harvested by humans, or are burned either for energy or in wildfires. All of these processes are fluxes that can cycle carbon among various pools within ecosystems and eventually release it back to the atmosphere. Viewing the Earth as a whole, individual cycles like this are linked to others involving oceans, rocks, etc. on a range of spatial and temporal scales to form an integrated global carbon cycle (Figure 1.2).

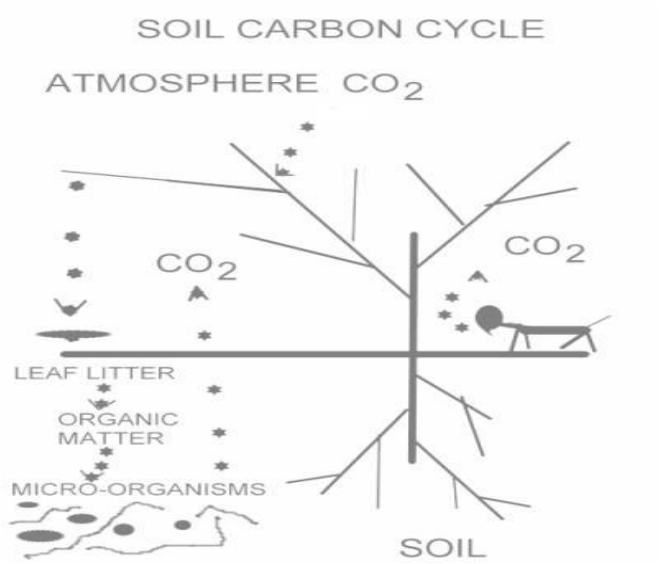


Fig 1.1 Carbon continuously moves between the atmosphere, plants and soils through photosynthesis, plant respiration, harvesting and decomposition.

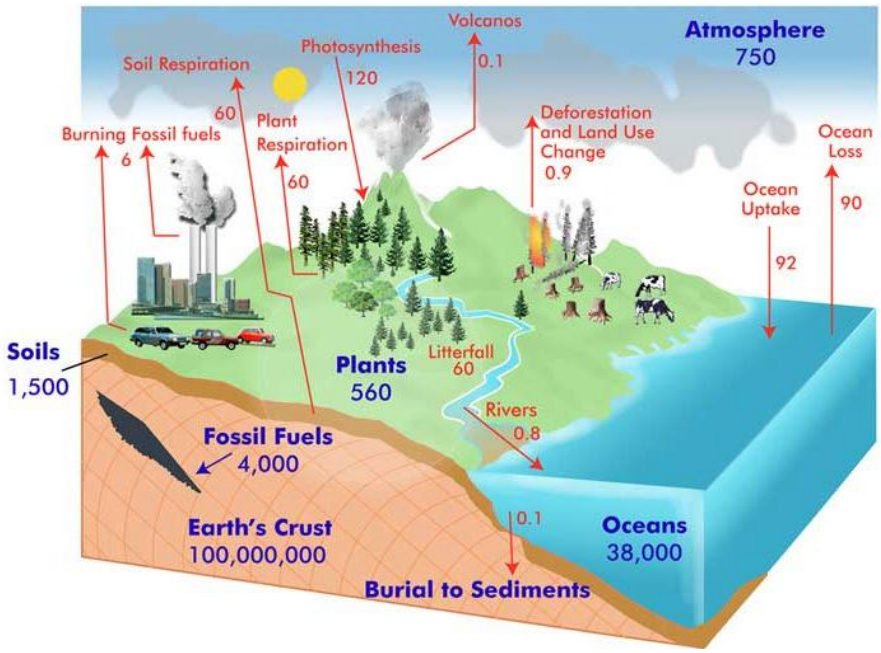


Figure 1.2. Global C cycle

Globally the release of carbon from soils into atmosphere is about 62 Pg/yr, while only about 60 Pg/yr enter the soils from the atmosphere The land use practices and fossil fuel burning contributed to increase from 290 to 370 ppm (the air concentration) during the past century alone the levels of carbon dioxide emissions *(Neff et al., 2002).*

The Earth's carbon reservoirs naturally act as both *sources*, adding carbon to the atmosphere, and *sinks*, removing carbon from the atmosphere. If all sources are equal to all sinks, the carbon cycle can be said to be in equilibrium (or in balance) and there is no change in the size of the pools over time. Maintaining a steady amount of CO_2 in the atmosphere helps maintain stable average temperatures at the global scale. However, because fossil fuel combustion and deforestation have increased CO_2 inputs to the atmosphere without matching increases in the natural sinks that draw CO_2 out of the atmosphere (oceans, forests, etc.), these activities have caused the size of the atmospheric carbon pool to increase *(Elliott et al., 1994).*

When the soils are added with fresh and decomposable tissues (often containing water soluble compounds like sugars, amino acids) an immediate increase in metabolic activity among the soil microbes is stimulated. In this case, carbon compounds are enzymatically oxidized to produce carbon dioxide, water, energy and decomposer biomass, moreover the essential nutrients such nitrogen and phosphorous are released and/or immobilized by specific reactions. In temporal terms there are two kinds of inputs of organics into soil: (i) one-time or occasional or (ii) permanent (continuous) *(Kuzyakov, 2010; Fontaine et al., 2003).* The pulse inputs are typical for the breakdown of microbial, root and animal cells, decomposition of above-ground litter with subsequent leaching of dissolved organic matter (DOM), and root exudation. Because of the ready availability of soluble organics, such inputs produce hotspots of microbial activity in which the turnover rates are much higher than they are outside of these zones. The continuous inputs is typical for the slow decomposition of dead roots, leaf and shoot residues, and for some rhizo-deposits. In all these cases, the substrates are less immediately metabolisable and, therefore, utilized slowly and over longer periods. Because of the low availability, it is likely that the array of extracellular enzymes generated to degrade these organics may be more efficient at decomposing SOM in comparison with the largely intracellular enzymes that breakdown the easily available substrates *(Fontaine et al., 2003).*

Abiotic factors such as temperature, soil moisture, and pH are main drivers of C turnover in soil and act indirectly mainly by affecting microbial activity that drives the mineralization of soil organic matter (SOM) and plant residues

(Cook et al., 2007). Beyond these abiotic factors, however, many biotic factors directly affect C mineralization in soil. The term priming effect (PE) was introduced to describe changes in the SOM decomposition effected by the addition of organic or mineral substances *(Jenkinson et al., 1985)*. These changes are due to changes in the microbial activity as a response to altered amounts and availability of C. There might be various mechanisms for the changes in microbial activity in soil after adding organic or mineral substances *(Fontaine et al., 2003).* Consequently, interest in the soil organic matter and its relationship to agricultural management has developed another important function of soil: its role in the worldwide C budget and climate change. The influence of the climate (moisture, temperature, radiation) on decomposition of soil carbon has been investigated but the effect of the increase in reactive nitrogen, coming largely from agricultural fertilizers, on the soil carbon dynamics remains uncertainty. Neff at al. (2002) used ^{14}C and ^{13}C to show that nitrogen addiction accelerates decomposition of light soil carbon fractions, but this result suggested that current models of terrestrial carbon cycling do not contain the mechanisms needed to capture the complex relationship between nitrogen availability and soil carbon storage. Human activity, in particular agricultural manage, fixes more N_2 into biologically available forms each year than all natural processes, causing a wide cascading environmental response**.** On average soil contains three times much C as does terrestrial vegetation and if changes N availability alters soil C turnover, net C sinks from increased plant growth could be significantly increased or reduced depending on the direction of the soil response. By increasing soil organic matter levels and labile C and stocks the nutrient supplying potential of soil will be improved and long term ecological sustainability may be economically achieved.

Land use change can induce emission or sequestration of carbon depending on a range of soil and environmental properties and land management practices. In agricultural soils, the carbon stock is affected by changes or management practices. Soil C changes can show negative or positive effects of different management which are strongly linked to microbial and enzyme activities because they regulate soil quality and functioning by their involvement in organic matter dynamics, nutrient cycling and decomposition processes *(Lagomarsino et al., 2010;Nannipieri et al., 2000).*

Soil Carbon dynamics are affected by microorganism through their involvement in organic decomposition and therefore any change in N inorganic content could affect soil C sequestration *(Nannipieri et al., 2000*).

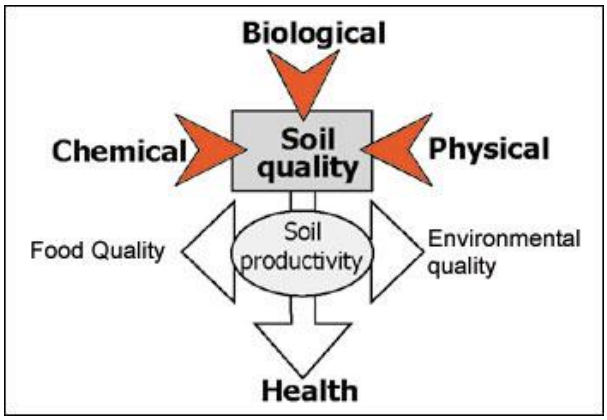
1.4 Soil quality

Soil is natural substrate for growth and productivity of most of the plants that live on the hearth because they get all the essential nutritional elements from it for their own development; consequently each nutritional element present into the soil in a bio-available form is potentially destined to entry in the animal and human food chain. To preserve the soil quality it will be important to assure the best management and with right amount of fertility elements in order to guarantee the best production.

The terms soil quality and soil fertility are currently used interchangeably in the scientific literature and popular press, the soil quality has a profound effect on the health and productivity of given ecosystem and the environments related to it. Soil quality has been difficult to define and quantify and several definitions more or less simply were proposed over the time. *Per* example for Power and Myers (1989) soil quality is the ability of soil to support crop growth which includes factors such a degree of this aggregation, organic matter content, soil depth, water holding capacity, pH changes, nutrient capacity. For Pierce and Larson (1991) soil quality is simply "Fitness for use".

How it is shown in the Figure 1.3 below, the major issues that define soil quality are:

- Productivity: The ability of soil to enhance plant and biological productivity.
- Environmental quality: The ability of soil to attenuate environmental contaminants, pathogens, and offsite damage.
- Animal health: the interrelationship between soil quality, plant, animal, and human health.



- **Fig 1.3. Properties for *Soil quality* concept**

The most common used soil quality definition in the last years was advanced by Doran and Parking in the 1993, how "the ability of soils to interact with the ecosystem in order to maintain the biological productivity, the environmental quality and to promote animal and vegetal health".

Soil is a dynamic, living, natural body that plays many key roles in terrestrial ecosystem. The components of soil include inorganic mineral matter (sand, silt, and clay particles), these matter are highly complex media with a diversity of substrates that vary in both the energy required for their breakdown and their total N content *(Barford et al., 2008).*

Agricultural development naturally takes place first on the best land. Whether at the scale of the individual farm or a whole country, the tendency is to use the best land first. When there is a need to increase agricultural production it is usually directed to maximize production in the areas which have the best potential. But as demand increases for the products of the land - food, fuel, shelter, and clothing - it is necessary to make increasing use of land which is less suitable for agriculture, or land in less favorable climates. There are always strong links between measures for soil conservation and measures for water conservation, and this applies equally in semi-arid areas. Many measures are directed primarily to one or the other, but most contain an element of both. Reduction of surface run-off by structures or by changes in land management will also help to reduce erosion. Similarly, reducing erosion will usually

involve preventing splash erosion, formation of crusts, breakdown of structure, thus increasing water infiltration, and so help the water conservation. Soil is a living organism and needs to be appreciated as such, as a dynamic system that works in concert with plants. In living soil around 100 elements and billions of microorganisms work together in synergy. Microorganisms are precisely what differentiates a living soil from a dead soil. Therefore, maintaining soil health, that is, the life of the soil, is the truly rational place to begin in healthy sustainable farming practice.

Land practices have different effects on different organism, for example some agricultural practices as extensive tillage reduce the diversity of the soil organism as well as their abundance. Problems arising from conventional management in agriculture, as frequent pesticide applications, excess of inorganic fertilizer usage, declining of soil organic matter, and soil erosion have led to the development and promotion of organic farming management systems that take account of the environment and public health as main concerns.

The ability of a soil to provide nutrients for plant growth to obtain a certain crop yield is the fertility. No matter what program of soil fertility are used, but what is taken from soil must to be returned or eventually a price will have to be paid.

For valid comparison of soil qualities across variations in climate, soil and management, we must define reference guidelines and soil quality indicators that enable interpreting relationships between measured soil attributes and soil functions. It need to identify biological indicators of soil quality related to soil biological health.

An understanding of biological processes is important for the management of farming systems, particularly those that rely on organic inputs of nutrients. Transformation of soil organic matter is associated with the activity of microorganisms and enzymes in soil.

Microbial activity and, in part, enzymatic activity in soil are controlled by physical and chemical conditions, although the major limiting factors for microorganisms are temperature and water content. Soil microorganisms constitute an active component of the soil organic pool, controlling the breakdown of organic matter and, hence, the release of nutrients and their

availability for other organisms. The microbial biomass also acts as a small but labile reservoir of nutrients that contributes to maintaining long-term agricultural sustainability *(Benedetti et al., 2000).* The microbial biomass, rather than total amounts of organic C, has been suggested as a useful and more sensitive measure of a change in organic matter status. Changes in microbial biomass C can provide an early indication of short-term trends in total organic C of soils. The study of the relationship between microbial biomass C (Cmic) and total organic carbon (TOC), and the metabolic quotient, which is the rate of CO_2 per unit of biomass and time, can provide understanding of the biological and chemical changes that occur under different agricultural practices *(Anderson and Domsch, 1990).* Soil enzyme production as a result of microbial metabolism is a sensitive indicator of soil microbial activity. Extracellular soil enzyme activity may be indirectly regulated through increased production and secretion by microbes or directly through physical-chemical conditions. Recent studies comparing conventional and organic farming have shown an increase in organic matter, nutrients content, and microbial biomass (Cmic and Nmic) in organically managed soils *(Edmeades, 2003).* The response of soil microorganisms to organic and conventional amendments is often studied in the short term, but less is known about the long-term effects.

Estimate economic impacts of improving soil quality and evaluate how soil quality can be used to estimate economic return from public investment in conservation practices such as the Conservation Reserve Program *(Huang et al., 2002).*

1.5 Nitrification inhibitors

Nitrification in soil is an important process as it is responsible for the conversion of relatively immobile NH_4^+-N into NO_3^--N accompanied with the production of several gases N forms, including N_2O. The NO_3^- produced by nitrification may then be lost from the agricultural system by subsequent leaching or, trough de-nitrification process, it may be converted to N_2O and N_2 gases. N_2O is a potent greenhouse gas and stratospheric ozone destroyer and in order to comply with the Kyoto Protocol on gas emission, EU countries must

reduce 8% the levels by 2012. Various mitigation options to reduce N_2O emissions have been proposed, an effective strategy to reduce the N_2O emission from agriculture would be inhibition of nitrification and de-nitrification process into soil. In the last years the use of nitrification inhibitors (Nis) in combination with nitrogenous fertilizers offers several environmental advantages, compared to the application of conventional N fertilizers *(Zerulla et al., 2001).* Nitrification inhibitors (Nis) are compounds that delay the bacterial oxidation of ammonia to nitrite by depressing the activity of *Nitrosomonas* bacterial in the soil (first step of nitrification) for a certain period of time, whereas the second step of nitrification normally is not influenced. Thus the efficiency of N is increased while in the same time NO_3 losses, due to either leaching or nitrification are decreased. The use of nitrification inhibitors appears to be related more to the maintenance of nitrogen within the system, than to increase NH_4^+ nutrition *(Pasda et al., 2001).*

Most of N fertilizers used is ammonia based, and much of this is oxidized rapidly to nitrate (NO_3^-), and the gaseous N_2O emitted during nitrification can be a major source of N_2O from arable soil; it has been reported that Nis is very effective in reducing NO_3^- and N_2O losses under cool conditions. *(Zerulla et al., 2002).*

In the literature *(Chenet al., 2008; Di and Cameron, 2004)* are reported significant reduction in NO_3^- formation in soil and N_2O loss has also been reduced by the addiction of nitrification inhibitors.

The use of Nis in combination with nitrogenous fertilizers offers several environmental advantages, compared to the application of conventional N fertilizer *(Pasda et al., 2001).*

Though slow and controlled-release and stabilized fertilizers can contribute to improve nutrient efficiency, minimizing negative environmental effects, it has to be kept in mind that errors in field and crop management cannot be compensated for by the use of these special fertilizer types.

However, to justify their price, these fertilizers must also offer economic advantages, it has been shown that specific Nis may improve N utilization from fertilizers by crops, thus leading to higher yields with the same fertilizer rate, or to reduction of N application rates. *(Pasda at al., 2001; Zerulla et al 2002).*

In the last years it has increased the interest in a new relatively stable Nis, the 3,4-Dimethylpyrazole phosphate (DMPP) that is highly effective when applied to soils in conjunction with N fertilizes. Recently the attention has been paid to the possibility of using inhibitors with animal slurries. The combination of an N fertilizer and 3,4 DMPP is now available on the market as ENTEC® ENTEC® ® granules *(Zerulla et al., 2001).*

1.6 Bio - stimulants

The term bio- stimulant defines a substance that has a positive impact on plant health, but is neither a plant nutrient nor a pesticide. Bio -stimulants are natural (nutrient) and organic (bio-stimulant) substances which stimulate plants to meet their maximum, healthy potential and cause regeneration of healthy soil. A bio- stimulant is an organic material that, when applied in small quantities, enhances plant growth and development such that the response cannot be attributed to application of traditional plant nutrients. Bio -stimulants have been shown to influence several metabolic processes such as respiration, photosynthesis, nucleic acid synthesis and ion uptake. They are not fertilizers meant to correct a severe nutrient deficiency, but are mixtures of one or more things such as microorganisms, trace elements, enzymes, plant hormones, and seaweed extracts *(Benedetti, 2010)*. They may enhance nutrient availability, water-holding capacity, increase antioxidants, enhance metabolism and increase chlorophyll production.

Some might refer to them as a natural organic fertilizer because of the way bio -stimulants affect the growth of plants. Bio-stimulants allow growers to decrease the use of fertilizers up to and more than 50% because of their healthy natural affects on the soil around plants.

Some commercial products increases soil microbial activity causing increased soil nutrient availability in a healthy natural way, others determine a triple action effect of three to prime specific complex: organic complex (which stimulates biological activity in the rizhosphere), complex root system, complex (balances the forms of nitrogen in the soil).

OBJECTIVE OF THE RESEARCH

To improve soil fertility it must to increase soil organic content, optimize the water use, and minimize the contamination of surface water through sustainable management, thus it needs to develop news fertilization solutions according to an environmental point of view.

Animal manure is frequently used to fertilize specially maize crop; ammonia N present in, or mineralized from manure, can be rapidly nitrified. Nitrous oxide resulting from de-nitrification has a detrimental effect on the ozone layer and contributes to the greenhouse effect and N leaching may eventually lead to nitrate concentrations in ground water exceeding the European Community (EC) standard for drinking water.

In Europe, the SRWC are commonly fertilized with urban wastewaters, that is an important solution for the recycling of nutrients. This application in a *pedo*-climatic environment typical of the Mediterranean area, where scarce water resources are present, could be a valuable step towards sustainable management. Recycling of manure from intensive animal industries in the agro-ecosystem could increase soil organic matter and nutrient contents, but needs appropriate treatments before it is applied to soil, to preserve the soil and the groundwater from the nitrate pollution and eventually obtain the European Nitrate Commission derogation. In fact, the application of slurry on the soil in Mediterranean areas, could be interesting fertilizer practice for environmental and economic problems related to the disposal of these waste materials and water recovery *(Plaza et al. 2004).* The combined use of slurry with Nis is a new frontier in organic fertilization and were not reported at the time of application experiences in the Mediterranean environment. The most studies published have investigated the effect of combined use on emissions and few studies were focused on effect of chemical indicators on agricultural soil. My research was focused on slurry and a Nis molecule addition in short rotation woody crops by investigating the effect on soil biochemical and microbiological properties linked at carbon cycle and by verifying the effect on

plant production. The final objective was to propose it as a really promising approach to the sustainable management on Mediterranean environmental. .

The use of slurry in agriculture crops as Short Rotation forestry together with Nis should be beneficial for nutrient recycling, soil quality, plant productivity, gas emission reduction and could offer economic advantages to make them alternative to conventional fertilizers.

The connection between the relative availability of different N forms and the impact on C cycling is not well understood and has only recently begun to be explored *(Nordin et al., 2001).*

To evaluate the significance of nitrification on C turnover, the interaction on the functional group of nitrifying bacterial *via* chemical inhibition should highlight its importance for the C cycle *(Austin et al., 2006).*

The objective of my research was to compare the effectiveness of controlled N release with inhibitor of nitrification, as DMPP, in addition to a slurry in a Mediterranean environment and to investigate the effect on the carbon cycle in a short rotation forestry, with particular attention to the soil ecosystem. The application of fresh organic matter can produce immediately effect on soil properties, including the microbial activity. The nutrients cycles in the soil are driven by the activities of different communities which continuously influence physical structure, nutrient availability and organic matter turnover of soil (*Gregorich et al., 1996*; *Plaza et al., 2004*). Microbial biomass C content, enzymes activities, microbial respiration are appropriate and sensitive indicators that can be used to monitor the microbial response to the organic amendment *(Plaza et al., 2004)*. The comparison with soil microbiological dynamics background and the integration with field experiments could help to evaluate the positive or negative influence of addiction of Ni at slurry on soil ecosystem in a SRWC system.

