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MICROBIAL DIVERSITY AS SOIL QUALITY INDICATOR IN AGRICULTURAL SOILS

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Summary

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Abstract

Soil quality is the capacity of a specific kind of soil to function, within natural or managed ecosystem boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality, and support human health and habitation. Soil organisms are assumed to be directly responsible for soil ecosystem processes, especially the decomposition of soil organic matter and the cycling of nutrients. Since soil quality is strongly influenced by microbe-mediated processes, and function can be related to diversity, it is likely that microbial community structure will have the potential to serve as an early indication of soil degradation or soil improvement.

Here I tried to define the impact of different management practices on the fate of microbial guilds having fundamental role in soil healthiness and functions. Moreover, I attempted to manipulate experimentally soil microbial diversity to ask a central ecological question: is there a relationship between survival of exotic species and diversity? Answering this question would contribute to the debate on biodiversity and soil functioning, unfortunately still open after decades of theoretical and experimental works.

Understanding of the microbial fate, and subsequent ecosystem functional modifications, are necessary to drive political decisions about the best practises to apply at the different territorial scales. The final goal is always improving soil quality and conservation practices.

-CHAPTER I-

GENERAL INTRODUCTION

1.1 State of art

The key role of agriculture now and in the future is the supply of safe food at 'reasonable' prices. Over the past 40 years while world population has nearly doubled, food prices have dropped substantially in real terms and food production per capita has increased by nearly 25%. These developments have been possible through farmers, scientists and agricultural research investment raising crop yields and livestock productivity and improving farm management practices. The productivity improvements for agriculture have also been achieved through using less labor, inputs and land. There are concerns, however, that the scale of agricultural expansion is going to place greater pressure on the environment. Some consider that current farming practices are leading to the degradation and depletion of the natural resource base upon which farming depends, namely soils, water, natural plant and animal resources (OECD, 2001). In deciding priorities for conservation, there is an urgent need for criteria that help us to recognize losses with potentially serious consequences (Grime, 1997)

During the last decade, the concept of soil quality has been developed by a large number of institutions (e.g. FAO, OECD, USDA and so on) to help in recognizing practices helping conservation projects.

1.2 Soil quality and indicators

Soil quality is how well soil does what we want it to do. More specifically, soil quality is the capacity of a specific kind of soil to function, within natural or managed ecosystem boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality, and support human health and habitation (www.soils.usda.gov)

People have different ideas of what a quality soil is. For example:

- for people active in production agriculture, it may mean highly productive land, sustaining or enhancing productivity, maximizing profits, or maintaining the soil resource for future generations;
- for consumers, it may mean plentiful, healthful, and inexpensive food for present and future generations;
- for naturalists, it may mean soil in harmony with the landscape and its surroundings;
- for the environmentalist, it may mean soil functioning at its potential in an ecosystem with respect to maintenance or enhancement of biodiversity, water quality, nutrient cycling, and biomass production.

Soil quality cannot be measured directly, so we evaluate indicators. Indicators are measurable properties of soil or plants that provide clues about how well the soil can function. Indicators can be physical, chemical, and biological properties, processes, or characteristics of soils. They can also be morphological or visual features of plants. Good indicators are relevant, sound and cost-effective. A relevant indicator is directly related to the most important aspects of the goal, is self-explanatory, is sufficiently sensitive for its purpose, and can be used to develop and monitor actions. A sound indicator is acceptable to experts in the field, regardless of their backgrounds. Thus, it is science-based and sufficiently accurate, precise and robust for its intended purpose. For an indicator to be cost-effective, the value of its information must be greater than its cost. In general, this means required data is readily available, computation is relatively easy, and the data is required or synergistic with other needs.

Soil quality indicators are useful to policy makers to: monitor the long-term effects of farm management practices on soil quality; assess the economic impact of alternative management practices designed to improve soil quality, such as cover crops and minimum tillage practices; examine the effectiveness of policies addressing the

agricultural soil quality issue; and improve policy analysis of soil quality issues by including not only environmental values but also taking into account economic and social factors.