Moreover, since increasing attention is devoted to the bio-nutrition of soil, to facilitate nutrient availability and uptake, we investigated also the combined effect of bio-stimulant and *zoo*-technical waste application. It is generally accepted that bio-stimulants are a new possible tool for fertilization, the scientific community continues to find more evidence of the efficacy of biological products for the plant health, cost reduction and their contributions to the air and water above ground.

We studied in a simulation experiment under controlled moisture and temperature the effect on soil functional activity of the addition of bio-stimulant, DMPP and their association at the slurry with particular attention at the C cycle. Thanks to this experiment we obtained a measure of C mineralization, microbial C biomass and enzymes analysis which relates to microbial activity.

The soil samples were collected in June 2009 at the CRA Research Unit for Intensive Wood Production , site in Rome. The Rotation Forestry is a 4 years old one where are cropped different clones of *Eucaliptus (E. gomphocephale, E bridgesiana, E camaldulensis, E camaldulensis x E bicostata, E camaldulensis x E. grandis*).

Biochemical properties of soil, such as microbial biomass (MBC), microbial respiration, and enzymatic activities were chosen as indicators of changes in the soil organic matter biochemical alteration.

In particular, microbial biomass is the labile portion of the organic fraction in soils and serves as both an important source of and sink for plant available nutrients *(Dalmonech et al., 2010).* MBC has been suggested as an integrative signal of the microbial significance in soils because it is one of the few fractions of soil organic matter (SOM) that is biologically meaningful, easily measurable, and sensitive to management or pollution.

Soil enzymes play an essential role in catalyzing reactions necessary for organic matter decomposition and nutrient cycling. They are involved in energy transfer, environmental quality and crop productivity.

Management practices (as application of fertilizers) may have different effects on various enzymes and microbial activities of soil. N fertilization may affect soil enzymes activities because organic matter modifies the development of the enzyme-producing microbial community, management practices that minimize the addiction of organic matter to the soil may reduce enzymes activities and thus affect the ability of soils to supply nutrients needed for plant growth.

Moreover enzyme activities can provide indication for quantitative changes in SOM. It is known that the activities of most enzymes increase as native SOM content increases, reflecting larger microbial communities and stabilization of enzymes on humic materials.

In the present research, enzyme activities and microbial biomass are used to investigate the response of microbial community to the application of slurry addicted with a nitrification inhibitor (Ni) or with a Bio-stimulant product in an agro ecosystem constituted by a SFR stand.

Understanding of microbial process is important for management of agro ecosystem particularly those rely on organic inputs of nutrients. Soil microorganisms constitute an active component of the soil organic pool, controlling break down of organic matter and hence, the release of nutrients and their availability for other organisms *(Melero 2006).*

Were added microbiological and structural analysis to investigate the nitrification inhibitor effect and slurry on soil microbiology community, as the structure of microbial communities has direct and indirect effects on the decomposition of organic matter and on plant growth. Fungi and bacteria has been suggested to influence the biogeochemical fluxes especially that of C, in fact drive a important rule into soil for the decomposition of organic matter *(Bååth 2001b).*

The two groups of decomposing microorganisms are also often thought to have different efficiencies of C sequestering in soil. This is based on the hypothesis of a lower N requirement of the fungal biomass, in addition to its higher growth efficiency , and the lower biodegradability of the fungal cell wall.

The fatty acids of cell membrane phospholipids (PLFA) is another indicator used for the biochemical study of the total biomass and structure of living soil microbial communities, and their determination can be used to highlight environmental changes, and are more discriminating than other methods *(Frostegård et al., 1996; Kaur et al., 2005).*

Bacterial growth was estimated using leucine (Leu) incorporation in the cell bacteria extracted from soil and the fungal growth in soil was measured using the acetate into ergosterol incorporation *(Bååth 2001a).* Moreover, it was investigated the effect of temperature on microbial dynamics (especially bacterial growth) after re-wetting soil.

The microbiological response after treatments on soil treated was determined by respiration analysis (GC), bacterial growth (leucine incorporation), fungal growth (acetate in ergosterol incorporation) and on the biomass concentration and composition (PLFA). The analysis of variance was used to investigate the

microbial variables differed between the begin and the end of treatments. The number of treatments was used to investigate their influence on the measured microbial variables. Were used also the PCA (principal component analysis) to analyze the PLFA data and to verify existing relationship after treatments between the components of PCA at the begin and at the end of the observations.

Although urea-based fertilizers are the most common global source, they are susceptible to loss as ammonia (NH_3) gas when left on the soil surface. Ammonia losses from fertilizer can represent a significant economic loss and can have a negative effect on air quality , ecosystem productivity and human health.

A field experiment was conducted at the intensive Eucalyptus crop located in Central Italy. The NH_3 and CO_2 emission were studied because their presence in air causes environmental problem, such a direct damage to vegetation and contributes to global warming. In the first case, several soil processes are considered to be responsible for N emission, including autotrophic and heterotrophic nitrification. The objectives of this experiment was to estimate the influence of addiction of Ni at slurry on the gas NH_3 flux with a pilot experiment and to indicate possible correlation with CO_2 emission. Two treatments with different level of N fertilization with Ni were applied, one was the bovine slurry application. NH_3 emissions were measured with a simple chamber method for determining ammonia in the field (Drager-Tube Method, DTM) *(Pacholski et al., 2006)* and the carbon loss from the soil, CO_2 emissions, with a portable dynamic closed –chamber infra red gas analyzer system *(Pumpanen et al., 2004*) (EGM-4).

At the end of the first growth season, in order to estimate different productive responses of trees to treatments, values of basimetric area were calculated to point out productivity advantages of combination of animal manure and new fertilizer with slow and controlled release as 3,4 DMPP. Thus it has been possible to give indication for the sustainability and economical advantages of combination of animal manure and new fertilizer characterized by N controlled release, as those containing 3,4 DMPP.

CHAPTER 2: MATERIALS AND METHODS

2.1 Soil characteristics, sampling, and bovine effluent

The experiment was conducted at the intensive Eucalyptus crop of CRA-PLF (Rome, central Italy 41° 54' 33.55" N, 12° 21' 37.83" E). The SRWC is a 4 years old where are a crop of different species and clones of *Eucalyptus (E. gomphocephala, E bridgesiana, E camaldulensis, E camaldulensis x E bicostata*). The site is characterized by a Mediterranean climate with an average annual temperature of 14° and an average annual rainfall of 755mm and a summer drought period 6 months long during the year of experimentation. The system covers a total area of 1200 m^2 and each the plants is 0,50 cm distant to the next one on the row and 4 meter between rows. Experimental plantation was harvested when the plants were 3 years old, the experimentation was done during the first growing season after the harvesting. The soil classified as *Luvisols* for WRB classification (World Reference Base, 2006), having a sandy loam texture (sand 63%, silt 16% , clay 21%). In the first 30 cm it showed pH 7.5, total N content 0.06 %, total organic C content 1.03 %, available P 8 ppm, available K content 622 ppm. More information are reported in the Table 2.1 below. A composite sample was collected at six different points still recognizable, plant material was removed by hand-picking, the soil was air dried, sieved at 2mm and stored at room temperature. The bovine effluent was collected from a bovine farm located in Rome, Casalotti, close to the SRWC (to minimize the energy cost). The mean composition was the following: N content 0.32 % NH_4^+0.17 content NO_3 0.0025, total organic carbon 5.7 %, other information are reported in the Table 2.1.

Tab 2.1. Soil Physical and Chemical Proprieties

	0-30cm	30-60cm	60-90cm
%Sand	63.00	63.00	63.00
%Silt	16	16	16
%Clay	21	19	20
%Skeleton	Negl	Negl	Negl
pH	7.5	7.7	7.7
Organic Matter	1.03	0.22	0.22
% Total N	0.06	0.02	0.02
ppm Assimilable P	8	5	5
ppm Exchangeable P	622	641	649
ppm Exchang Mn	490	510	575
ppm Exchangeable Na	251	375	391
% Carbonate	1,38	/	/

Tab 2.2. Effluent Physical and Chemical Parameters

Parameters	Units	Value
Moisture	%	93.0
Dry	%	7.0
Total N	%	0.32
Ammonia N	%	0.17
Nitric N	%	0.0025
Density	g cm^{-3}	1
Cu	mg kg^{-1}	71
Zn	mg kg^{-1}	117

2.2 The dimethyl pirazole phosphate (DMPP)

Nitrification inhibitor DMPP is a compound that delays the bacterial oxidation of ammonia to nitrite by depressing the activity of *Nitrosomonas* bacterium in the soil. It reduces N-leaching and increases N- plant uptake efficiency. During the active phase of DMPP (4 to 8 weeks, depending on soil temperature and soil humidity) the transformation of ammonium to nitrate is delayed. As a result N-availability is further adapted to the plants´ requirements and N-efficiency is increased .

The nitrification inhibitor dimethyl pirazole phosphate, 3,4 DMPP used in the research, was in form of a nutrient solution containing N_{tot} 1.76% (1.1% NO_3, 0.66% NH_3).

2.3 The Bio-stimulant

Commercial bio stimulants products increase soil microbial activity causing increased soil nutrient availability in a healthy natural way. Bio-stimulants are compounds that when in contact with the rhizosphere hydrolyse, thus they release substances and activate the carboxylic acid. The former act by stimulating the bacterial population associated with roots and increasing the rhizosphere concentration phytoregulation. The organic acid facilitates the nutrients transport thanks to the complexation capacity.

The N fertilizer with bio stimulant properties, used in the experiment, is a commercial granular products containing 20 % N (12 % in ammonium form, 4 % as ureic N).

2.4 Laboratory experimental design: microcosms preparation

Soil samples were pooled together by quartering to minimize any interference due to heterogeneity of the site. Samples were preconditioned at 30°C at the water holding capacity 10 day before to start with the incubation. The N treatment was at the rate of 250 mg N kg^{-1} soil, by using combination of 3,4 DMPP (Nib), Effluent (Ef) and Bio-stimulant (Bio) with the following procedures: Nib, Bio, Ef, Nib+ Ef, Bio+ Ef. Control soil samples (C) were treated in a equal amount of distilled water. All the samples (in tree replicates) were incubated for 1 month in a thermostatic chamber at 30°C (the best period to performance active phase of DMPP at 30°C) *(Irigoyen et al., 2010).* The weight of each samples was recorded at the beginning of the treatments and soil moisture was adjusted twice a week with de ionized water. The soils sampled for analysis were air-dried (to keep natural conditions) and re-wetted before to start with the microbiological analysis.

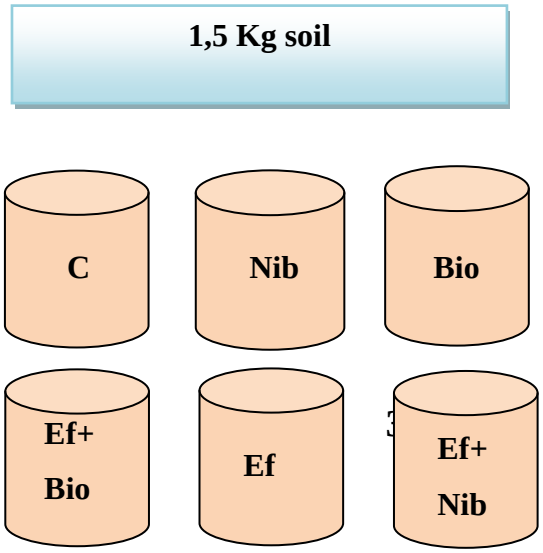


Fig 2.3. Experimental design

2.5 Chemical and Biochemical analysis

Total organic carbon , soil respiration and carbon of microbial biomass

Total organic carbon % (TOC) was determined by wet oxidation with 2N potassium dichromate solution in acid environment for 10 minutes at 160°C, according to Springer and Klee (1954) in three replicates in the soil and in the effluent at the beginning of incubation.

Basal respiration was measured on days 1, 2, 4, 7, 10, 14, 17, 21, 28 three replicates for each treatment, 25 gr of soil were incubated in a hermetically sealed polyetilende flask with a backer content 5 ml of 0.1 M NaOH; the alkaline trap for CO2. Excess NaOH was then titrated with 0.1 N HCl. Cumulative C-mineralization was computed by summing the amount of C respired during the incubation. Microbial biomass determination has been suggested as an integrative signal of the microbial significance in soils because it is on of the few fractions of soil organic matter (SOM) that is biologically significant and easily measurable and, more important, it is sensitive to management and pollution. *(Lagomarsino et al., 2009*). The Microbial biomass content (MBC) was determined at the begin of incubation, on days 2, 7, 14 and at the end, by the fumigation-extraction method (Jenkinson and Powlson,

1976). The MBC then was calculated from the difference in CO_2-C evolved (MC) between fumigated and un fumigated samples *(Voroney and Paul., 1984).* The amount of C-CO_2 produced per unit of microbial biomass C, was determined to calculate the metabolic quotient (qCO_2), while the microbial quotient (q_{mic}) was computed as the ratio between microbial biomass C and the total organic carbon measured. Samples pH was determined in three replicates in water with soil : water ration of 1:2.5, at all the sampling times.

Enzymatic Activity

The microbial performance clue was investigated measuring the activity of soil's enzymes, in particular β glucosidase (βG) β xylosidase (XYL) and α glucosidase (αG), which are key C-enzymes involved in cellulose, hemicellulose and start degradation; N acetyl glucoseaminidase (NAG) and leucine aminopeptidase (LEU), which are involved in N cycling through the release of amino sugars from chitin and amino acid from polypeptides respectively; acid phosphatase (AP) which is a crucial enzyme involved in organic P transformation; and MUF acetate (MUF) which is an endo-cellular enzyme *(Dalmonech et al., 2010).*The activities were measured at the beginning of incubation, on days 2, 7, 14 and at end of incubation, and detected colorimetrically using 96 well microplates and model substrates that yield 4-methylumbelliferone or 7 amino -4 methyl coumarin as products. The measure were made with a SCHIMADZU UV-VIS 1240 spectrophotometer the pNP (p-nitrophenol) released after enzymatic reaction at 400mm wavelength. The activities were expressed in unitis of nmol of product g^{-1}dry soil $h^{-}1$ *(Marx et al., 2001)*

2.6 Microbial Analysis

Bacterial Growth

Bacterial growth was estimated using Leucine (Leu) incorporation in the cell bacteria extracted from soil using the homogenization centrifugation techniques *(Baath et al.,2001a).* Since the samples were airdried, in order to stop any processes, we rewetted the soils two days before the measurments.

This has earlier been shown to restore microbial activity *(Iovieno and Bååth., 2008).* The soil samples were mixed with 20 ml of distilled water and shaken on a vortex for 3 min. The soil suspension was then centrifuged at 3000rpm (which corresponds to 1,000x g) for 10 min. Then, 1,5 ml of the supernatant with extracted bacteria and ^{3}H-leucine (final concentration 275 nM leucine) was added to microcentrifugation tubes. After incubation for 4 h, bacteria were killed by adding 75µl Tricloric Acetic Acid (TCA). Time zero controls were killed before adding leucine.The samples were washed with 5% TCA and, 80% ethanol, and 0.2 ml 0.l M NaOH were added and treated for 30 min at 90^{o}C. Ultima Gold scintillation cocktail was added and the radioactivity measured using a scintillator. The relative radioactivity incorporated into bacteria as DPM (disintegration per min) was used to estimate a relative growth rate, in that a high incorporation rate indicates high growth rates and low incorporation rates low growth rates. In this way a toxic effect can be measured by comparing a soil with added toxicant with a soil without a toxicant. We mesured the bacterial growth using 4 replicates for each treatment at the beginning of experiment, after 2, 7, 14 days and at end of incubation.

Fungal Biomass and Growth

The soil's fungal growth was mesured using the acetate into ergosterol incorporation method modified by *(Baath, 2001b).* We added 1-^{14}C Acetic Acid combined with unlabeled sodium acetate. resulting in a final acetate concentration of 220 µM, and having a 4 h incubation at room temperature. Ergosterol was extracted, separated and quantified using HPLC with a UV detector. The fungal biomass was estimated assuming 5 mg erosterol g^{-1} fungal biomass. The ergosterol peak was collected and the relative amount of acetate incorporated into fungal ergosterol as DPM was used as measure of fungal growth. We mesured the fungal biomass and growth in 3 replicates for each treatment at the beginning of experiment, on days 2, 7, 14 and at end of incubation.

Biomass composition of the community (PLFA)

The phospholipids fatty acids (PLFAs) are principal constituents of cell membrane and are a soil microbial variable frequently used to study the microbial community structure in soil *(Frostegård et al., 1996).* The PLFA pattern was determined mainly according to Forstegård et al. (1993). Briefly, 1 gr of soil were stored frozen (-20°C) before the analysis. The soil were extracted for 2 h in a one phase mixture of chloroform, methanol and citrate buffer (1:2:0.8, v:v:v). After dividing the extract into two phase by adding chloroform and buffer, the lipid-containing phase was collected and evaporated under N_2. The lipid material was fractionated on columns containing silic acid into neutral, glycolipids and polar lipids. The polar fractions containing phospholipids was collected for further analysis. Methyl nonadecanoate fatty acid (19:0) was added as an internal standard. The phospholipids were methylated by mild alkaline methylation before being analyzed on a gas chromatograph. The PLFAs chosen to indicate bacterial biomass were i14, 14:0, i15, a15, 15:0, i16, 16:1w9, 16:1w7c,16:1w5, 16:0, br17, 10:Me16a, 10Me16b, i17, a17, 17:1w8, cy17, 17:0, br18, 10:Me17, 18:2w6, 18:1w9, 18:1w7, 18:1, 18;0, 10:Me18, cy19, 20:0. The quantities of the fatty acids were obtained using 19:0 as the internal standard.

Tab 2.3 PLFA and specific microbial group

Microbial Group	PLFA Marker	Reference
Fungi Biomass	18:2w6,9; 18:1w9c	Federle 1986
Bacterial Biomass	Amount of : il4:0, il5:0. a15:0, 16:1w7t, 16:1w9, 16:1w7, 10Me-16:0, i17:0, a17:0, cy17:0. 17:0, 10Me-17:0, 10Me-18:0 and cy19:0	Kaur 2005; Šnajdr et al., 2008
Gram-negative	Amount of: cy17:0, 16:1ω7, 18:1ω7	Kaur 2005; Forstegård 1993
Gram-positive	Amount of il5:0, al5:0, i16:0, il7:0, and a17:0	
Actynomicetes	Amount of10Me16:0, 10Me17:0, e 10Me18:0	
F/B ratio	Amount of Fungi/bacteria acids	Frostegård et al., 1996

2.7 Field Experiment

A experiment was conducted on intensive Eucalyptus crop located in Central Italy(Rome), in Research Unit for Intensive Wood Production, (41° 54' 33.55" N,12° 21' 37.83" E) in June 2011. In the our interest crop are cultivated clones of *Eucaliptus (E. gomphocephale, E bridgesiana, E camaldulensis, E camaldulensis x E bicostata*). Experimental plantation was harvested when the plants were 3 years old, the experimentation was done during the first growing season after the harvesting. A completely randomized block design with three replicates was used for each treatment. Three treatments were applied, one treatment was the bovine effluent (Ef) application, the second was the bovine effluent with the addition of 3,4 DMPP (Ef+Ni), the third was slurry with a granular commercial fertilizer (ENTEC® 46) containing 24%N and 3,4 DMPP, (Ef+ENTEC), finally the control was not fertilized. The fertilizer application

once in the June with tanker furrowing plowing. Respectively, there were added 170 kg N ha^{-1} in the slurry trial (Ef) and with the combination of 3,4 DMPP solution at the effluent (Ef+Nib). In the trial (Ef+ENTEC) the amount of N added was 240 kg N ha^{-1}. The fertilization interested all the clones of *Eucaliptus* but were investigated the effect of Ni and effluent on *Eucaliptus camaldulensis and E camaldulensis x E bicostata* .

A meteorological station was put downwind of the field measuraments pole, according to the wind direction, in order to avoid perturbance on NH_3 measurements. Soil sampling were done in October to determine possible C microbiological biomass and Organic Carbon variations with respect to the initial values.

NH_3 volatilization and CO_2 emissions

A simple chamber method for determining ammonia in the field (Drager-Tube Method, DTM) *(Pacholski et al., 2006)* was used to ammonia volatilization . The volume of each ammonia chamber is 370m2, the air is enriched with NH3 volatilization from the soil surface. The pump rate is about 11min-1 corresponding to an air exchange rate approximately 1 vol min-1. The chamber method is showed in the Figure 5 below. Ammonia flux were calculated according with the equation : FNg = volume *|conc.|*10-6 *pNH3*UN*UF*UZ *(Pacholski et al., 2006)* where FNg is NH3 flux (mg N m-2 h-1) volume is air volume sucked throught the chambers |conc.|* : value of NH3 vol.conentration (volume ppm); pNH3 is temperature-dependent density of NH3 (mg1-1); UN is molecular weight conversion factor of NH3 to N; UF is surface area conversion factor (m^{-2}): $U_{Z\ is}$ time conversion factor (h^{-1}). *(Pachoski et al., 2006).* A single measurement took about 3 min. The taming of measurement was chosen taking into consideration the ammonia diurnal variation in emission, low ammonia fluxes in the night .The measurements were carried out twice time per day with 3 replicates on each measurement plot. The readings of the NH_3 concentrations on the treatment Drager Indicator Tubes have a coefficient of variation between 10-15% as indicated by the manufacter.

During one month the CO_2 emissions from the soil were measured every day in the first week and 2 time at week successively in three randomized area for each plot. In the event of rain the measurements of the CO_2 emissions were performed 48h after rainfall *(Mancinelli et al., 2010)*. A portable dynamic closed –chamber infra red gas analyzer system (EGM-4) was used *(Pumpanen et al., 2004*). It was placed on the small PVC collar which are located in the soil, the collar were put in the furrows covered in tree fixed point per plot. To minimize the effect of soil furrow, soil respiration was determined in the control soil with and the without furrow. Each measurement tooks about 3 min. The soil temperature was measured by the soil chamber simultaneously to the CO_2 analysis at 5 cm depth by using the "STP -1 soil temperature Probe" connected with EGM 4.

Fig 2.4.Chamber method for determining ammonia in the field – (Drager-Tube Method, DTM)

Fig 2.5.EGM 2.4 used to study CO_2 emissions

Soil sampling were collected in three point per plot after 5 months the fertilization. Total organic carbon and carbon bacterial biomass were determined to compare different efficiency of the treatments .

Plant productivity analysis

To determine the productivity of plants during the first growing season and get a picture of the potential effect of the treatments we decided a non-destructive method. We used sectional area (SA) to establish plants productivity on the first growing season. Is generally assumed that diameter is positively correlated with height with the volume and the weight. The diameters were taken with a digital caliper to 130 cm from the soil and for each diameters were calculated the sectional area and the SA for each replicate was calculated including the 20 plants of row.

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2.8 Statistical analysis

The variance analysis was used to show difference between the treatments at different time for each biochemical and microbial determination and to investigate if the microbial variables differed between the beginner and the end of treatments. The multivariate analysis were used to investigate difference on the biochemical and microbiological response at the treatments. The number of treatments was used to investigate their influence on the measured microbial variables. Discriminating analysis contributed to investigate the changes of enzymatic activity and a clear picture of effect between the treatments. We used also the PCA (principal component analysis) to analyze the PLFA to describe if there are relationship between the components of PCA and beginner and end of the treatments. The statistical analysis were done with a STATISTICA 7 software.

To estimate the plant productivity we used a Kruskal-Wallis method to test the equality of the mediane of different groups.

The treatments were used to investigate they influence on the plant productivity and on this way we described if treatment C was showed difference from A+B on the clones.

CHAPTER 3: Preliminary investigation on biochemical response under 3,4 DMPP solution on two different soils from Mediterranean Area

3.1 INTRODUCTION

Soil mineralization is usually considered as one of most important properties of soil because carbon mineralization is related to ecosystem productivity, soil fertility, and regional and global carbon cycles. A large number of published field and laboratory studies have examined how nitrogen (N) affects belowground communities and processes after N addition to soils and then measuring how microbial respiration, biomass and microbial community composition respond *(Söderström et al., 1983; Fog, 198; Waldrop et al., 2004; Treseder, 2008; Janssens et al., 2010)*. The forms of N added varies across studies, yet we know little about how different N forms may impact microbial processes. The use of N inhibitors (Nib), could help to better understand the N availability impacts on soil respiration.

On this study was investigated the effect of Nib on soil mineralization and structural composition in two soils with different chemical and physical properties. The "Casalotti" (CAS) soil is from a experimental intensive arboriculture at short rotation site in Rome, whereas "Celimontana" (CEL), from urban green area in Rome, is an high biological fertility soil. The soils were sampled both an average depth of 0-30 cm, air dried, sieved to 2 mm for chemical and biochemical analyses. The soils were enriched with 0.5 mg kg^{-1} of a nutrient solution containing 3,4DMPP. The N content solution was N-NO_3 1.45 % (p/v), N-NH_3 0.73 %. 3,4 DMPP is a N inhibitor that can slow the reduction of ammonium in the soil through inhibition of the bacterium *Nitrosomonas* (see Materials and Methods, Chapter 2). Basal respiration was measured for 28 days in both soils at 20°C and 30°C with three replicates for each treatment as reported in chapter "Material and Methods". The fatty acids of cells membrane phospholipids (PLFA) are biochemical indicators used to study living and total biomass of soil microbial community structure, and their determination can be used as sensible environmental stresses, as they are more

discriminating than other methods *(Zelless, 1999; Moore-Kucera and Dick, 2005).* The Phospholipids in this case were extracted from fresh soils and purified in SPE (silica gel column) and esterified with alkaline methanolisys using the procedure suggest by Zelles (1992). The methyl esters of PLFA have been identified in GC/MS (86°C 4 min -6min, 280° 5minut). The mass spectra were compared using a mix of standard microbial quality esterified. On this way, were done a particular focus on some acids suggested as environmental indicators and indicator of fungal biomass, acid-Octadecadienoico 9.12 (*Moore and Kucera., 2005*), (18:2 w6) *(Frostegård et al., 1996).*

To better understand if there was a short term response of soil at the 3,4 DMPP treatment, were determined the activities of four enzymes by fluorimetric way (Material and Method, Biochemical Analysis) as a sensor of activities linked to the cycle of carbon (cellulase, and xylosidase βglucosidase) and to the N cycle of the N-acetyl glucosamide *(Dornbush et al., 2007).*

3.2 RESULTS

Chemical and physical properties of soils

The chemical and physical properties of soils are reported in the Table 3.1. At the beginning, were estimated the best quantitative of 3,4 DMPP to add in soils in order to detect the anti-nitrification effect, so carbon mineralization tests were conducted with different concentrations of solution. On second time were repeated the soil respiration at two different temperatures (20°C and 30°C) to estimate the relationships between 3,4 DMPP solution and temperature on C mineralization in soil.

How reported in Figure 3.1A, application of 3,4 DMPP in low concentration didn't affect on soils. On the contrary, the addiction of 0.5 mg Kg^{-1} of 3,4 DMPP solution at 20°C showed that microbial metabolism responses are different between the two soils (Figure 3.1B). In the CEL soil the nitrogen inhibitor solution implied a decrease of microbial respiration during the first weeks of treatment. Otherwise, the CAS soil reported a significant ($P<0,01$)

increased effect of Nib running the 48 hours after the addiction to back at the control value of mg C-CO_2 respired. The metabolic quotient reported difference values evidencing changes at 20°C, exactly in CAS+ Nib and in CEL+ Nib values were lower than controls (Table 3.3).

The Carbon mineralization conduced at 30°C showed different responses of the soils (Figure 2, A and B). In CEL soil, Nib involved an immediately significant effect ($P<0,01$) on soil respiration running the first week. In CAS soil this effect did was not significant with comparable values between C and Nib. In the Table 3 are showed the biochemical parameters of soils after one months of treatment at 30°C. The statistical analysis showed that cumulative respiration values (R_{cum}) were significantly different between groups. CAS and CEL had different value, but the addiction of Nib entailed the same decrease on R_{cum} in two soils at the end of incubation. MBC didn't change with Nib addiction. The metabolic quotient showed that at 20° C the lowest values on the soils treated and no difference values at 30° C, while the mineralization quotient evidenced changes in the soils response, in CAS+ Nib was higher and in CEL+ Nib was lower than controls (Table 3.3).

PLFA extraction

The abundance *per cent* (A%) of phospholipids fatty acids were detected on soils treated, as they are sensible indicators of structural changes of microbial community. After 24 hours of addiction of Nib solution, PLFA were extracted from the soils and the Total PLFA didn't significantly differed after 24h in the treated soil (Table 3.3). Moreover, in the Table 3.3 below are reported the A% of each acid analyzed and the changes recorded after 24 hours for some single PLFA. In CAS there were found significant difference of the 9,12-Octadecadienoic acid (Z,Z), (18:2w6), mentioned as a biomass fungal indicator *(Kaur et al., 2005; Bardgett et al., 1999)* and of Esadecanoic acid (C16:0) used as total indicator of microbial biomass *(Frostegard et al 1996)* (Fig. 3). The Fungi/Bacteria ratio PLFA (Fig. 3.3) decreased in CAS+Nib and increased in CEL. Bardgett (1999) used this PLFA to study the microbial community in terrestrial ecosystem grazing subject or after fire and found F/B decrease. The A% of cyclopropanoic acids, 9,10 methylenehexadecanoate and 9,10

methyleneoctadecanoate (Cy17:0-Cy 19:0) didn't result significantly different between the tests. The total of PLFA markers of Gram- didn't differed.

A preliminar screening of the enzymatic activities showed difference respect to the soils controls just for B glucosidase (Figure 3.4).

Tab 3.1: Main soils properties (0-30 cm depth). Standard deviation (DS) are reported on the brackets.

	CAS	CEL
Sand (%)	63.0 (0.1)	67.0 (0.3)
Silt (%)	16 (0.2)	22 (0.1)
Clay (%)	21 (0.1)	11 (0.2)
pH	7.5 (0,4)	7.9 (0.7)
Organic Matter (%)	1.89 (0,1)	4.31 (0.9)
TOC (%)	1.07 (0,2)	2.51 (0.3)
Total N (%)	0.06 (0.00)	0.15 (0.2)
C/N	17.8 (0.6)	16.73 (0.8)

Tab 3.2. Soils Biochemical parameters after 28 day of incubation at 30°C and 20°C. Different letters within the same parameters indicate significant differences according to Posthoc test (P<0,05).

	END of incubation	SO	R_{bas} mgC-CO_2g^1	R_{cum} $mgCO_2$	MBC mg Cg^{-1}	qCO_2 mgC-CO_2h^{-1}kg MBC^{-1}	q_{mic} µg MBC mg TOC^{-1}
30°C	CAS	1.84^{ns}	0.40^{a}	282.11^{a}	$95,53^{a}$	0.17^{a}	2.63^{a}
	CAS+Nib	1.84^{ns}	0.95^{a}	388.75^{b}	$100,14^{a}$	0.20^{a}	3.63^{a}
	CEL	4.59^{ns}	11.30^{c}	856.49^{c}	$307,27^{b}$	1.56^{b}	5.90^{c}
	CEL+Nib	4.59^{ns}	7^{b}	583.09^{b}	250^{b}	1.55^{b}	4.51^{b}
20°C	CAS	-	4.60^{a}	294.11^{a}	-	2.04^{a}	-
	CAS+Nib	-	4.56^{a}	292.85^{a}	-	0.94^{a}	-
	CEL	-	5.24^{c}	426.49^{b}	-	2.38^{b}	-
	CEL+Nib	-	5.65^{b}	329.09^{b}	-	1.09^{b}	-

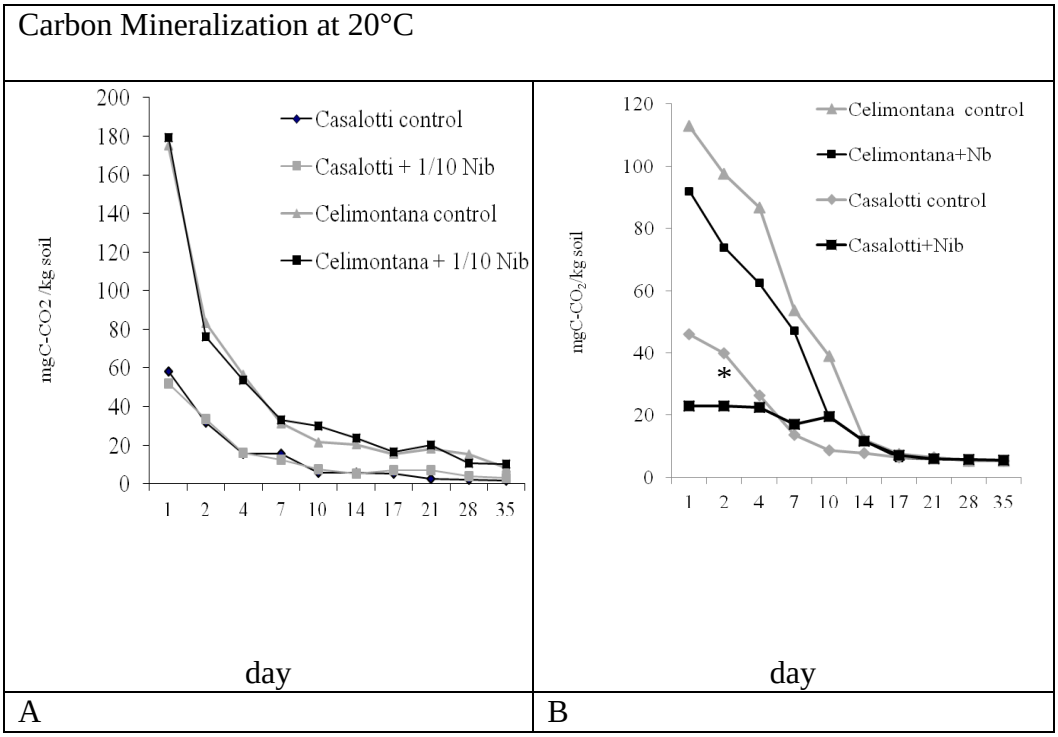


Fig 3.1. Carbon mineralization in Casalotti and Celimontana soil at 20°C with 0.05 mg/Kg of 3,4 DMPP solution (A) and 0.5 mg/kg 3,4 DMPP (B). The asterisks denote where the treatments with Nib are significantly different in the two soils.

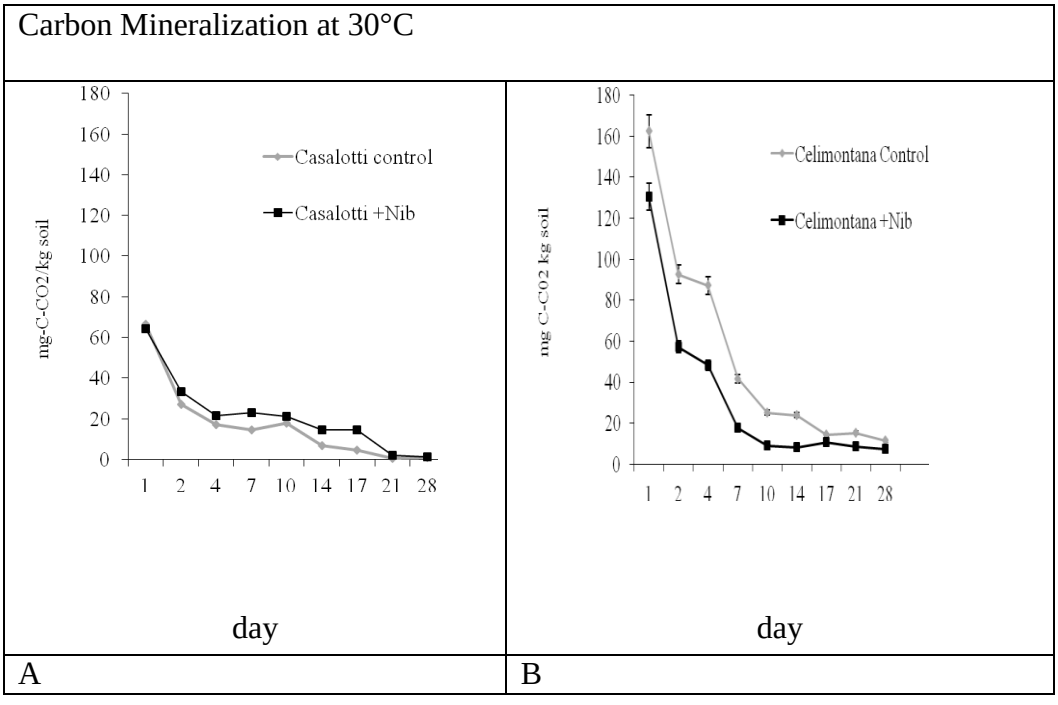


Fig 3.2. Carbon mineralization at 30°C with 3,4 DMPP solution in CAS soil (A) and CEL soil (B). The asterisk denote where the treatment with Nib are significantly different from the control.

Tab 3.3. A% PLFA on the soils. Standard deviation (DS) are reported on the brackets.

		CAS		CEL	
c	PLFA	C	Nib	C	Nib
Total		95.29(15.7)	106.23(16.78)	98.17(4.5)	99.25(13.42)
G+	i14:0	2.18(0.1)	12.19(1.17)	1,92(0.39)	4.10(0.9)
G+	i15:0	6.13(0.48)	1.95(0.1)	3.99(0.01)	2.80(0.08)
G+	a15:0	5.03(0.78)	10.23(0.67)	3.30(0.05)	5.62(0.01)
Bacteria	15:00	0.27(0.01)	0.00 /	0.88(0.1)	0,00 /
G+	I16:0	2.53(0.1)	0.81(0.1)	2.20(0.4)	2.13(0.01)
G-	16:1w7c	9.68(1.2)	17.28(1.3)	9.63(2.3)	16.20(4.5)
Bacteria	C16:0	23.56(2.3)	15.60(0.1)	22.89(9.1)	22.21(6.4)
ACT		1.00(0.01)	1.09(0.2)	1.56(0.04)	2.27(0.02)
G+	17:00	1.87(0.1)	1.89(0.2)	2.01(0.6)	2.88(0.01)
G-	i 17:0	3.06(0.23)	1.66(0.2)	2.45(0.8)	1.84(0.01)
Bacteria	Cy17:0	2.12(0.34)	2.57(0.3)	2.53(0.8)	1.81(0.01)
TF	18:2w6,	1.52(0.2)	0.62(0.6)	1.81(0.1)	2.25(0.04)
G-	18:1n9C	14.70(0.34)	17.12(1.45)	12.26(0.1)	10.94(1.4)
G-	18:1n9 t	7.68(2.1)	3.68(1.1)	12.94(0.3)	6.79(2.2)
Bacteria	18:00	4.74(0.78)	3.12(1.1)	6.16(0.4)	4.61(1.1)
ACT	10Me	2.67(0.001)	4.48(1.2)	3.93(0.3)	5.03(1.3)
G-	Cy19:0	4.09(1.1)	6.86(1.01)	6.27(1.3)	4.30(0.2)
Bacteria	19:00	0.95(0.4)	1.78(0.2)	0.75(0.03)	1.90(0)
Bacteria	20:00	1.55(0.98)	0.92(0.05)	0.70(0.4)	1.60(0.04)

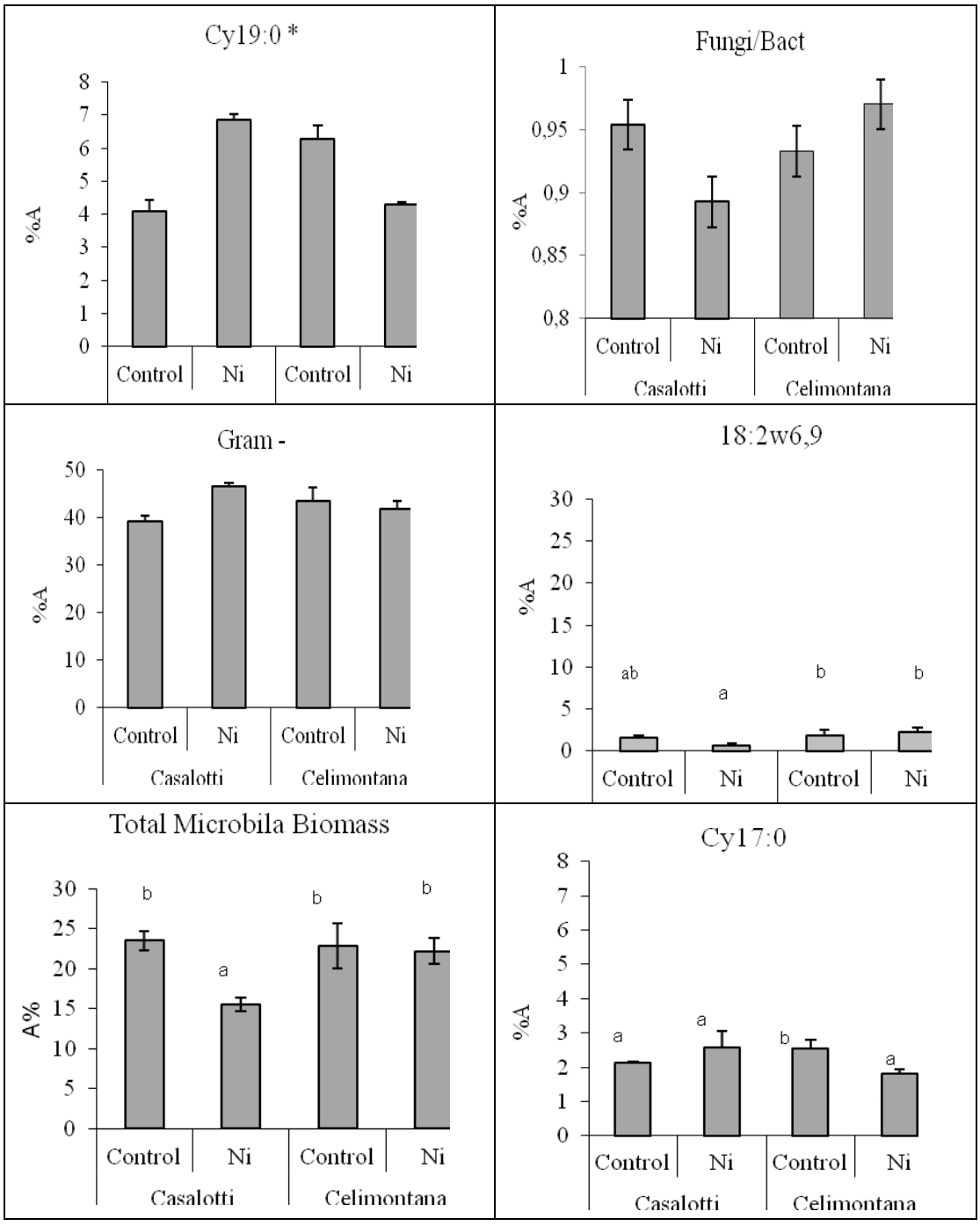


Fig 3.3. Specific PLFA for Fungal and Microbial Biomass in general, Gram-, and Cy17,Cy19 used as environmental indicator on CAS and CEL soil with Nib solution after 24 hours. Different letters within the same plot indicate significant differences according to LSD test (P<0,05).