Most of the European countries, USA, Canada and so on developed their own parameters to evaluate soil quality. Since the early 1990s, there has been a considerable effort in the United States to develop soil ratings based on measured soil properties for the comparison of land management systems (Karlen et al. 2001). Many potential parameters of soil quality, measurable at various scales of assessment, have been proposed (Table 1)

Table 1 Potential biological, chemical, and physical indicators of soil quality, measurable at various scales of assessment (from Karlen et al. 2001).

Biological	Chemical	Physical
	Point-scale indicators	
Microbial biomass	pH	Aggregate stability
Potential N mineralization	Organic C and N	Aggregate size distribution
Particulate organic matter	Extractable macronutrients	Bulk density
Respiration	Electrical conductivity	Porosity
Earthworms	Micronutrient concentrations	Penetration resistance
Microbial communities	Heavy metals	Water-filled pore space
Soil enzymes	CEC and cation ratios	Profile depth
Fatty acid profiles	Cesium-137 distribution	Crust formation and strength
Mycorrhiza populations	Xenobiotic loadings	Infiltration
	Field-, farm-, or watershed-scale indicators	
Crop yield	Soil organic matter changes	Topsoil thickness and color
Weed infestations	Nutrient loading or mining	Compaction or ease of tillage
Disease pressure	Heavy metal accumulation	Ponding (infiltration)
Nutrient deficiencies	Changes in salinity	Rill and gully erosion
Growth characteristics	Leaching or runoff losses	Surface residue cover
	Regional-, national-, or international-scale indicators	
Productivity (yield stability)	Acidification	Desertification
Species richness, diversity	Salinization	Loss of vegetative cover
Keystone species and ecosystem engineers	Water quality changes	Wind and water erosion
Biomass, density and abundance	Air quality changes (dust and chemical transport)	Siltation of rivers and lakes

Countries within the European Union have made considerable efforts to develop agro-environmental indicators. In contrast to North America, most efforts have focused on environmental impact rather than on production, particularly for water quality as

affected by excess nutrients or pesticide use. Another area of considerable interest in Europe is the conservation of agricultural lands for biodiversity, wildlife habitat and aesthetics (Dabbert et al. 1999; Meudt 1999; OECD 1998).

1.3 Microbial soil quality indicators

Biomass, community structure, and specific functions of soil microorganisms appear to be of major importance for general soil functions and if detectable could serve as sensitive soil quality indicators. Since microbial soil communities strongly depend on the conditions of the habitat they colonize, microbiological characteristics of a soil may provide indicators, which integrate short-, middle- and long term changes in soil quality. As soils display a multitude of biological characteristics and many of them may not be accessible, specific indicators have to be chosen.

Oberholzer and Höper (2000) have proposed a reference system for the evaluation of agricultural soil based on the most applied soil microbial parameter, i.e. soil microbial biomass, the metabolic quotient (qCO2), and the carbon quotient (qC).

In addition to these approaches, more recently developed techniques such as analyses of total soil DNA (Pace, 1986; Widmer et al., 1999; Amann, 2000), soil fatty acids (FA, e.g. phospholipid FA (PLFAs), FA methyl esters (FAMEs), and archaeal ether FAs (AEFAs) (Zelles, 1999; Widmer et al., 2001; Gattinger et al., 2002) and community level substrate utilization (CLSU) (Bochner, 1989; Garland and Mills, 1991; Garland, J. L. 1996; Widmer et al., 2001) allow for a more detailed investigation of soil microbiology.

Each of these approaches offers a focus on specific aspects of microbial soil characteristics and represents an independent analysis of differences or changes in soil microbial community structures or functions. For the DNA approach, total DNA is directly extracted from soil (Bürgmann et al. 2001). In several studies it has been