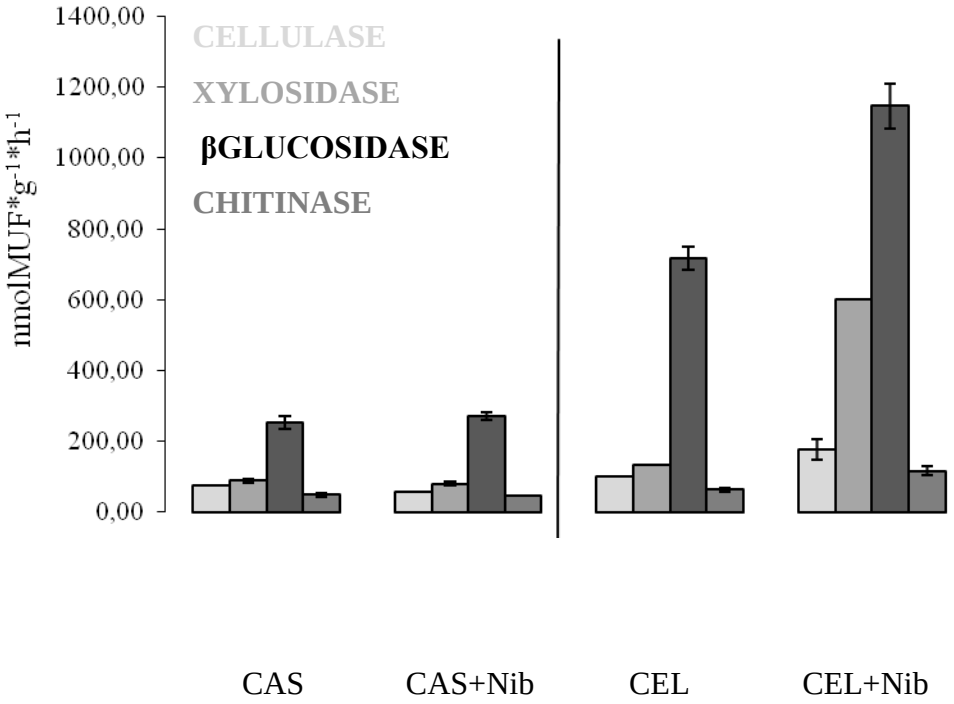


Fig 3.4. Preliminary enzymatic activities (chitinase, β glucosidase, xylosidase and cellulose) on Celimontana and Casalotti soils.

Under 24h of 3,4 DMPP treatment, there were not differences for CAS soil while the activities of CEL showed changes. Exactly cellulase, xylosidase and β glucosidase increased after 24 hours (Fig.3.4.).

3.4 DISCUSSION

The soil C mineralization showed a different effect of 3,4 DMPP solution on soils with different chemical and physical properties. The soil CAS, with a low organic C and N content showed a different mineralization daily trend with respect to the temperature, increase being generated at higher temperature (30 ° C) which probably was dictated by the contribution of low N content in the solution added. On the contrary, the solution with Nib in the soil CEL determined decrease of C mineralization at both the temperatures by inhibiting soil respiration. The analysis of soil respiration, conducted on these two soils

with different fertility and organic nutrients content, showed that 3,4 DMPP has a different effect depending on the metabolic activity and chemical characteristics of soil and this effect is in general more evidenced at low temperature.

The soil microbial carbon content did not change significantly after 28 days from the addiction.

The nutrient solution with 3,4 DMPP produced changes in the CAS patterns of acids considered, in particular, on single acids. The CAS soil showed decrease in the markers of fungal ratio F / B, or even the C16, an indicator of microbial biomass, suggested a quick change in the balance between microbial soil fungi and bacteria due to treatment, probably as biochemical response of the microbial community *(Frostegard et al., 1996).* The enzymatic activities related to the carbon cycle showed different response in the two soils: β-glucosidase activity and xylosidasi, which are associated with nutrient availability *(Gang et al., 2008),* were high in Celimontana and increased after the treatment, while in the soil Casalotti they did not undergo significant changes.

We can therefore conclude that the treatment with 3,4 DMPP has an immediately effect on the soil and on the microbial community; in particular, through the use of PLFA there were indications of the presence of a microbial community more sensitive to treatment on CAS, where there are significant changes in biomarkers after 24 hours. Enzymatic activities considered didn't report short term significant changes on the soils. Further analysis, with combined use of 3,4 DMPP inhibitor can provide more information about the structural and functional changes of soil microbial communities and follow relationships between interference with the nitrogen cycle and carbon cycle on short term. This part of the research is reported in the following Chapter 4 "Effect of organic fertilization combined with nitrification inhibitor 3,4 DMPP on soil microbial function in a closed experiment.

CHAPTER 4: Effect of organic fertilization combined with nitrification inhibitor 3,4 DMPP on soil microbial function in a closed experiment

4.1 INTRODUCTION

The microbial biomass has been suggest as an integrative signal of microbial significance in soil, because it is one of the few fractions of soil organic matter (SOM) that is biologically important and easily measurable and sensitive to management or pollution *(Lagomarsino et al., 2009).* The SOM is both a source and a sink for nutrients, it participates in the main biogeochemical transformation of C, N, P, S and it contributes to soil structure and stabilization. For this reason microbial biomass is used as indicator in many soil-monitoring programs. Moreover soil enzymes can provide indication for quantitative changes in SOM and C cycling. In fact it is known that the activity of most native enzymes increases as native SOM increases *(Lagomarsino et al., 2009).* Enzymes allow microbes to access the nutrients present in complex substrates and catalyze decomposition and nutrient mineralization. For example, enzymes like β glucosidase and acid phosphatase have been used as indicator of changes in quantity and quality of SOM *(Masciandro and Ceccanti., 1999; Allison and Vitousek., 2005),* N actelyglucosaminidase activity is considerated important for C cycling because it participates in the chitin process to convert in amino sugars (one of most sources mineralizable N in soils) (*Enkenler and Tabatai.., 2003),* N acetyl glucosaminde and arylsulphatase are considered as indirect indicators of the presence of fungal biomass (sulphate esters are present only in fungal cells) *(Dick et al., 2000*).

An estimation of the microbial metabolic efficiency can be done with the use of metabolic quotient (qCO_2) which relates microbial respiration per biomass unit and reflects microorganism maintenance energy. The quotient appears to provide more sensitive indications of soils changes than either activity or population measurements alone. The metabolic quotient (qCO_2) reflects the maintenance energy requirement of soil microbes *(Anderson et al., 2003)* and can be a relative measure of how efficiently the soil microbial biomass is

utilizing C source, as well as the degree of substrate limitation for soil microbes (*Anderson et al., 2003; Dilly and Munch., 1998*). The percentage of MBC to TOC (q_{mic}) could be used as a stability indicator for quick recognition of an environmental change, it reflects the contribution of microbial biomass to soil organic carbon and indicates the substrate availability to the soil microbes, being value below 2% a signal of SOM depletion *(Anderson et al., 2003).*

As represents the presence of viable microorganisms and their oxidative capability, MBC can be a valid approach to evaluate the significance of microbial activity in the cycling of elements in soils under different fertilization. The aim of this study is to estimate the effect of the addition of a slurry on the functions of the microbial community of a Mediterranean soil and to check in time the effect on these activities exerted by the addition on the slurry of a nutrient solution containing one molecule with anti nitrifying action. In fact, the potential interference of N availability with soil carbon dynamics is less known.

Indeed, the addition of easily degradable organic matter results in an expected increase in microbial biomass and enzyme activities related to it, while are not well known the effects of inhibition of N availability on soil microbial activity. We prepared four treatments, adding respectively 250 kg ha^{-1} of N (Chapter 2-Material and Methods): soil control (C), soil with the nitrification inhibitor (Nib), soil with bovine effluent (Ef) and soil with effluent and anti nitrifying solution (Ef+Nib). We determined the pH of soil, the mineralization of soil carbon and the carbon of microbial biomass, and likely changes over time in different view. The pH of soil has been called the master variable, since it can have an overriding effect on numerous process and properties - chemical, physical and biological *(Bredy and Weill., 1999).* The activities of eight enzymes were determined by fluorescence, in particular, were measured the activities of cellulase, and xylosidasi βglucosidasi (C cycle), leucine and N-acetyl β glucosaminidase (NAG) (N cycle), acid phosphatase and P for the aryl sulphatase to the cycle of S, with the addition of acetate esterase to intracellular activity. At the end of the incubation period, the effects of processing on the characteristics of soil showed clear correlations. The mineralization triggered by fresh organic matter to soil (animal manure) was significantly reduced when adding the anti nitrification product during the incubation period, as well as the

change in the microbial pool size, estimated by measuring the microbial biomass carbon, appeared to be considerably lower when the slurry was added to the anti nitrification solution. The enzymatic activities related to the cycles of carbon and nitrogen in this study showed lower activity in soils treated with slurry and anti nitrification product than in treatment with the only slurry, and they were related to the measured pH values in time. In this study, all the parameters after the addition of the anti nitrifying at the slurry were significantly lower and the changes of enzyme activities, during the weeks after the treatment, followed the trend of carbon mineralization. The main effect of Nib on biochemical variables was examined through analysis of variance for N fertilization (one way-ANOVA), followed Post hoc test. Correlation between different variables were computed using Pearson's correlation statistics. Enzymatic discrimination among treatments were computed using the discriminant function analysis (DFA). DFA is a multivariate statistic that uses *a priori* classification to maximize the power of separation of those variables that generate the greatest difference between the experimental groups. DFA was followed by determination of Mahalanobis distances for computing the effective separation. All statistics were produced with STATISTICA Version 7 (data analysis software, Stat.Soft. INC 2004).

4.2 RESULTS

Chemical and Biochemical analysis

The pH of soil was monitored during the experiment and changes occurred since the first days in the treatments with N inhibitor (Nib) (Table 4.1). Cumulative C mineralization (R_{cum}) increased with Ef treatment, corresponding to 1.4 mg C g^{-1} added and the soil with Ef+ Nib treatment showed value significant lower than Ef effect (Figure 4.1b). The daily respiration exhibited comparable values between the control soil and Nib and Ef+ Nib treatments; after 2 days, the Ef+ Nib started to respire at higher rates than the control, but lower than Ef (Figure 4.1a). MBC significantly changed after the treatment. Addiction of effluent increased significantly the MBC of 40% compared to the soil control. The effluent arranged with Nib showed an effect on MBC at the end of incubation with a decrease of 50% of MBC compared to the effluent.

All the treatment showed significant decrease of MBC after 7 days of experiment (Table 4.2). Microbial respiration did not show significant differences between the treatment with Nib and the Control, but increased significantly with the effluent addiction. MBC, basal respiration, and qCO_2 showed different values at the end of incubation (Table 4.3). The basal respiration per unit of microbial biomass (qCO_2) significantly increased with Ef and Ef+ Nib at the end of incubation. The q_{mic} showed greater values at the end of the treatments, the highest increase as expected was reported in Ef and this value was reduced in Ef+ Nib (Table 4.3).

Tab 4.1. Value of pH in water and KCl during the experiment. Standard Deviation are reported on brackets

	Day1		**Day 4**		**Day 7**		**Day 14**		**Day 28**	
	pH (H_2O)	**pH (KCl)**	**pH (H_2O)**	**pH (KCl)**	**pH (H_2O)**	**pH (KCl)**	**pH (H_2O)**	**pH (KCl)**	**pH (H_2O)**	**pH (KCl)**
	Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean
C	7.04(0.4)	6.8(0.19)	6.84(0.01)	5.7(0.2)	7.08(0.1)	6.85(0.1)	6.85(0.1)	6.1(0.1)	7.03(0.2)	6.46(0.1)
Nib	5.5(0.1)	5.2(0.2)	6.2(0.1)	5.6(0.13)	6.4(0.2)	5.3(0.2)	6.3(0.2)	5.98(0.2)	6.5(0.13)	6.1(0.2)
Ef	6.9(0.2)	6.6(0.14)	7.57(0.2)	6.9(0.2)	7.24(0.14)	6.5(0.1)	6.84(0.14)	6.21(0.14)	7.23(0.23)	6.87(0.14)
Ef+Ni	6.59(0.1)	6.1(0.1)	6.4(0.1)	5.7(0.19)	6.8(0.2)	5.1(0.1)	7.1 (0.1)	6.89(0.1)	6.87(0.25)	6.1(0.1)

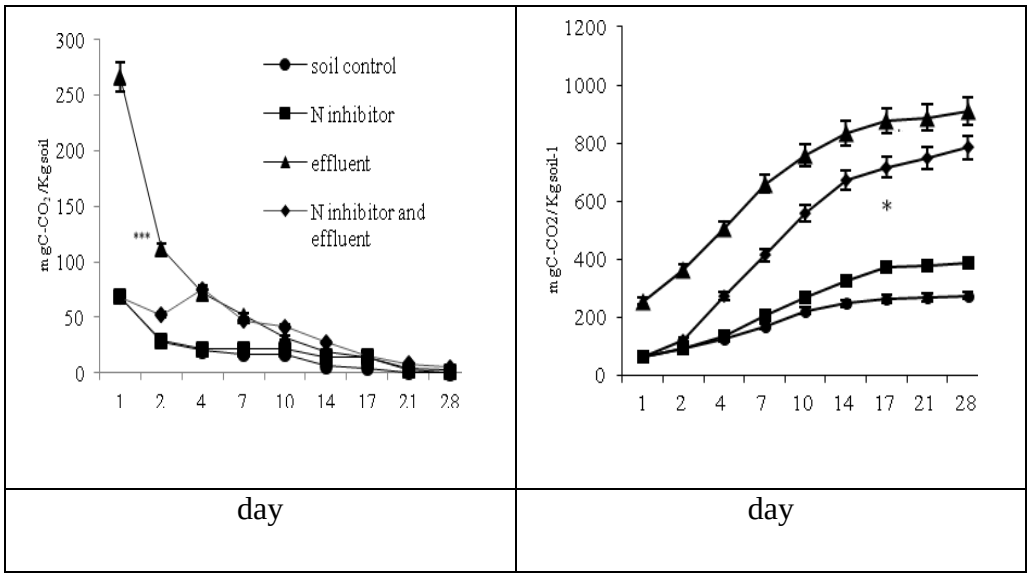


Fig 4.1 Daily and Cumulative respiration for each treatment during incubation time. Asterisk denotes where Ef+Nib treatment is different from the other groups C, Nib, Ef (P<0,05 Post hoc test).

Tab 4.2. Microbial Carbon Biomass is reported for each treatments during the experiment. The letters denote significant differences between the treatments (P<0,05 Duncan test).

	MBC (µg C g^{-1})	MBC (µg C g^{-1})	MBC (µg C g^{-1})	MBC (µg C g^{-1})	MBC (µg C g^{-1})
	Day 1 Mean	Day 4 Mean	Day 7 Mean	Day 14 Mean	Day 28 Mean
C	116.45^{ns}	107.38^{ns}	89.54^{ns}	74.28^{ns}	98.82^{ns}
N i	62.05^{a}	110.8^{b}	91.73^{b}	80.99^{b}	103.7^{b}
Ef	196.67^{b}	194.86^{b}	134.48^{a}	207.5^{b}	264.41^{b}
Ef +Nib	209.48	154.93	137.78	219.17	150.42

Tab 4.3 Biochemical parameters during the experiment. Standard erro (ES) are reported on brackets.

	MR_{basal} (µgC-CO_2 $g^{-1}h^{-1}$)	qCO_2 (g C-CO_2h^{-1}kg MBC^{-1})	q_{mic} (µg MBC mg TOC^{-1})
C	0.39(0.03)	0.17(0.02)	2.14(0.4)
Nib	0.95(0.09)	0.20(0.04)	3.65(0.2)
Ef	3.13(0.8)	0.65(0.15)	8.50(1.3)
Ef+Nib	5.17(0.9)	0.78(0.03)	7.34(0.7)

Enzymatic activities

The enzymatic activities are express per unit of MBC (specific activities) which represent a measure of microbial physiological capacity. Putting the control enzymatic activity as 100, xyl, βG, aryl (Fig 4.2 A, C, F), activities were significantly higher after Ef treatment and back at control activities at the end, while Leu effect was clear after 4 days (Fig 4.2 E). The other enzymes showed that the use of Ni decreases this activity keeping the same trend. Cell (Fig 4.2 B) and Pho activities don't were significantly changed during the treatment and NAG activity become to increase significantly since the last ten days (Fig 4.2 D, G). The Muf activity trend showed a decrease after seven days with each treatments (Fig 4.2 H).

Among several biological properties, the βG activity was the only parameters correlated with pH (Table 4.4). βG was also positively correlated with MUF activity and C microbial biomass (Table 4.4).

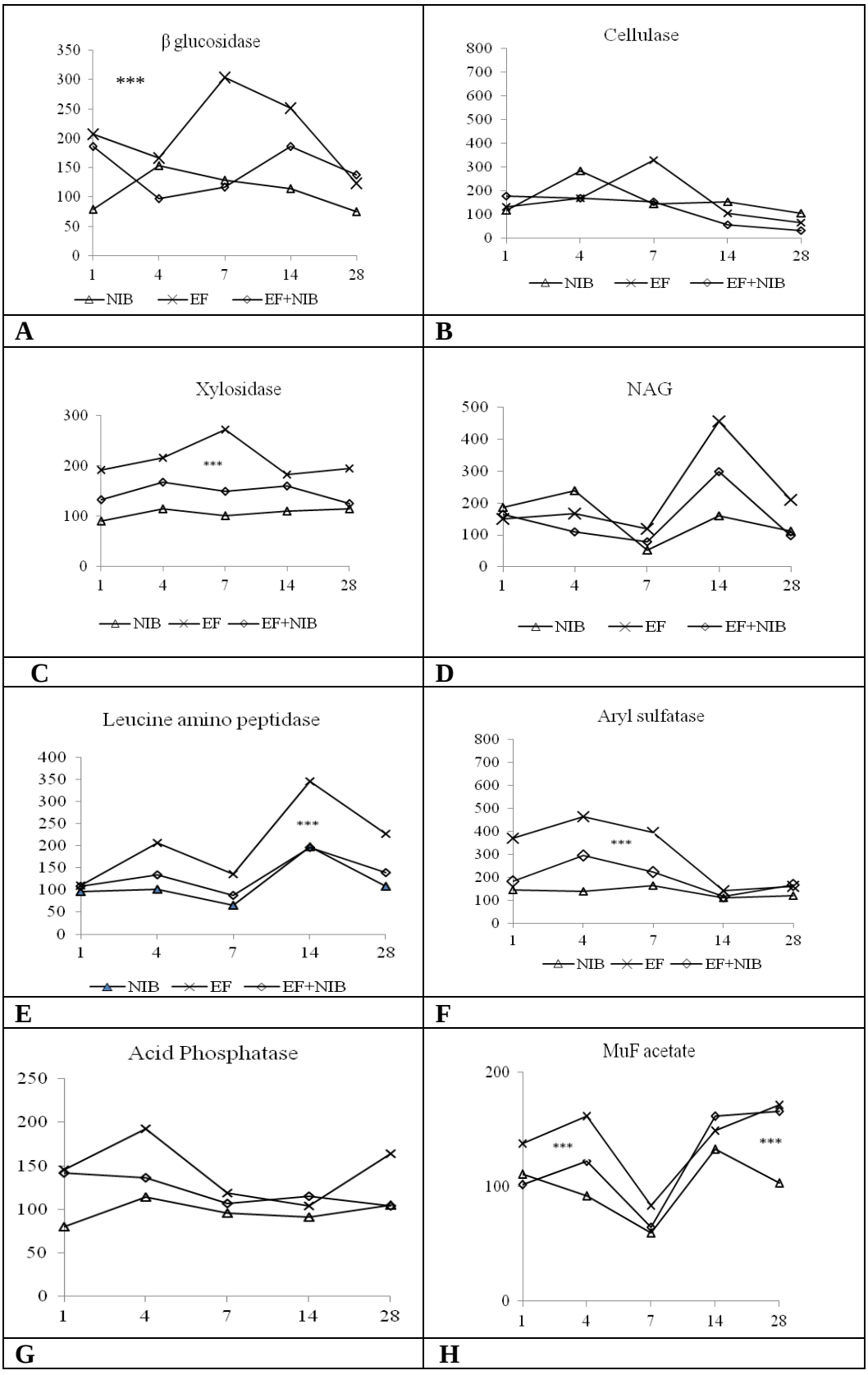


Fig 4.2. Pattern of the enzymatic activity (EA) of A)β-glucosidase B) Cellulase C) β-xylosidase D) N-acetyl β - glucosaminidase (NAG) E) Leucine amino peptidase F) Aryl sulphatese G) Acid phophatase H) MUF acetate. Asterisk denote where treatment EF+Nib is different from the other groups C, Nib, Ef (P<0,05, Post hoc test).

Tab 4.4. Pearson correlation coefficient r computed for the biochemical variables at the end of incubation. Correlation are significant at P<0,05

	Leu	Cel	NAG	BG	Xyl	Aryl	Pho	MUF	Cmic	pH	Rcum
Leu		0.68	ns	ns	0.55	Ns	0.81	ns	Ns	ns	ns
Cel	0.70	-	ns	-	-	-	0.60	-	-	-	-
NAG			ns	-	0.87	0.87	-	-	-	-	-
βG	ns	-	ns	-	-	-	ns	0.78	0.76	0.57	-
Xyl	0.55	-	0.87	-	-	0.91	ns	-	Ns	-	ns
Aryl	ns	-	0.87	-	-	0.91	-	-	Ns	ns	-
Pho	0.81	0.57	ns			ns	ns	-	-	-	ns
MUF	-	-	ns	0.78	-	ns	ns	-	0.57	-	
Cmic	-	-	ns	0.76	-	-	ns	0.57	-	-	0.61
pH	-	-	-	0.57	-	-	-	-	-	-	-
Rcum	-	-	-			-	-	-	0.61		-

Statistical analysis

DFA was performed on enzymatic activities including data at the beginner and the end of incubation. The multivariate analysis detected two significant roots, explaining 96% of the total variance between groups: the analysis yielded a clear separation, particularly between groups belonging to treatment Ef+Nib, Ef and control, as reported in Fig 4.3. The EF+Nib and Ef groups were discriminated from control and Nib (distance between group P<0.01), whereas there were no significant differences between the treatment Nib and the Control.

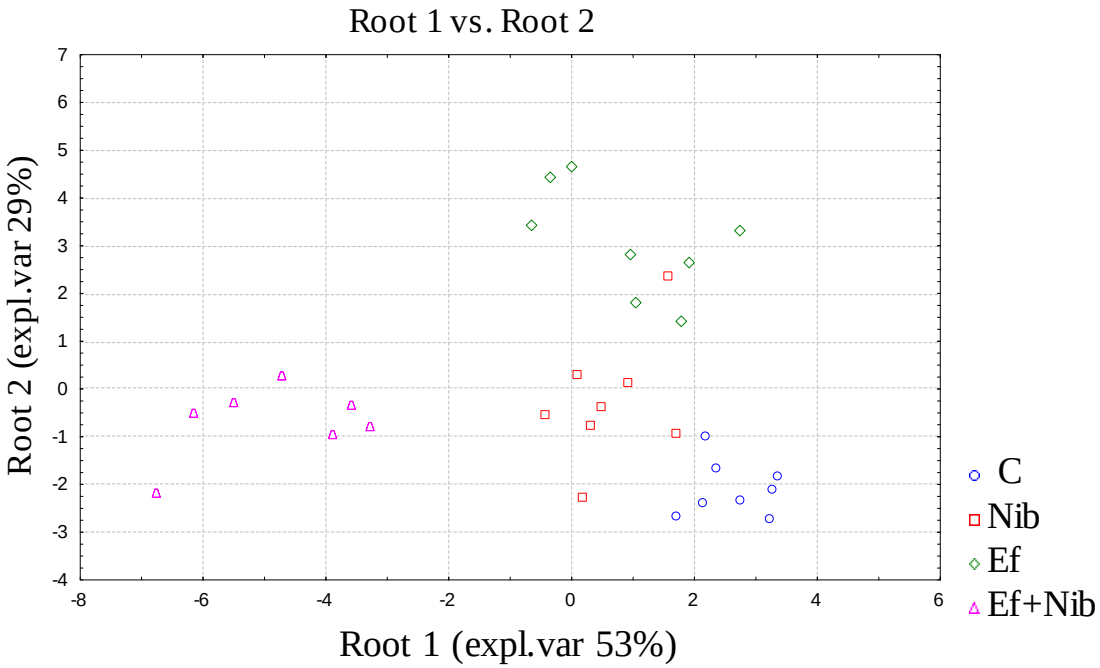


Figure 4.3. Correlations of variables to the two roots of the DFA analysis applied to enzymatic data (correlations in bold are significant at P<0,05).

Tab 4.5. Correlations of variables to the two roots of the DFA analysis applied to enzymatic data

ENZYMES	ROOT 1(64%)	ROOT 2(32%)
LEU	0.00	0.50
NAG	-0.57	-0.16
CEL	-0.39	0.25
βG	-0.63	0.13
XYL	0.63	0.54
ARYL	-0.99	0.16
PHO	0.00	0.69
MUF	-0.42	0.27

NAG, βG and ARYL were the enzymes that contributed most to discrimination (Table 4.5) with a negative correlation to the first root (which explained 64% of variance). Concentration of the other enzymes were correlated to the second root (which explained the 32% of variance) and PHO, XYL and NAG showed the highest correlations coefficients.

The treatment Ef, displaced all groups negatively along root 1, hence towards a higher ARYL, βG activity. It also shifted the groups Ef+ Nib negatively along root 2, hence yielding a trend towards a lower activity of PHO and XYL.

4.3 DISCUSSION

Biochemical properties are known to be highly sensitive to soil's fluctuations and monitoring trends and changes over time is essential for their application. In this study significant fluctuations of almost all biochemical properties were found, revealing a high degree of sensitivity to short term-changes. In fact, the effect of all the treatments on biochemical indicators was direct at the beginner of the experiment suggesting a quick response of microbial functions. The considerable increase of MBC, q_{mic}, $qCO_{2,}$ in Ef treatment was due to the easily available organic matter added to soil, and the decrease of values with Nib treatments suggested that the microbial functions are influenced by the nitrification inhibitor. The increase of enzymatic activity of βG, CEL, XYL, which are key C –enzymes, with Ef and the lowest value with Ef+Nib could response to changes of soil physico-chemical properties, that affected soil microorganisms. The response of biochemical function changed during the incubation time with Nib, the value of C mic, MUF and NAG activity resulting lower after 7 days than beginner and the end of incubation. The positive correlation between βG and pH suggested that the changes in the pH value of soil after treatments with Nib should be important for microbial biomass. The DFA showed that the effect of Ef and Ef+Nib treatments on soil are separated on two roots and they were significantly different from the control. With this experiment we can assume that on this soil, the combined use of nitrification inhibitor and animal manure have a positive effect on soil microbial functions and consequently on soil carbon dynamics; in the specific Nib slows the

increase of functional activities determined by Ef and resets it close to soil control values. Further investigation is required, both to verify the results in yield experiment and to robustly assess the influence on soils coming from other ecosystems. The microbial response at treatments and the experiment on the first season growth of field application are reported in following Chapters 5 and 6, respectively.

CHAPTER 5: Microbial dynamics under organic combined fertilization in a Mediterranean Soil from Short Rotation Forestry.

5.1 INTRODUCTION

Soil is a complex matrix and bacteria exist in or on the surface of soil aggregates; therefore, the ability to separate these cells from soil components is vital for studying biodiversity *(Trevors, 1998)*. Soil microorganisms play important roles in soil quality and plant productivity. Our knowledge of soil microbial diversity is limited in part by our inability to study soil microorganisms. Torsvik et al, *(1990)* estimated that in 1 g of soil there are 4000 different bacterial ‘‘genomic units’’ based on DNA–DNA re-association. Traditionally the analysis of soil microbial communities has relied on culturing techniques; in recent years molecular methods have provided a new approaches of phylo-genetic diversity of communities, and techniques based on PCR have been used to overcome the limitations of culture-based methods; however, they are not without their own limitations *(Kirka et al., 2004)*. If the method of cell extraction used is too gentle, Gram-negative, but not Gram-positive bacterial cells would be lysed. If the method is too harsh, both Gram-negative and Gram positive cells may be lysed but their DNA may become sheared *(Wintzingerode et al., 1997).* Lysis efficiency also varies for different fungal cells. The variation in the ability to break open cells or fungal structures can lead to biases in molecular-based diversity studies. A biochemical method that does not rely on culturing of microorganisms is phospholipids fatty acid (PLFA) analysis. This method provides information on the microbial community composition based on groupings of fatty acids *(Ibekwe and Kennedy., 1998).* Fatty acids make up a relatively constant proportion of the cell biomass and signature fatty acids exist that can differentiate major taxonomic groups within a community. Therefore, a change in the fatty acid profile would represent a change in the microbial population. A environmental changes create condition that can be stressful for microorganism, microorganisms must have the other

hand, both the bovine effluent (Ef) and Bio-stimulant (Bio) had higher pH values than control soil. There were little pH variations over time.

5.2 RESULTS

pH

he pH of soil was monitored during the experiment. A decrease with around 0,3-0,5 units occurred from the first measurement (day 3) and throughout the incubation in the treatments with N inhibitor (Nib treatments) (Table 5.2). On the other hand, both the bovine effluent (Ef) and Bio-stimulant (Bio) had higher pH values than control soil. There were little pH variations over time.

Tab 5.1. **pH variations during incubation time; standard error (n=3) is reported in brackets.**

	Day 3	**Day 6**	**Day 9**	**Day 16**	**Day 30**
C	7,0 (0,01)	7,2 (0,01)	7,2 (0,01)	7,1 (0,01)	7,2 (0,01)
Nib	6,7 (0,01)	6,7 (0,03)	6,7 (0,03)	6,7 (0,01)	6,7 (0,01)
Bio	7,5 (0,12)	7,6 (0,03)	7,8 (0,03)	7,8 (0,01)	7,4 (0,03)
Ef	7,5(0,05))	7,6 (0,08)	7,6 (0,05)	7,6 (0,03)	7,3 (0,01)
Ef+Nib	7,2 (0,01)	7,4 (0,06)	7,4 (0,1)	7,4 (0,01)	7,4 (0,01)
Ef+Bio	7,5 (0,08)	7,6 (0,05)	7,4 (0,08)	7,4 (0,1)	7,4 (0,01)

.

Microbial growth and activity

Respiration rates was positively stimulated by Ef (Fig. 5.1) the combined use both of Nib and Ef and Bio +Ef generated a significant ($P<0,05$) inhibition on this increased (Fig. 5.1); The single treatment of Bio and Nib didn't showed

significant difference to soil (Fig. 5.1). A significant positive correlation (P<0,001) was showed between pH and soil's respiration (Fig 5.4b).

Bacterial growth was stimulated by Ef addiction and declined with the addition of Nib (p<0,05); the single use of Nib drastically inhibited the growth (Fig 5.2a). Also the Bio treatment decreased bacterial growth significantly (p<0,01; Fig 5.2b), although not as much as the Nib treatment..There was a significant correlation between pH and treatments with Nib (Fig 5.5a). Bacterial growth for the treatments with Nib were positively correlated with soil respiration (p<0,01; Fig 5.5b) while on bio-stimulants treatments did not showed significant correlations between the analysis.

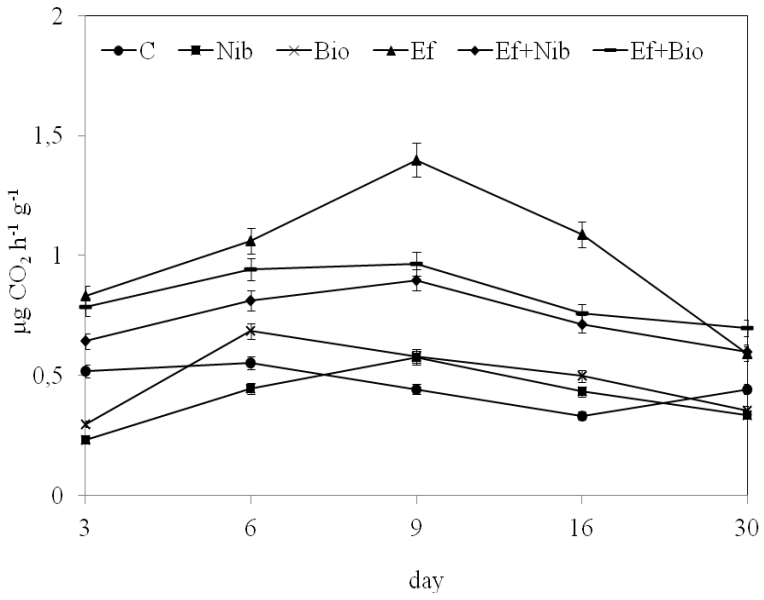
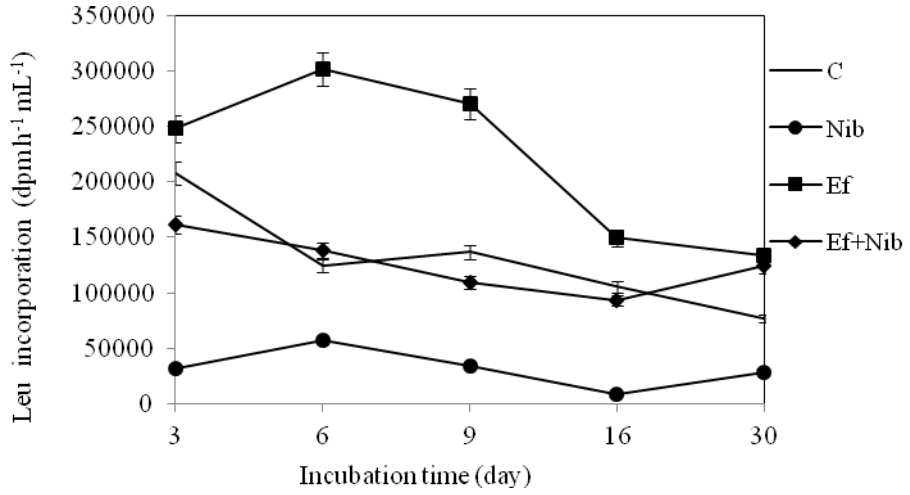


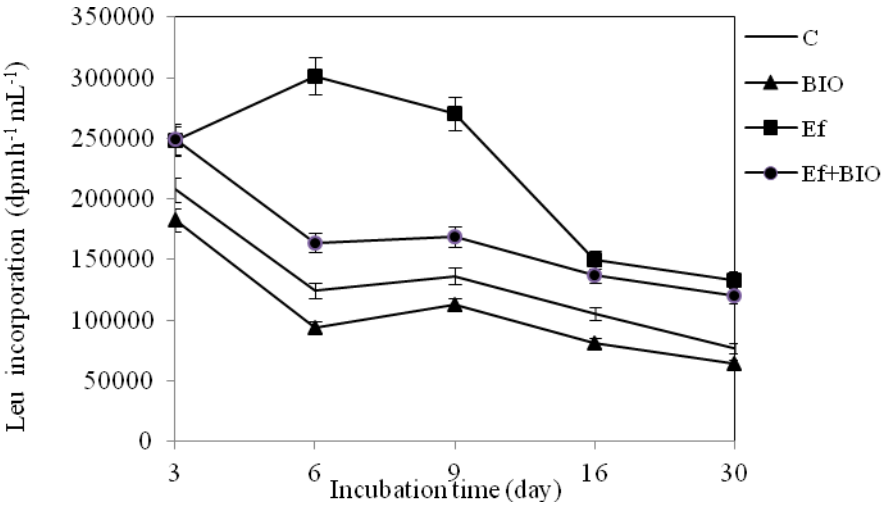
Fig 5.1 Respiration rates for each treatment during incubation time soil. Asterisk denotes where Ef+ Nib and Ef+ Bio treatments are different from the other groups C, Ni, Ef (p<0,05).

Fungal growth was highest after Ef treatment, and decreased with the application of Nib. However, the combined use Ef and Nib or Bio did not differ from the control (Fig 5.3a,b). On bacterial growth the interaction of Nib and Bio togheter with Ef are significantly different from the treatments alone. When the soil was pretreated with Nib the Ef addiction led a significant reduction in the first 10 day compared with Ef alone (p<0,01) and the combined use of Bio and Ef showed a significant slower growth after 6 and 9

day to come back again at the same Ef values. The treatments interaction on fungal growth did not showed difference from single use of Ef; Ni+ Ef reported significant different value from Nib at the beginner ($p<0,01$), after 9 days up to end of incubation ($p<0,05$), the Bio+Ef was significant different from Ef on the first 9 days ($p<0,05$).This indicated that bacterial growth responds was more evident than fungal growth to the application.

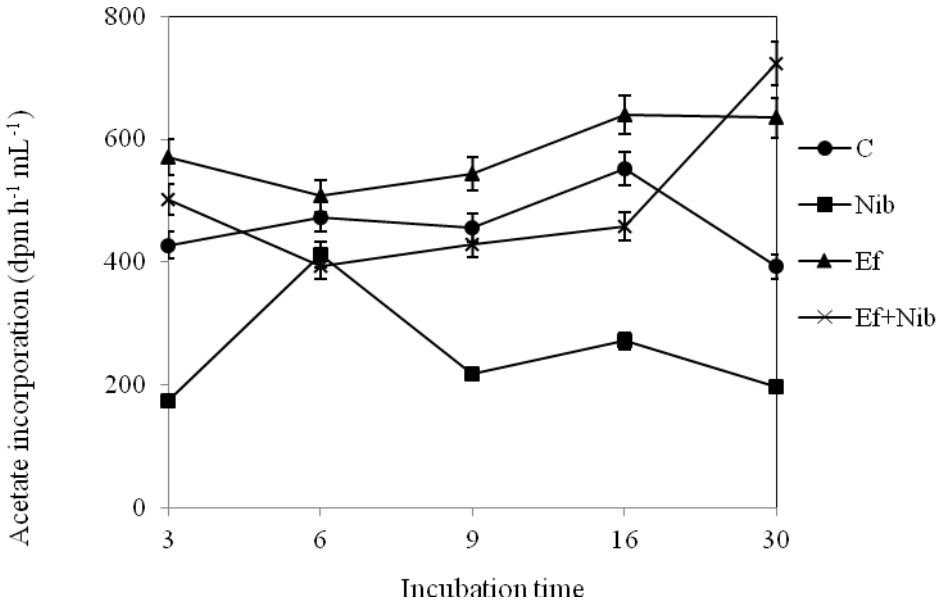


a)

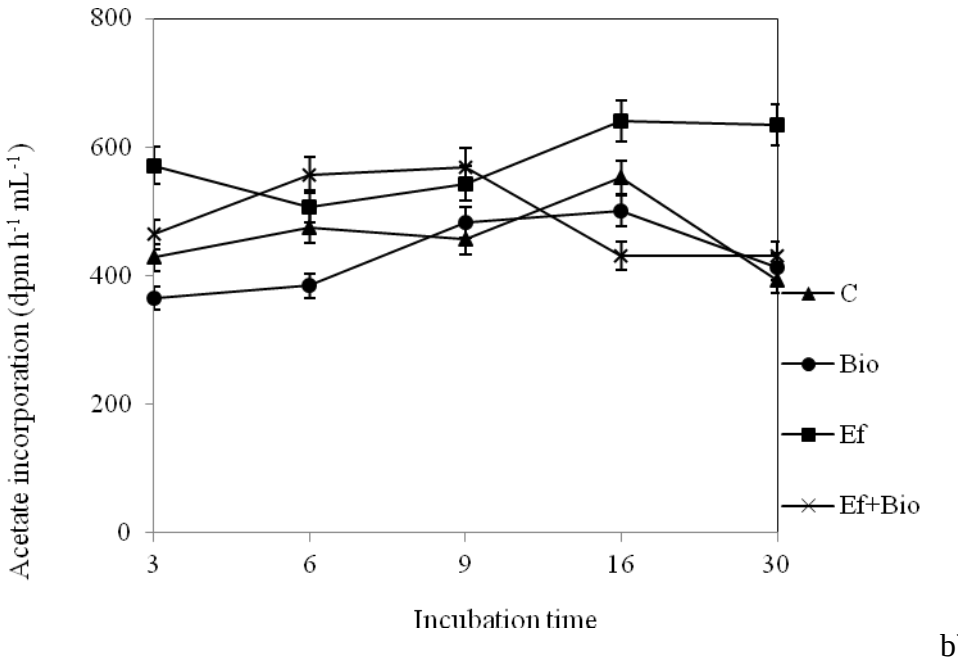


b)

Fig 5.2. Bacterial growth daily trend measured using leucine (Leu) incorporation in soil (a) treated with Bovine Effluent and Ef combined with Nib (b) treated with a Biostimulant, and Ef combined with Biostimolant. Measurements were made on n=4, scale bars denote SE.



a)



b)

Fig 5.3. Fungal growth measured using acetate (Ac) incorporation in soil expressed as compared soil control *per cent* (a) treated with Bovine Effluent and Ef combined with Nib (b) treated with a Biostimulant, an Ef combined with Biostimolant. Measurements were made on n=3, scale bars denote SE.