shown, that information on soil DNA contents can provide additional information on MSCs. In addition, extracted soil DNA can be further analyzed for specific marker gene pools. Combinations of specific polymerase chain reaction (PCR) amplification of target gene pools and genetic fingerprinting techniques, such as restriction fragment length polymorphism (RFLP; Widmer et al., 1998; Widmer et al., 1999) denaturant gradient gel electrophoresis (DGGE; Muyzer and Smalla, 1998; Van Elsas et al., 2001) or single strand conformation polymorphism (SSCP; Schwieger and Tebbe, 1998; Stach, et al., 2001) are applied. These analyses yield DNA banding patterns (genetic fingerprints), which can be quantified and compared among different soil samples. The numbers, relative migration positions, and intensities of specific bands in the patterns can be quantified and interpreted in relation to microbial community structures in a soil. Specific genes can be isolated and their DNA sequences determined which allows identification of the organisms at the genus level and in certain cases even at the species or sub-species level. For the FA approach, the total soil FA-fraction is obtained and quantitatively analyzed by gas chromatography (GC). Identities of FAs can be determined by use of reference systems or mass spectrometry (Laczkó et al., 1997; Zelles, 1999; Widmer et al., 2001). The result of these analyses is a GC profile with identified and quantified peaks for specific FAs (e.g. a PLFA fingerprint). Differences in FA fingerprints may be interpreted with respect to alterations of microbial community structures and microbial

physiologies in a soil. For analyzing community level substrate utilization (CLSU) fingerprints, soil suspensions containing total soil microbial communities are inoculated to multi-well plates containing a selection of specific substrates (Garland and Mills, 1991; Di Giovanni et al., 1999 Widmer et al. 2001). Growth of microorganisms in the wells is indicated by a specific dye, which can colorimetrically be quantified. The result of this analysis is a substrate utilization fingerprint of the different substrates (CLSU

fingerprint). This data can be interpreted in relation to metabolic activities of specific populations or communities in a sample. Therefore, all three approaches yield fingerprint-type data sets that may be used for further qualitative or numeric analyses. It will be important to establish protocols which allow to compile these data for comparative analyses and which allow to perform an objective characterization and comparison of soil microbial communities (Widmer *et al.* 2001). In addition, these down stream analyses may be used to test new and possibly improved fingerprinting approaches for their value in soil quality analyses.

1.4 Why choose microbial soil quality indicators?

Soil organisms are assumed to be directly responsible for soil ecosystem processes, especially the decomposition of soil organic matter and the cycling of nutrients (Wardle and Giller 1996). These processes are regarded as major components in the global cycling of materials, energy and nutrients. For example, the soil biomass (25 cm top soil layer) is known to process over 100,000 kg of fresh organic material each year per hectare in many agricultural systems. This processing includes the decomposition of dead organic matter by the microbes as well as the consumption and production rates in the soil community food web.

Since soil quality is strongly influenced by microbe-mediated processes, and function can be related to diversity, it is likely that microbial community structure will have the potential to serve as an early indication of soil degradation or soil improvement.

Therefore, there is growing evidence that soil microbiological and biological parameters may possess potential as early and sensitive indicators for soil ecological stress or reparation (Dick, 1992; Dick, 1994; Dilly and Blume, 1998), as is the case of soil enzyme activities and exopolysaccharides, soil microbial biomass, composition of soil microflora, that were used as potential biochemical/biological indicators of soil quality

(Dick, 1994; Fauci and Dick, 1994; Filip, 1998). For instance, Islam and Weil (2000) concluded that total microbial biomass, active microbial biomass and basal respiration per unit of microbial biomass showed the most promise for inclusion in an index of soil quality, based on soil samples of contrasting management systems obtained from long-term replicated field experiments and pair field samples in mid-Atlantic states.

1.5 Biodiversity and ecosystem functioning: a key issue

Although apparently obvious, the relationship between species composition and ecosystem functioning is difficult to quantify. When species disappear, others can become more dominant and take over a link in the process. It is possible that a process will continue while species composition has changed or degraded. So the preservation of biodiversity can not be guaranteed solely by measuring process values. Many processes are too general or insensitive as an early warning indicator.

Moreover, the wiew that "biodiversity begets superior ecosystem function" is not shared by all ecologists (Grime, 1987; Givnish, 1994). The most obvious conflict is between works on natural and synthesized ecosystems. Important works showed that ecosystem processes were determined much more by the functional characteristics of component organisms than by species number (Leps et al., 1982; MacGillivray et al., 1995). Other studies showed that the greatest deterioration in ecosystem processes occurs as diversity declines from moderate to very low value (McGrady-Steed et al., 1997; Naeem et al., 1994; Tilman et al., 1996).