This is also seen when cumulative values were calculated over the whole 30 day period (Fig.5.4). The results was standardized to the control in order to enable comparisons between the methods. The treatments without Ef did not showed a positive increase on the soil; in the specific cases the soil respiration

and fungal growth did not changed from the control while the bacterial growth was significantly inhibited by Nib. The treatments with organic application and Nib and Bio led highest value on respiration increasing the microbial response compared to control clear also with bacterial that grow up with significant more ($p<0,05$) with Ef and Ef+Bio than Ef+Nib .The Fungal growth did not significant response at the end of experiment between the treatments.

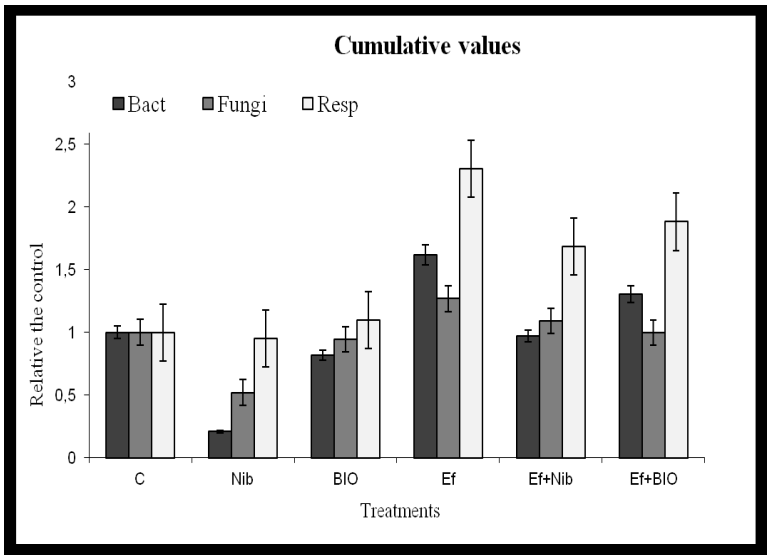


Fig 5.4 Cumulative values standardized to the control. Measurements were made on n=3, scale bars denote SE

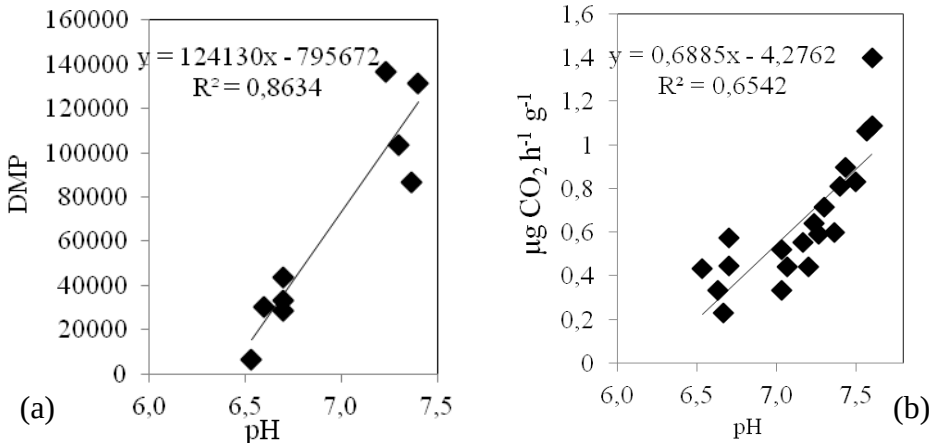


Fig 5.5 a) correlation between pH in water and bacterial growth using leucine incorporation on the treatments with Nib during the incubation time b) correlation between pH in water and soil respiration during the incubation time for each treatments.

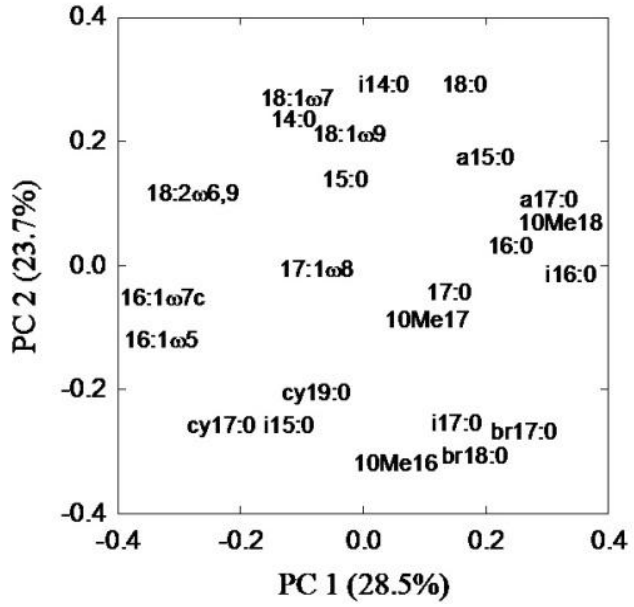
Phospholipid fatty acid analysis

PLFA were extracted from the soil after 14 and 28 days incubation. No significant difference were found between the days and thus only mean values for the two samplings are reported. The microbial biomass, estimated as total PLFAs, was 4,19±0,97 nmol g^{-1} in the control soil, after Nib addiction subsisted a decreasing for all groups while the treatments with Ef involved a little increase of the values that are reduced by Bio and Nib addiction in the table below are showed the nmol g^{-1} calculated for each microbial groups at the end of experiment.

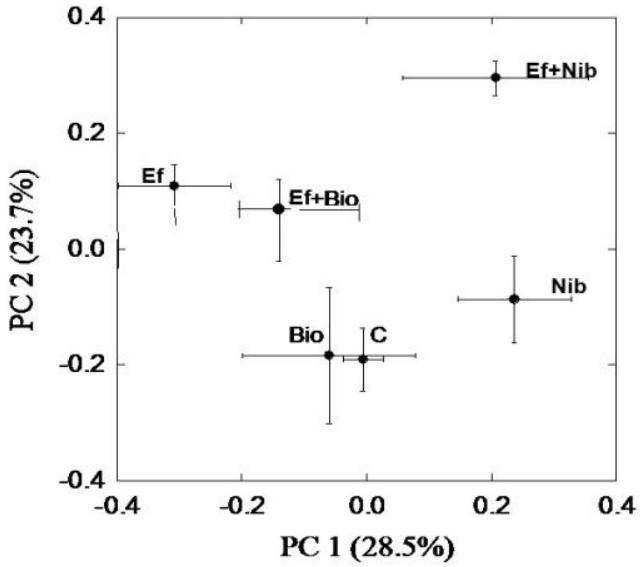
Tab 5.2 nmolg^{-1} PLFA extracted from the soils (at the end of experiment).

nmol g^{-1}	**C**	**Nib**	**Bio**	**Ef**	**Ef+Nib**	**Ef+Bio**
Total PLFA	4.19(0.97)	3.09(0.89)	4.09(0.69)	4.89(0.93)	3.82(1.20)	4.35(1.08)
Fungal PLFA	0.08(0.01)	0.04(0.09)	0.06(0.02)	0.10(0.02)	0.06(0.01)	0.09(0.02)
Bact. PLFA	2.17(0.5)	1.89(0.67)	2.10(0,76)	2.23(0.56)	1.78(0.28)	2.24(0.30)
G+ bacteria	0.93(0.25)	0.88(0.23)	1.02(0.11)	1.15(0.18)	0.87(0.31)	1.10(0.23)
G- bacteria	0.56(0.19)	0.56(0.2)	0.66(0.1)	0.71(0.13)	0.52(0.20)	0.68(0.16)
Act	0.37(0.03)	0.38(0.06)	0.42(0.04)	0.37(0.07)	0.36(0.06)	0.38(0.08)
F/B	0.04	0.02	0.03	0.05	0.03	0.04

Principal component analysis (PCA) of mol% of the different PLFAs was used to analyze the importance of different treatment on the soil PLFA pattern, without the total amounts confounding the results (Fig 5. 7 a,b). The first PC explained 28,5% of the variation, while the second component explained a 23% variation. PC1 has a significant treatment-effect ($F=7.6$, $p<0.01$) with Nib significant different from C ($p<0.01$) and BIO ($p<0.05$). Using multiple regression, pH was significantly related to PC 1 ($P < 0.001$). PC2 was significantly affected by Ef ($F=28.4$, $p<0.001$). PC 2 was partly explained by soil respiration and bacterial growth, with no significant correlation to pH (Table 5.2).



a)



b)

Fig 5.7. Principal component analysis of the PLFA pattern of soils from different treatment. PC 1 and 2 accounted for 28,5% and 23,7% of the variance, respectively.(a) loadings of the different PLFAs, (b) Scores of the different treatment (bars indicate SEs).

Tab 5.3 Axis Correlation of variables to the two axis of the PCA analysis applied to PLFA data.

	Axis1	Axis 2
pH	0.67*	0.5
Ergosterol	0.74**	0.50
Resp	0.43	0.94***
Bacterial	0.37	0.82***

Fungal biomass (ergosterol)

The fungal biomass (estimated as ergosterol concentration in the soil) was unchanged during the experiment (Table 5.4) and any difference were found during the incubation and between the treatments.

Tab 5.4 Ergosterol content as indicator of fungal biomass.

Ergosterol (µg g^{-1} dw of soil)					
	Day 3	Day 7	Day 10	Day 17	Day 30
C	0.55 (0.02)	0.52 (0.05)	0.48 (0.12)	0.49 (0.17)	0.47 (0.10)
Nib	0.49 (0.01)	0.56 (0.14)	0.53 (0.11)	0.49 (0.11)	0.53 (0.09)
BIOS	0.48 (0.1)	0.54 (0.08)	0.43 (0.09)	0.50 (0.09)	0.52 (0.07)
Ef	0.57 (0.1)	0.58 (0.05)	0.58 (0.06)	0.60 (0.06)	0.66 (0.08)
Ef+Nib	0.53(0.09)	0.47 (0.06)	0.52 (0.04)	0.54 (0.04)	0.48 (0.1)
Ef+Bio	0.52 (0.12)	0.59 (0.13)	0.44 (0.14)	0.41 (0.11)	0.64 (0.1)

5.3 DISCUSSION

Microbial dynamics are highly sensitive indicator to soil's fluctuations and changes *(Lagomarsino et al., 2010)* and in this study microbial fluctuations were found during the treatments revealing a high degree of sensitivity to short term-changes suggesting a quickly response of microbial functions. Mainly similar results of the methods: Nib decreased while Ef increased. BIO slightly negative for bacteria and changed PLFA. The respiration rate increase with the labile organic matter (effluent) and the combination with Nib and Bio involved a significantly activity decrease in according with reported on the Chapter 4 (biochemical response).Growth based methods have earlier been shown to be more sensitive than biomass-based, which also was found here. Drastic effects on bacterial growth, with little effects on PLFAs of bacterial groups, and fungal growth being affected but not ergosterol (biomass) Although respiration is the most common way to study microbial activity in soil, it lacks in resolution. For example, in a gradient of different pH respiration was little affect, while fungal

and bacterial growth changed dramatically *(Rousk and Bååth ., 2007).* This was also seen in this study, where for example the negative effects of Nib was much more evident for bacterial growth then respiration. The negative effect of Nib could be a direct toxic effect, but low pH has been shown to affect bacteria negatively *(Rousk and Bååth ., 2007)* However, it is likely not to be solely a pH effect since the decrease in pH was only one unit (and still being neutral), and the negative effect on bacterial growth on pH is usually seen at low pH (<pH5). Furthermore, respiration also slightly negative, and respiration is usually not affect by only one unit change around neutral pH. Thus, Nib is not only affecting nitrification, but appeared to have non-target effects on other bacteria. The addiction of easy degradable organic matter (Ef) was a substrate for the microbes increasing growth and activity especially on a soil very poor from the beginning .Bio did not significant difference during the incubation for the bacterial growth and showed a slightly negative during the first week for fungal. Leucine incorporation measures a mean growth of all bacteria in the soil. Still, within the bacterial group the different treatments affect subgroups differently. This is seen by the PLFA study where PC1 indicated changes within the community due to Nib. Bacteria, having the PLFAs a17 and i16, became relatively more common in treatments with Nib, while bacteria, having the PLFAs 16:1ω7 and 16:1ω5, became relatively less common. Thus, Nib did not only decrease bacterial growth and activity, it also altered the bacterial community composition. However, PLFA measurements only indicates that changes have occurred, and further studies are needed to pinpoint which species that are affected. With this closed experiment we can assume that on this soil, poor in nutrient, the combined use of N inhibitor and animal manure have an effect on the microbial dynamics and consequently on soil carbon dynamics; the increase of activity generated by labile carbon source was significantly reduced with the soil treatment of Nib. The biostimulant increase the soil respiration without upset the control soil dynamics. Further investigation is required, both to verify the results in yield experiment and to robustly asses the influence on soil from other ecosystem.

CHAPTER 6: Field experiment: Influence on gas emissions (NH_3 and CO_2) and plant productivity of short rotation woody crops organic fertilization combined with nitrogen inhibitor

6.1 INTRODUCTION

Short Rotation Woody Crops (SRWC) is a method of cultivation of selected material of fast growing species (poplar, willow, eucalyptus, black locust) to produce in short time large quantity of biomass for energy purposes (calorific or/and electric energy). In a SRWC very high plant density per hectare is used (from 5-10,000 to 15,000 plants per hectare), the rotation age is 2-3 years: in the first rotation the plantation is high forest managed in the following is coppice managed. In general after 4-5 rotations the plantation is removed and another is established *(Facciotto et al., 2007).*

In Mediterranean climate conditions in central-southern Italy characterized by very long summer drought period (growing season) and very difficult soil (clay soil) *Eucalyptus camaldulensis* lake Albacutya provenance is at the moment one of the best choice for conventional plantations in general and in particular for SRWC. Some Eucalypt clones have been recently selected by CRA-PLF of Rome more productive than *E. camaldulensis,* for two of these clones, Viglio and Velino, have been requested the European Union plant *patent (Mughini 1996, Facciotto et al., 2007).*

In Italy for a Eucalyptus SRWC two cultivation models are suggested: 1) 5,000-5,500 p ha^{-1} for 3-4 rotation cycles of 2-3 years; 2) 1,100-1,600 p ha^{-1} for 2 rotation cycles of 5-6 years. The second one is intermediate between the first and the conventional eucalypt plantation (1,100-1,600 p ha^{-1} for 2-3 rotation cycles of 10-12 years).

The SRWC with eucalyptus, especially those with cuts every 2-3 years, require amount of water and of nutrient greater than those of the crops of conventional *Eucalyptus*. In fact, in Brazil it is estimated that a plantation *E. grandis* at a density of 5.3 / ha to 2.5 years of age (a SRWC) in the tissues absorb epigeal per hectare for N, P, K, Ca, Mg, respectively, 256; 28; 273; 125 and 33 kg for a

production of 85.70 tons of biomass. This is estimated in three times higher than that of a plantation conventional (600 to 1600 plants per ha with rounds of 8 years). The same for water contribution that is estimated to be 4 times upper *(Poggiani et al., 1983).* More generally it is estimated that a SRWC *Eucalyptus* requires an average of at least 100 kg of N year, the quantities are very variables because they depend on the stationary condition, species and clone used, system density, shifts adopted. A recent study carried out in Congo in clonal plantations of eucalyptus (density 600 p / ha, turns 7 years) showed that up to 2 years (the age of the cut in SRWC) nutrients absorbed by plants reduce the availability in the soil, at the end of the third round instead it creates an intense recycling process between plant and soil *(Laclau et al., 2000).*

The use of irrigation and fertilization to meet the demand of this type of crop is not feasible because is a very uneconomic crop *(Sperandio et al., 2004, Facciotto et al.. 2003).* Irrigation can be used as a rescue intervention and inorganic fertilizer plant and after the cuts *(Facciotto et al., 2007).* For this reason, the fertilization with manure where available may be an attractive option because allows you to properly dispose of the sewage and to give to the plant nutrients from those elements necessary for growth.

In effect the main limiting factor under the difficult Mediterranean climate, is long dry season, in costal central Italy precipitation can varies with an average annual rainfall of 580 mm.

Moreover, irrigation is not believed to be economically and environmental viable, although it is well documented that SRWC has a high water demand *(Lindroth et al., 1996; Hunth et al., 1997).* Instead, SRWC should be planted on soils that can adequately supply the trees with water.

To satisfy the need for agro ecosystem sustainability and the demands of society, most cropping systems must evolve towards more environmentally friendly practices.

The recycling of animal manures from intensive zoo-technical industries in the agro-ecosystem could increase soil organic matter and nutrient contents, with relative increase of plant productivity; moreover could be a good solution to help to solve the environmental and economic problems related to the disposal of these waste materials *(Melero et al., 2006)* and preserve water for irrigation

in Mediterranean area to assure high productivity, to minimize production costs and to limit off-site effect of fertilizer application.

The application of zoo-technical waste needs to appropriate treatments before it is applied to soil, to preserve the soil and the ground water, such as nitrate pollution. Uncontrolled application of slurry to soil can generate an excess of nitrates in the soil and consequently in the ground water, salts, trace metal, undesirable xenobiotic organic compounds and green house emissions.

The European Union Directive (91/676/EEC) was promulgated in the 1991 and has dictated basic agronomic principles regarding the use of animal manure source, zoo-technical and waste water from small food companies. It is one of the earliest pieces of EU legislation aimed at controlling pollution and improving water quality. While nitrogen is a vital nutrient that helps plants and crops to grow, high concentrations are harmful to people and nature. The agricultural use of nitrates in organic and chemical fertilizers has been a major source of water pollution in Europe and generally, farming remains responsible forever 50% of the total nitrogen discharge into surface waters.

Recently the attention has been paid to the possibility of using inhibitors nitrification (as 3,4 DMPP) with effluent to minimize and to solve the environmental problems of animal manure.

As reported in Chapter 2, Materials and Methods, paragraph *NH_3 volatilization and CO_2 emissions*, NH_3 and CO_2 fluxes were monitored for a month after the treatment. A simple chamber method for determining ammonia in the field (Drager-Tube Method, DTM) *(Pacholski et al., 2006)* was used to estimate ammonia volatilization.

The CO_2 emission from the soil was measured every day in the first week and 2 time a week thereafter in three randomized area for each plot. Were determined the carbon loss from the soil with a portable dynamic closed – chamber infra red gas analyzer system *(Mancinelli et al., 2010; Pumpanen et al., 2004*) (EGM-4) carbon dioxide emission. The soil temperature was measured by the soil chamber simultaneously to the CO_2 analysis at 5 cm depth by using the "STP -1 soil temperature Probe" connected with EGM.

The second objective of this experiment was to investigate significant differences between treatments with bovine manure with and without addiction of a nitrification inhibitor, 3,4 DMPP (Nib) in a SRWC to establish whether the

3,4 DMPP in addiction to effluents offers agro-forestry advantages and to estimate also if the hybrid clones keeps the greater production and adaptability than the *E. camaldulensis*. Many studies *(Chen et al., 2008; Di and Cameron, 2004)* reported significant reduction in NO_3^- formation in soil and N_2O loss has also been reduced due the addiction of nitrification inhibitors (Nib).

6.2 RESULTS

The experiment was conducted in June 2011. A randomized block design with three replicates was used for each fertilized trials. Three treatments were applied: one treatment was the bovine effluent (Ef) application, the second was the addiction to the bovine effluent of 3,4 DMPP (Ef +DMPP) and the third was the effluent with the fertilizer ENTEC® 46 that contains 3,4 DMPP (Ef +ENTEC). The control was not fertilized (C).

The fertilizer was applied once in June, by furrowing soil with a tanker and following by the addition of 170 kg N ha^{-1} to soil to respect the European Normative. In the application with ENTEC® 46 an agronomical dose was applied, 240 kg N ha^{-1}.

NH_3 emissions

The ammonia flux was detected only for three days after treatment, and this lost of flux after 92 hours should be due to the effect of the disposal procedure, i.e. the burial of fertilizers into the soil.

On Figure 6.1 different results for the treatment are shown. The data of ENTEC® 46 plus Ef were normalized for N content of 170 kg ha^{-1}, the same rate of Ef treatment. Any difference was detected with respect to EF treatment. On the contrary, the addition of 3,4 DMPP at soil showed a real decrease during 52 hours after the treatment, that was significant for ANOVA test at 26 hours.

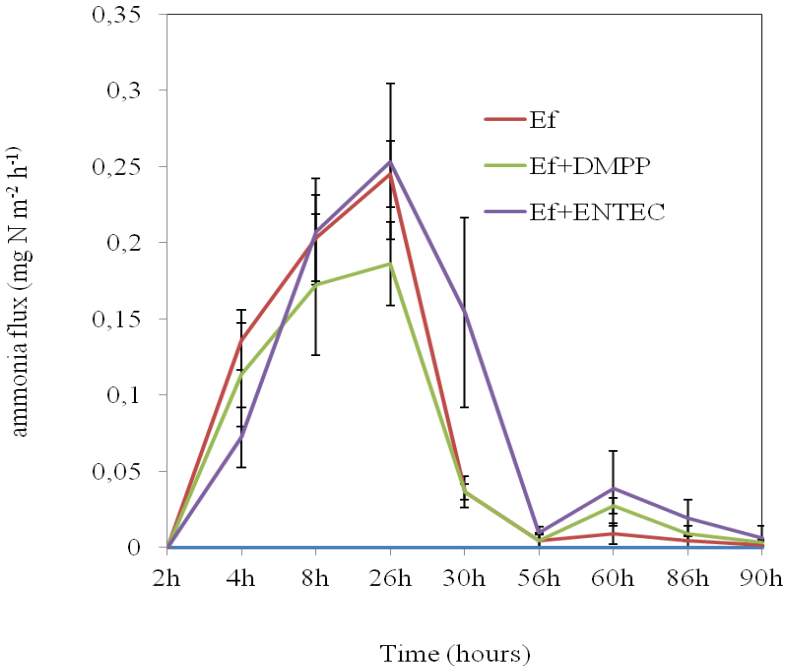


Fig 6.1 Ammonia Flux during 90hs later of treatment.

CO_2 emissions

The mean values of the CO_2 flux during the 1-month study period (June –July 2011), were higher than the baseline of the control plot, in particular they increased with Ef to 5 kg CO_2–C ha^{-1} d^{-1} and then decreased during the first week reaching 0.8 kg CO_2–C ha^{-1} d^{-1} after 1 week (Fig. 6.2).

A significant effect of the Ef +3,4DMPP and Ef + ENTEC treatments on the CO_2 flux was observed at the peak of first day ($p<0,01$), and during the week after. The decreasing trend of the CO_2 flux, observed in the Ef + ENTEC was not significantly different from the decrease showed with Ef +3,4DMPP (Fig. 6.2). The temperature of soil was monitored on morning (22–27 °C) and on the warmest hours (28–32 °C) (Fig. 6.3). In according with the Mediterranean climatic conditions (cool-humid winter and hot-dry summer) the water soil content did not change (8%).

On the Fig 6.4 were correlated the soil temperature means with the CO_2 fluxes showing a positive correlation between temperature and organic fertilization (R=0.84). For the temperature range taken with this experiment the CO_2 emissions increased during the time (one month) with lower temperature (23° C) after Ef addition, while decreased at highest temperature (29° C). On the contrary for the treatment with 3,4 DMPP and ENTEC® the correlation was not so significant (R=0.34 and R=0.17 respectively). In particular, for Ef +3,4 DMPP we did not found any correlation. This different trend should be explicated because the emissions were kept down from the 3,4 DMPP (with best performance) and ENTEC® 27, independently by the temperature effect, suggesting the effective action of the investigated nitrification inhibitor in that field temperature range.

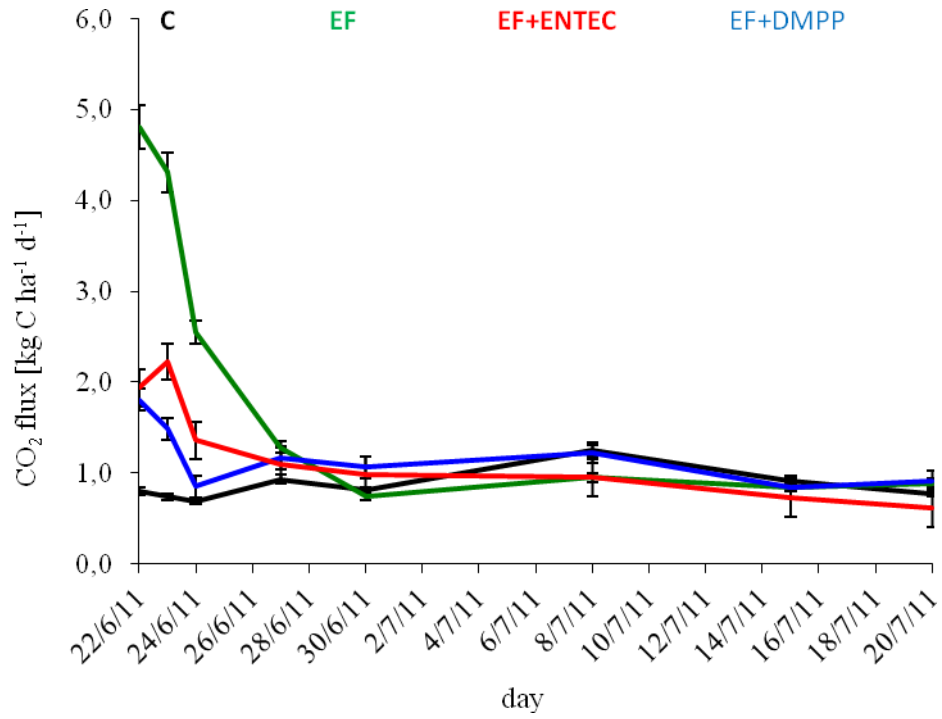


Fig 6.2 Daily average variation of soil CO2 flux (kgCha−1 d−1) over the 1 st month of measurements. Bars are standard errors (n = 3).

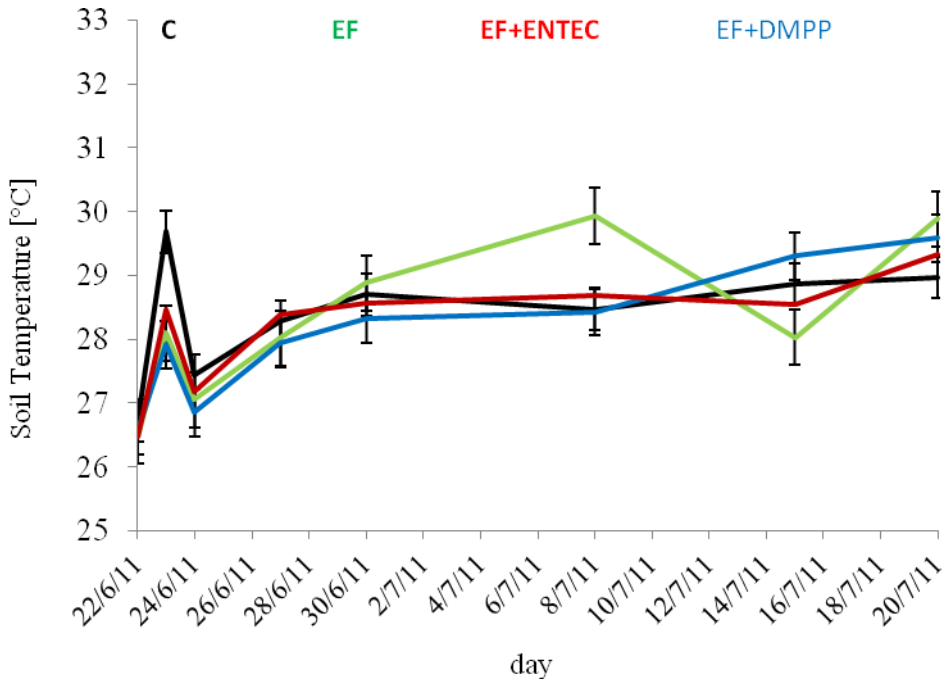


Fig 6.3 Daily average variation of soil temperature (◦C), over the 1st month of measurements. Bars are standard errors (n = 3).

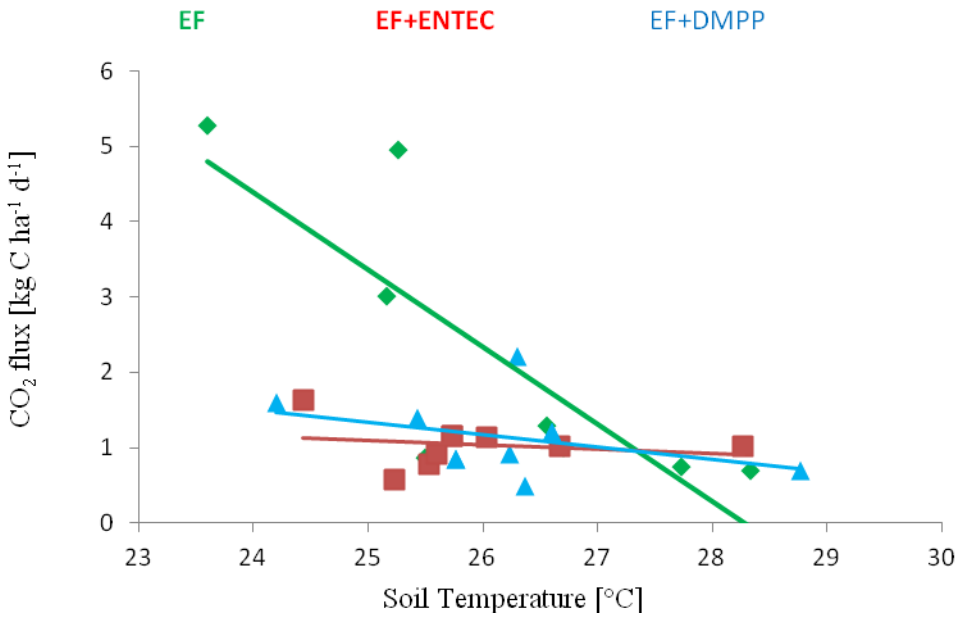


Fig 6.4. Regression line between means of soil temperature (◦C) and soil CO_2 flux, over the 1st month of measurements. Bars are standard errors (n = 3). The line-s equation for Ef was y = 0,1883x² - 10,835x + 156,39 R^2 = 0,713, EF+ENTEC y = -0,1637x + 5,43 R^2 = 0,1407 , EF+DMPP y = -0,0543x + 2,45 R^2 = 0,039 .

The effect of N inhibitors on organic fertilization was also underlined by estimating the difference of CO_2 emission (Δ) between treatment Ef and the treatments with Ef + 3,4 DMPP and Ef + ENTEC, respectively. The delta values are shown on the Fig 6.5 below. The Anova test showed that in the morning the effect was the highest at afternoon and that the first and second day the delta value of Ef+3,4 DMPP was significantly higher than Ef+ ENTEC ($p<0,05$).

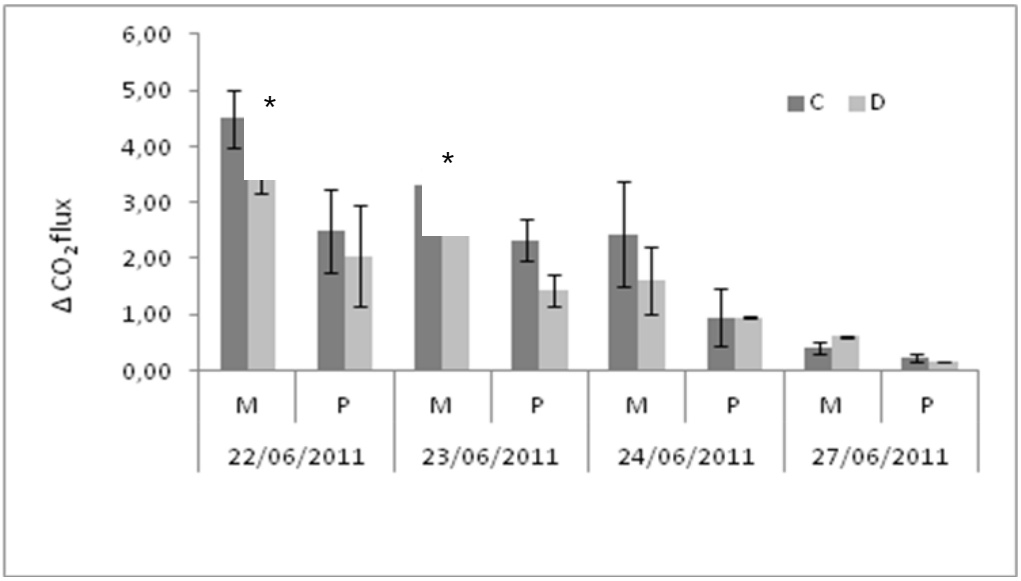


Fig 6.5. Δ of CO_2 flux. between treatment Ef and the treatments with Ef+ 3,4 DMPP and Ef+ ENTEC on morning (M) and afternoon (A). Asterisk denotes where Ef+3,4 DMPP treatment is different from Ef +ENTEC (P<0,05 Post hoc test).

Plant Productivity

To determine the productivity of plants during the first growing season and get a picture of the potential effect of the treatments we decided a non-destructive method. We used surface area (SA) to establish plants productivity on the first growing season. It is generally assumed that diameter is positively correlated with height, volume and weight. The diameters were taken with a digital gauge to 130 cm from the soil and for each diameter were calculated the basal area (SA) for each replicate, including 20 plants for each clone.

The data collected from meteorological station were used to Bagnouls-Gaussen (Fig 6.6) and showed for the last year a period of drought from April to October and this means that the plants were under water stress during 7 months.

The results showed an high variability of the SA for *E camaldulensis* (Table 6.1) with no significant effect of treatment (p>0,05). *E camaldulensis x E bicostata* (Viglio 358) is the clone with best performance *(Mughini et al., 2006)* of the system and the results evidenced a response at the treatments. On

the table 6.1 were reported the results of SA for Ef+ 3,4 DMPP, Control, and Ef. As can be saw, the SA (resulted from the calculation of 20 plants) did not change from the treatment where it is applied animal manure while the in treatment with Ef+ DMPP it increased .

A non parametric test was used to verify the effect of treatments on the plants productivities. With the Kruskall-Wallis were tested the equality of the median of different groups.

The Kruskall-Wallis test resulted significant with a median test equal to 79.18 for *E camaldulensis x E bicostata* . The non parametric test gave a better description of the effect of treatment Ef+3,4 DMPP on Viglio productivity. As reported in Figure 6.6 the third treatment growth up the means of SA that turned out to be significant ($p<0.05$).

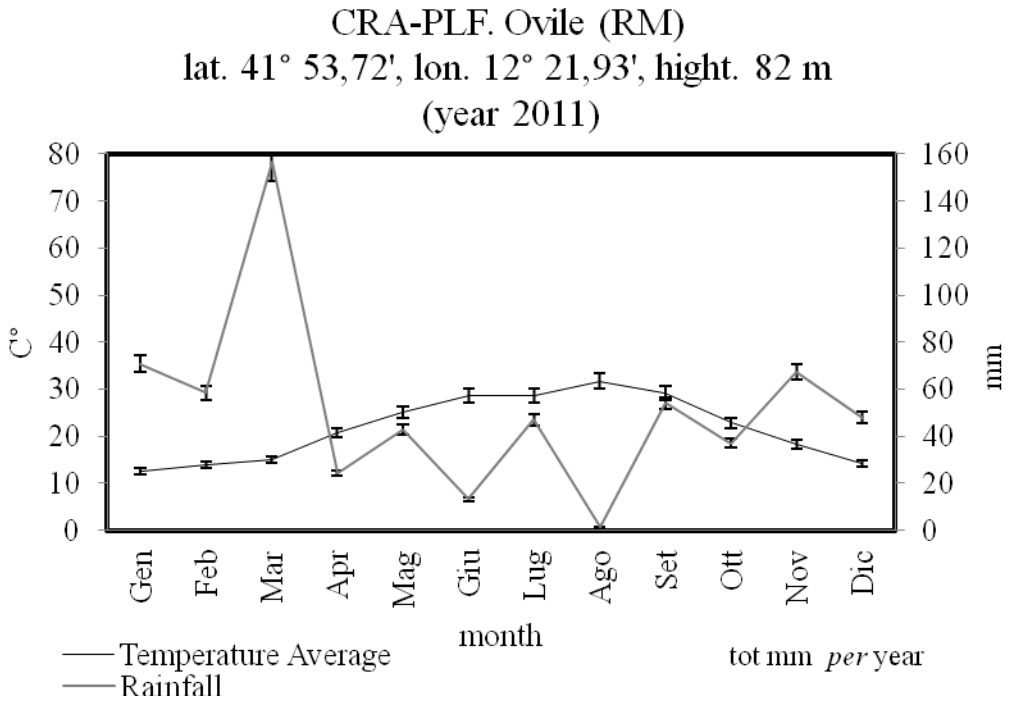


Figure 6.6. Bagnouls-Gaussen. for experimental site during the year 2011

Tab 6.1. SA of *E camaldulensis x E bicostata* (Viglio 358), the mean is made with 20 plants for each replicates.

	Treatment	SA	Mean	Std.Dev	ES
E cam	C	64.30	50.70	15.29	4.41
E cam	C	55.10			
E cam	C	32.71			
E cam	Ef	48.37	41.17	15.29	9.38
E cam	Ef	49.37			
E cam	Ef	49.43			
E cam	Ef+ 3,4 DMPP	42.63	40.67	16.25	7.74
E cam	Ef+ 3,4 DMPP	27.31			
E cam	Ef+ 3,4 DMPP	52.06			
Viglio	C	60.65	76.82	15.13	8.74
Viglio	C	79.18			
Viglio	C	90.64			
Viglio	Ef	63.20	72.56	8.26	4.77
Viglio	Ef	75.67			
Viglio	Ef	78.82			
Viglio	Ef+ 3,4 DMPP	95.37			
Viglio	Ef+ 3,4 DMPP	12.32			
Viglio	Ef+ 3,4 DMPP	97.22	107.30	19.09	11.02
Total			85.56	20.85	6.95

E camaldulensis x E bicostata (Viglio) is the clone with the best performance *(Mughini 2006),* thus the diameter measure was used to investigate the effect of treatment through the non parametric test Kruskall-Wallis. Ef+ ENTEC did not show a significant effect on the growth while differences between the treatments Ef+ 3,4 DMPP and Ef and Control were found (Figure 6.7).

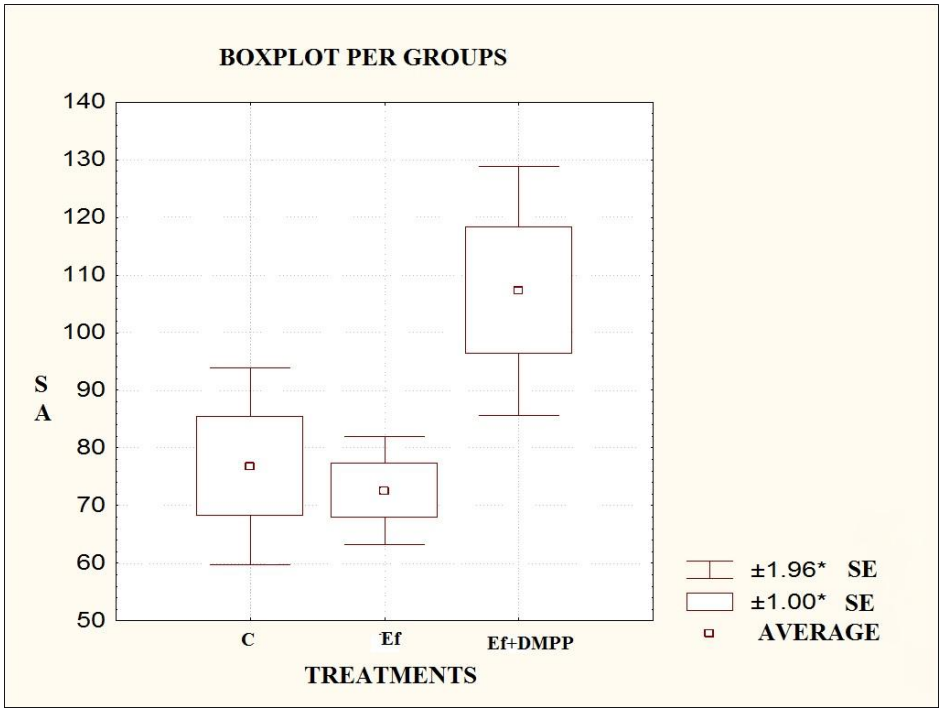


Figure 6.7. Boxplot for groups on SA and Treatment on Kruskall-Wallis test measured with STATISTICA.

6.3 DISCUSSION

The main effect of different organic fertilizations on NH_3 and CO_2 emissions was to significantly slow them by treatment with 3,4 DMPP. In detail, ammonia flux was detected only for 92 hours after all treatments and this confirms the good agricultural practice of furrow the soil and cover when spread manure. Moreover, the addiction of 3,4 DMPP gave good results because it has reduced the ammonia flux during 50 hours following the spill. Treatments with ENTEC® and 3,4 DMPP slowed also CO_2 emissions; 3,4

DMPP presented a significantly better performance in the first days, as shown by calculating the differences between the control and treatments.

The high genetic variability of *E. camaldulensis* (each experimental plants is a different genotype) interfered with the treatments effect as shown from the high variability of the SA values calculated**.** .

The estimate of the productivity has highlighted how in the clone Viglio, where is not acting the influence of genetic variation, the effect of treatment with 3,4 DMPP is significantly positive. A collateral deduction is that environmental variability can play a principal role on the assessment of effectiveness of results.

The statistical approach with non parametric-test gives a picture of the different effect of the treatment on plant productivity.

The animal manure application alone did not bring about a productivity response, this should be explicated by higher losses of nitrogen as NH_3 and by the drought season longer than 7 months.

The combined use of bovine effluent and 3,4 DMPP increased the productivity of clone Viglio, while did not exerted effect on *E. camaldulensis*.

This preliminary study evidenced that this strategy of fertilization could be a good solution to reducing the ammonia and carbon dioxide emissions and improving the productivity in agro-ecosystem of Mediterranean environment and preserve the soil use.

Further investigation repeating the experiment and calculating the biomass productivity after the cutting can give more information about efficiency of the proposed fertilization practice together with response of this agro-ecosystem.