A large body of ecological literature was developed thinking about vegetal and animal communities and only tentatively adapted to the microbial ones. In fact, limitations due to the cultivation methods and to the extremely high diversity of soil microbial communities, makes it difficult to use approaches based on determining the distribution of different types of organisms. Although utilization of molecular techniques are

increasing the capability to analyze microbial communities, we still have conceptual troubles in categorizing the constituents of a communities or in understanding the functional abilities of each individual organism type (Franklin and Mills, 2006). Relationship between microbial community structure and function presents another complication in the physiological versatility of many microorganisms. Functional redundancy of microorganisms was found being quite high in many experimental systems after artificial reduction in species diversity (Franklin and Mills, 2006; Wertz et al, 2006; Wertz et al., 2007). However, different ecosystem functions have to be considered in evaluating impact by microbial diversity, since many experimental study, for instance, showed an high positive correlation between diversity and invaders survival (Matos et al., 2005; Kennedy et al., 2002; Fargione et al., 2003; McGrady-Steed et al., 1997).

Certainly, biodiversity may represent a form of biological insurance against the loss of selected species (Folke et al., 1996) and in turn to the maintaining of specific functions. If this is the case, for instance, heavy pollution or disturbances select for a few resistant species. In such situations the ecological basis for processes may become very narrow. When the resistant species also disappear, or are inhibited, as a result of future and yet unknown human activities a process stops and the life support function is permanently affected. Due to redundancy of species, a process is assumed to continue to exist with fewer species, when species disappear, in which case the risk of instability and uncontrolled fluctuations will increase. Research is still necessary to understand the threshold afterwards further species losses could impair ecosystem functioning and then soil quality.

1.6 Management practices and impact on biodiversity in

agroecosystems

In agricultural systems, biodiversity performs ecosystem services beyond production of food, fibre, fuel, and income. Examples include recycling of nutrients, control of local microclimate, regulation of local hydrological processes, regulation of the abundance of undesirable organisms, and detoxification of noxious chemicals. These renewal processes and ecosystem services are largely biological, therefore their persistence depends upon maintenance of biological diversity (Altieri, 1994).

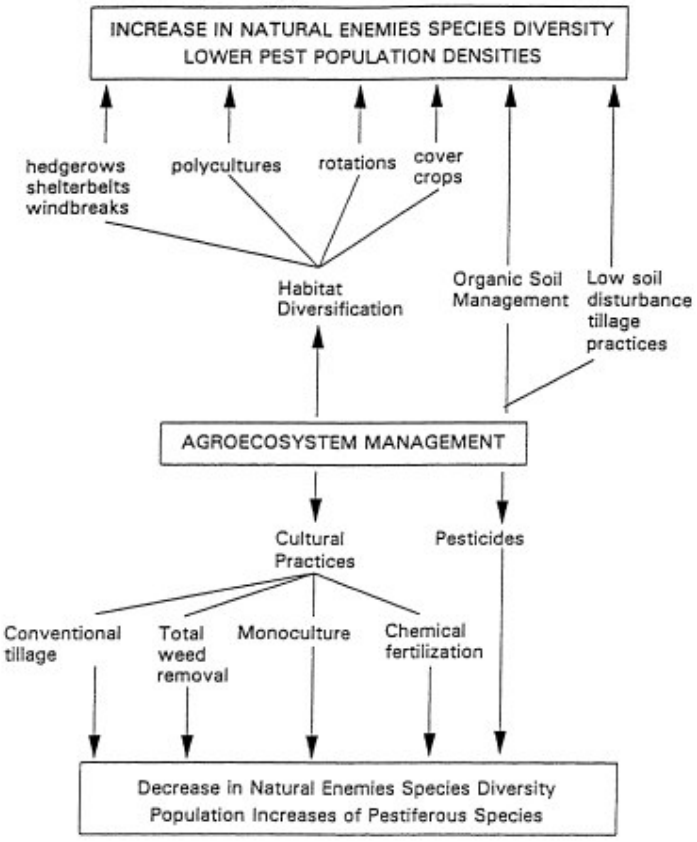

Fig. 1: The effects of agroecosystem management and associated cultural practices on the biodiversity of natural enemies and the abundance of insect pests (from Altieri (1999)