Chapter 7: Conclusions

The effect of organic fertilization combined with Nib on biochemical and microbiological soil properties in a closed experiment showed similar results from different methods (soil respiration, MBC, enzymatic activity, bacterial and fungal growth, PLFA). On the investigated Mediterranean soil, with low organic matter content, there was a positive effect on the microbial functions and consequently on soil carbon dynamics. With the closed laboratory experiment we can assume that on this soil, poor in nutrient, the combined use of N inhibitor and animal manure has a positive effect on the microbial dynamics and consequently on soil carbon dynamics. The addiction of easy degradable organic matter (Ef) was a substrate for the microbes increasing their growth and activity especially on a very poor soil. This increase was slowed down with Nib treatment. The positive correlation between respiration, bacterial growth, β-glucosidase activity and pH suggested that the changes in the pH value of soil after treatments with Nib should be important for microbial biomass.

The field experiment confirmed the laboratory results. The effect of organic fertilization on emissions of NH_3 and CO_2 was to significantly slow them by treatment with 3.4 DMPP.

The estimate of the productivity has highlighted how the combined use of bovine effluent and 3,4 DMPP increased the productivity of clone Viglio. Diversely from the Anova parametric test, the statistical approach with Kruskall-Wallis non parametric-test permitted to obtain a picture of the different effect of the treatments on plant productivity.

The research evidenced that this strategy of fertilization could be a good solution to reduce the water demand and to contain the potential negative effects of animal manure application on Mediterranean Rotation Woody Crops. Further field experiments after the cutting and the determination of nitrate contents in groundwater and leaves could give more information about the response of this agro-ecosystem and the effective application of this practice in Mediterranean environment.

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REFERENCES

1. **Allison, S. D., and P. M. Vitousek.** 2005- Responses of extracellular enzymes to simple and complex nutrient inputs. Soil Biology & Biochemistry 37, 937-944.
2. **Anderson, T.H. and Domsch, K.H.** 1990- Application of ecophysiological quotient ($qCO_2$and qD) on microbial biomass from soils of different cropping histories. Soil Biology and Biochemistry 10, 251-255.
3. **Anderson, T.** 2003- Modes of interaction in distance education: Recent developments and research questions. In M. Moore (Ed.) *Handbook of Distance Education.* p. 129-144 Mahwah, NJ.: Erlbaum.
4. **Austin, A.T., .Sale, E.O., Jackson R.B.** 2006- Inhibition of Nitrification Alters Carbon Turnover in the patagonia Steppe. Ecosystem 9,1257-1265.
5. **Bååth, E.** 2001a- Adaptation of a rapid and economical microcentrifugation method to mesure thymidine and leucine incorporation by soil bacterial . Soil Biology and Biochemstry 33, 1571-1574.
6. **Bååth, E., Petterson, M., Söderberg, KH.** 2001b- Estimation of fungal growth rates using 14C acewtate incorporation into ergosterol. . Soil Biology and Biochemistry 33, 2011-2018.
7. **Bardgett, R.D., and McAlister, E.** 1999- The measurement of soil fungal bacterial biomass rations as an indicator of ecosystem self-regulation in temperate meadow grasslands. Biological and Fertility. Solis 29, 282-290.
8. **Barford, W., Bloem, J., Hopkins, W., Benedetti, A.** 2008- Microbiological Methods for Assessing Soil Quality, a book.
9. **Bapiri, A., Rousk, J., Bååth, E.** 2010- Drying -Rewetting Cycles Affect Fungal and Bacterial Growth Differently in Arable Soil. Microbial Ecology 2, 419-428.

10. **Benedetti, A.** 1983- Fertilità biologica dl terreno e concimi a lento rilascio. Istituto sperimentale per la nutrizione delle piante Vol. XII – Roma pubblicazione n°3.
11. **Benedetti, A., Dell'Abate M.T., Mocali, S., Pompili, L**.2006- Indicatori microbiologici e biochimici della qualità del suolo. ATLAS (Atlante degli indicatori della qualità del suolo). MIPAF
12. **Benedetti, A.** 2010- Characterization of bio stimulanting properties in fertilisers using bioassays Proceedings of the 9th New Ag International Conference & Exhibition.
13. **Bergante, S., Faciotto, G., Minotta, G.** 2010- Identification of the main site factors and management intensity affecting the establishment of Short-Rotation-Coppices (SRC) in Northern Italy through Stepwise regression analysis. Central European Journal Biology 5, 522–530.
14. **Bisoffi, S., Facciotto G.** 2000- I cedui a turno breve (SRWC). Sherwood 59,21-24.
15. **Brady, N.C. and R.R., Weil**. 1999-The Nature and Properties of Soils. 9th Edition. Macmillan Publishing Company New York. 750
16. **Chen, D., Grace, O., Moiser, A.R**. 2008- N2O emission From agricultural lands : a synthesis of simultation approaches. Plant and Soil 309, 169-189.
17. **Ciancio, O., Nocentini S**. 2000- Eucalyptus in Italy: cultivation and management. Proceeding of the International Conference Eucalyptus in the Mediterranean basin: perspective and new utilization. Cannata (ed.),CNR, Italy and IUFRO 75-79.
18. **Cook, F., Orchard, V.,** 2007- Relationship between soil respiration and soil moisture. Soil Biology and Biochemistry 40, 1013.1018.
19. **Dalmonech, D., Moscatelli,A., Moscatelli, MC., Chiti, T.,Valentini, R.** 2010- Microbial preformance undere increasing nitrogen availability in a Mediterranean forest soil. Soil Biology and Biochemistry.
20. **De Franchi, A.S. Todaro, L., Di Tommaso, T, Moretti, N, Lovelli. S.** 2010- Short Rotation Forestry (SFR) in a Mediterranean Environment Under Limited Energy Inputs Italian. Journal of. Agronomy 4, 393-400
21. **Di, H.J., Cameron, K.C.** 2004- Effects of temperature and application rate of a nitrification inhibitor, dicyandiamide (DCD) , on nitrification

rate and microbial biomass in a grazed pasture system. Australian jurnal of soil research 42, 927.932.

22. **Dick, R.P., RPgs, R.A., Christ, J.D., Istok and pd F. Iyamuremye**. 2000- Nitrogen fractions and transformations in vadose zone sediments under intensive agriculture in Oregon.Soil Science 165, 505-515.
23. **Doran, J.W., Parking, T.B.** 1994- Defining and Assessing soil quality, Soil Science Society of America, 677 Sogo e Rd., Madison WI53711, USA. In defining Soil Quality for a .sustainable Environmental . ISS special publication n. 35 .
24. **Dilly, O., Munch, J.C.** 1998- Ratios between estimates of microbial biomass content and microbial activity in soils. Biology and Fertility Soils 27, 374–379.
25. **Edmeades, D.C.** 2003- The long-term effects of manures and fertilizers on soil productivity and quality: a review. Nutrient Cycle Agro-ecosystem 66, 165–180.
26. **Ekenler, M., and Tabatabai, M.A.** 2003- Tillage and residue management effects on B-glucosaminidase activity in soils. Soil Biol. Biochem. 35, 871-874.
27. **Elliott, E., Burke, I.C., Monz, C.A., Frey, S.D.** 1994- Terrestrial Carbon Pools . preliminary data from the corn belt and great plains regions. Soil Science of America sp 35.
28. **European Nitrate Directive** 91/676/EEC.
29. **Facciotto G., Bergante S., Mughini G., Gras, M., Nervo, G.** 2007- Le principali specie per la produzione di biomassa. L'Informatore Agrario 40, 36-37 .
30. **Facciotto, G.; Zenone, T.; Sperandio, G.** 2003 -Dalla colture da biomassa reddito incerto senza aiuti. L'Informatore Agrario 59, 10 91-93
31. **Fontaine, S., Mariotti, A., Abbadie, L.** 2003- The priming effect of organic matter: a question of microbial competition? Soil Biology & Biochemistry 35, 837-843.
32. **Frostegard, A., and Baath, E.** 1996- The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. Biology and Fertility Soil 22, 59-65.

33. **Frostegård, A., Bååth, E.** 1996- The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. Biology Gertility Soils 22, 59-65.
34. **Frostegård, A., Tundil, A., Bååth, E.** 1993- Phospolipid fatty acid analysis to estimate bacterial and fungal bioass in soil. Applied Enviromental Microbiology 59, 3605-3617.
35. **Gang, D., Z. Shi-Sheng and L. Yang**. 2008-Time series prediction using wavelet process neural network. Chinese Journal of Physiology 17, 1998-2000.
36. **Gleick., P.** 1997-Water in crisis: paths to sustainable water use. Ecological Application 8, 571-579.
37. **Grego, S., Lagomarsino, A.** 2008- Soil organic matter in the sustainable agriculture: Source or sink of carbon? In: Soil Carbon Sequestration Under Organic Farming in the Mediterranean Environment, Sara Marinari and Fabio Caporali (eds.).
38. **Gregorich, E.G., Ellert, B.H., Drury, C. F. and Liang, B. C.**1996- Fertilization effects on soil organic matter turnover and corn residue C storage. Soil Science Society of America Journal 60, 472–476.
39. **Hardcastle P.D**. 2006- A Review of the Potential Impacts of Short Rotation Forestry. LTS International Ltd.,Penicuik, UK.
40. **Huang, X.. Skidmore, E.L., Tibke, G.L**. 2002- Soil quality of two Kansas soils as influenced by the conservation reserve program. Journal of soil and water conservation 57, 344-350.
41. **Hunt, P.G., Rose, C.D., McIlvain-Simpson G, and Tejani, S.** 1997- The effects of daily intake of folic acid on the efficacy of methotrexate therapy in children with juvenile rheumatoid arthritis. A controlled study. Journal Rheumatology 24**,** 2230-2232.

    Ibekwe, A.M., Kennedy, A.C 2006- Phospholipids fatty acid profiles and carbon utilization patterns for analysis of microbial community structure under field and greenhouse conditions Microbiology Ecology 2, 152-163.
42. **Ioveno, P., Bååth, E.** 2008- Effect of drying and rewetting on bacterial growth rates in soil. Micorbial Ecology 65, 400-407.

43. **Irigoyen, I., Muro, J., Azpilikueta, M., Aparicio-Tejo P., Janssens, I.A.** 2010- Reduction of forest soil respiration in response to nitrogen deposition. Nature Geoscience 3, 315-322.
44. **Jenkinson, D.S., & Powlson, D.S.** 1976- Effects of Biocidal Treatments on Metabolism in Soil .1. Fumigation with Chloroform. Soil Biology & Biochemistry 8, 167-177.
45. **Jenkinson, D.S., Fox, R.H., Rayner, J.H.**1985- Interactions between fertilizer nitrogen and soil nitrogen e the so-called 'priming' effect. Journal of Soil Science 36, 425-444.
46. **Kaur, A., Chaudhary, A., Kausi, R**. 2005- Phospholipid fatty acid-a bio-indicator of environmental monitoring and assessment in soil ecosystem. Review articles, Current Science.
47. **Kirka, J.L., Beaudette, L.A., Hart, M., Moutoglis, P., Klironomos, J.N., Lee, H. and Trevors, J.T.** 2004-Methods of studying soil microbial diversity. Journal Microbiology Methods 58,169–188.
48. **Kuzyakov, Y.** 2010- Priming effects: Interactions between living and dead organic matter Soil Biology & Biochemistry 42 1363-1371.
49. **Laclau, JP., Ranger, J., Nzila, JD., Bouillet, JP., Deleporte, P.** 2003- Nutrient cycling in a clonal stand of Eucalyptus and an adjacent savanna ecosystem in Congo. Forestry Ecology and Management. 180, 527-544.
50. **Lagomarsino, A., Moscatelli, M.C., Di Tizio, A., Mancinelli, R., Grego, S., Marinari, S.** 2009- Soil biochemical indicators as a tool to assess the short-term impact of agricultural management on changes in organic C in a Mediterranean environment. Ecological Indicators, 9, 518-527.
51. **Lagomarsino, A., Benedetti, A., Marinari, S., Pompili, L., Moscatelli, MC., Roggero, P.P, Lai, R., Ledda, L., Grego, S.** 2010- Soil organic C variability and microbial functions in a Mediterranean agro forest ecosystem, Biology and Fertility of Soils, *in press*.
52. **Lal, R**. 2003- Soil erosion and the global carbon budget. Environmental Int. 29. 437-450.
53. **Larson, W.E., and F.J. Pierce.** 1991- Conservation and enhancement of soil quality. In: J. Dumanski, E. Pushparajah, M. Latham, and R. Myers, eds. Evaluation for Sustainable Land Management in the

Developing World. Vol. 2: Technical Papers. Proc. Int. Workshop., Chiang Rai, Thailand. 15-21 Sept. 1991. Int. Board for Soil Res. and Management, Bangkok, Thailand.

54. **Lamsfus, C.** 2003- Ammonium oxidation kinetics in the presence of nitrification inhibitors DCD and DMPP at various temperatures Australian Journal of Soil Research 41 (6) 1177 - 1183 .
55. **Lindroth, RL.** 1996- CO2-mediated changes in tree chemistry and tree-Lepidoptera interactions. In: Carbon Dioxide, Populations, and Communities. Physiological Ecology Series (eds Ko¨rner C, Bazzaz FA), pp. 105–120. Academic Press, San Diego, CA.
56. **Marx, M.** 2001- A microplate fluorimetric assay for the study of enzyme diversity in soils . Soil Biology and Biochemistry 33, 1633-1640.
57. **Mancinelli, R., Campiglia, E., Di Tizio, A,. Marinari, S.** 2010- Soil carbon dioxide emission and carbon content as affected by conventional and organic cropping systems in Mediterranean environment. Applied Soil Ecology 46 64–72.
58. **Marinari., S. Caporlai., F.** 2008-Soil Carbon Sequestration under Organic Farming in the Mediterranean Environment (eds.).
59. **Masciandro, G., Ceccant, B.** 1999-Assessing soil quality in different agro-ecosystem through biochemical and chemico-structural properties of the humic substances. Soil &Tillage Research**.**
60. **Melero, S., Porras, J.C.R., Herencia, J.F. and Madejon, E.** 2006-Chemical and biochemical properties in a silty loam soil under conventional and organic management. Soil Tillage Research, 9, 162-170.
61. **Mitchell, C.P., Stevens, E.A., Watters, M.P.** 1999- Short-rotation forestry – operations, productivity and costs based on experience gained in the UK Forestry Ecology and Management 121, 123-136.
62. **Mughini, G.** 2000- Eucalyptus breeding in Italy. In: Proceeding of the International Conference Eucalyptus in the Mediterranean basin: perspectives and new utilization. October 15-19, 2000 Taormina – Italy.
63. **Mughini, G.** 1996- Production of hybrid clones: Italy. In: Improvement of eucalypt management an integrated approach: breeding, silviculture

and economics, Final Report, (01.11.1993-31.10.1996), AIR3-CT93-1678, pp.10, Commission of the European Communities, Brussels.

64. **Mughini, G., Gras, M., Facciotto, G.** 2007 - Eucalyptus clones selection in Central-Soithern Italy for biomass production. 15th European Conference and exibition, Berlin, Germany ISBN -978-88-894°7-59-X. ISBN 3-936338-21.
65. **Nannipieri, P., Kandeler, E., Ruggiero, P**.2000- Enzyme activities and microbiological and biochemical process in soil. In: Burns, R.G., Dick, R.P.(Eds),Enzymes in the Environment: Activity, Ecology and Applications. Marcel Dekker, New York, pp1-33.
66. **Neff, .C.J., Townsend, AR., Gleixenr, G., Lehman, J.S. Turbull, J.B.W.D.** (2002) Variable effects of nitrogen additions on the stability and turnover of soil carbon. Nature 419, 915-917
67. **Nordin, A., Hogberg, P., Nashlom, T.** 2001- Soil nitrogen form and plant nitrogen uptake along a boreal forest productivity gradient. Oecologia 129, 125-132.
68. P**acholski, A., Cai, R., Nieder, J., Richter, X., Fan, Z., Zhu and M. Roelcke.** 2006- Calibration of a simple method for determining ammonia volatilization in the field-comparative measurements in Henan Province, China. Nutr. Cycl. Agroecosyst 74, 259-273.
69. **Pasda, G., Hahndel, R., and Zerulla, W**. 2001- Effect of fertilizers with the new nitrification inhibitor DMPP on yeld and quality of agricultural and horticular crops. Biology Fertility Soils 34,85-97.
70. **Plaza, C., Hernandez, D., Garcia-Gil, J.C., Polo, A.** 2004- Microbial activity in pig slurry-amended soils under semiarid conditions Soil Biology & Biochemistry 36, 1577–1585
71. **Pierce, F.J. and Larson, W.E.** 1993- Developing criteria to evaluate sustainable land management . p 7-14. In J.M- Kimble (ed) Proc. Of the 8thInternational Soil Management Workshop; Utilization of Soil Survey Information for Sustainable Land Use. May 1993. USDA-SCS National Soil Surv. Centre, Lincoln, NE.
72. **Poggiani, F., Couto, H. T. Z.** 1983 Biomass and Nutrient Estimates in Short Rotation Intensively Cultured Plantation of *Eucalyptus grandis*. IPEF 23, 29-36.

73. **Poggiani, F., Couto, H. T. Z.** 1983- Biomass and Nutrient Estimates in Short Rotation Intensively Cultured Plantation of *Eucalyptus grandis*. IPEF.23, 29-36.
74. **Power, J.F., and Myers, R.J.K.** 1989.-The maintenance or improvement of farming system in North America and Australia *In* soil quality in semiarid agriculture. Proc..of an Int. Conf. sponsored by the Canadian Int. Development Agency, Saskatoon,Saskatchewan,Canada.11-16June 1989. Saskatchewan Inst. Of Pedology Univ. Of Saskatchewan, Saskatoon, Canadan.
75. **Pumpanen J, Kolari P, Ilvesniemi H, Minkkinen K, Vesala T, Niinistö S, Lohila A, Larmola T, Morero M, Pihlatie M, Janssens I, Curiel Yuste J, Grünzweig JM, Reth S, Subke J-A, Savage KE, Kutsch W, Østreng G, Ziegler W, Anthoni P, Lindroth A and Hari P.** 2004-Comparison of different chamber techniques for measuring soil CO_2 efflux..Agricultural and Forest Meteorology 123, 159-17
76. **Niinisto, S., Lohila, A., Larmola, T., Morero, M., Pihlatie, M., Janssens, I., Curiel, J., Gru¨nzweig, J.M., Reth, S., Subke, J.A., Savage, K. Kutsch, W., Østreng, G., Ziegler, W., Anthoni, P., Lindroth, A.** 2004- Comparison of different chamber techniques for measuring soil CO2 efflux. Agricultural and Forest Meteorology 123, 159-176.
77. **Rengel, Z., Pineros, M., and Tester., M.** 1995- Transmembrane calcium fluxes during Al stress. Plant Soil 171, 125-130.
78. **Rousk, J., Baath, E.** 2007- Fungal and bacterial growth in soil with plant materials of different C/N ratios. FEMS Microbiology Ecology 62, 350-358.
79. **Schroder, J.J. Holte, L., Keulen, v.H, Steenvoorden, J.H.A.M** 1993 .Effects of nitrification inhibitors and time and rate of slurry and fertilizer N application on silage maize yeld and losses to the environment. Fertilizer Research 34, 267-277.
80. **Söderström, B., Baath, E., Lundgren, B.** 1983- Decrease in soil microbial activity and biomasses owing to nitrogen amendments. Revue Canadienne de Microbiologie. (Canadian Journal of Microbiology) 29, 1500-1506.

81. **Sperandio, G.** 2004- Convenienza economica delle colture forestali energetiche. *EM-Linea Ecologica* 5 26-31.
82. **Springer, U., Klee, J.** 1954- Prufung der Leistungfahigkeit von einigen wichtigen verfaren zur Bestimmung des Kohlenstoffe mittels Chromschwefelsaure sowie Vorschlag einer neuen Schnellmethode. Z. Pflanzenernahr. Dung. Bodenk. 64, 1-26.
83. **Treseder, K.,** 2008- Nitrogen additions and microbial biomass: a meta-analysis of ecosystem studies. Ecology Letters 11, 1111-112.
84. **Trevors, J.T.** 1998- Bacterial biodiversity in soil with an emphasis
85. on chemically-contaminated soils. Water Air Soil Pollution 101, 45 – 67.
86. **Torsvik, V., Goksoyr, J., Daae, F.L.** 1990- High diversity in DNA of soil bacteria. Applied Environmental Microbiology 56, 782 – 787.
87. **Waldrop., M., Zak, D., Sinsabaugh, R.** 2004- Microbial community response tonitrogen deposition in northern forest ecosystems. Soil Biology and Biochemistry 36, 1443-1451.
88. **Wick, B., Kuhne, R.F., Vlek, P.L.G.** 1998- Soil microbiological parameters as indicators of soil quality under improved fallow management systems in south-western Nigeria. Plant Soil 202, 97-107.
89. **Word reference base for soil resources** 2006, A framework for international classification, correlation and communication, World Soil Resources Reports 103.
90. **Zelles, L.** 1999- Biological and Fertility Soils 29, 111–129
91. **Zerulla, W., Barth T., Dressel J., Erhardt K., Von Locquenghien K.H., Pasda G., Rädle M., Wissemeier A.H.** 2001-3,4-Dimethylpyrazolephosphate (DMPP) – a new nitrification inhibitor for agriculture and horticulture. Biology and Fertility Soils 34, 79-84
92. **Zerulla, W., Pasda G., Händle R., Wissemeier A.H** 2002- The new nitrification inhibitor DMPP (ENTEC®) for use in agricultural and horticultural crops — an overview. Plant and Soil Science 92, 10-750-751.