When these natural services are lost due to biological simplification, the economic and environmental costs can be quite significant. Modern agriculture implies the simplification of the structure of the environment over vast areas, replacing nature's diversity with a small number of cultivated plants and domesticated animals. A number of management techniques are known to sustain soil biodiversity, increasing, in turn, soil quality. Fig. 1 shows that there are many agricultural practices and designs that have the potential to enhance functional biodiversity, and others that negatively affect it. Organic farming is becoming a major tool for sustaining the soil quality degraded by intensive use of synthetic chemicals for increasing crop production and therefore, use of bio-agents as biofertilizers or biopesticides is an integral part of organic farming especially in vegetable cultivation (Srivastava et al., 2007). A comparative study of organic and conventional arable farming systems was conducted in The Netherlands to determine the effect of management practices on chemical and biological soil properties and soil health (van Diepeningen et al., 2005). In such experiment, soils from thirteen accredited organic farms and conventionally managed neighbouring farms were analyzed using a polyphasic approach combining traditional soil analysis, culture-dependent and independent microbiological analyses, a nematode community analysis and an enquiry about different management practices among the farmers. Organic management, known primarily for the abstinence of artificial fertilizers and pesticides, resulted in significantly lower levels of both nitrate and total soluble nitrogen in the soil, higher numbers of bacteria of different trophic groups, as well as larger species richness in both bacteria and nematode communities and more resilience to a drying–rewetting disturbance in the soil (van Diepeningen et al., 2005).

Agricultural practices that maintain adequate soil organic matter content favour the proliferation of soil biota (Reid, 1985). For example, the simple practice of adding straw mulch on the soil surface increased soil organic matter and the number of living

organisms as much as threefold (Teotia et al., 1950). Similarly, the application of organic matter or manure enhanced earthworm and microorganism biomass as much as fivefold (Ricou, 1979). Also, when organic manure was added to agricultural land in Hungary, soil microbial biomass increased tenfold (Olah-Zsupos and Helmeczi, 1987). Because increased biomass generally is correlated with increased biodiversity (Elton 1927; Odum, 1978; Sugden and Rands, 1990), it is logical to assume that the increase in biomass of arthropods and microbes represents an increase in biodiversity (Pimentel et al., 1992).

Physical disturbance of the soil caused by tillage and residue management is a crucial factor in determining soil biotic activity and species diversity in agroecosystems. Tillage usually disturbs at least 15–25 cm of the soil surface and replaces stratified surface soil horizons with a tilled zone more homogeneous with respect to physical characteristics and residue distribution. The loss of a stratified soil microhabitat causes a decrease in the density of species that inhabit agroecosystems. Such soil biodiversity reductions are negative because the recycling of nutrients and proper balance between organic matter, soil organisms and plant diversity are necessary components of a productive and ecologically balanced soil environment (Hendrix et al., 1990). Reduced tillage (with surface placement of residues) creates a relatively more stable environment and encourages development of more diverse decomposer communities and slower nutrient turnover. Available evidence suggests that conditions in no-till systems favour a higher ratio of fungi to bacteria, whereas in conventionally tilled systems bacterial decomposers may predominate (Hendrix et al., 1990). As opposed to conventional tillage, in reduced tillage nutrient reserves are stratified, with concentrations of organic matter and microbial populations being greatest near the soil surface. Stratification of crop residues, organic matter, and soil organisms often slows cycling of N as compared with conventional tillage with the moldboard plow. Increased microbial immobilization

of soluble N in the surface of reduced tillage soils may require modified fertility or tillage management practices for optimal growth and yield of grain crops (Paoletti et al., 1994).

A crop-rotation system with grass and other suitable plant associations included may well be in the position to make the best use out of the soil by mobilizing and, at the same time, renewing continuously its biotic potential. For instance, microbial diversity was significantly higher under wheat preceded by red clover green manure or field peas than under wheat following wheat (continuous wheat) or summer fallow. These results indicate that legume-based crop rotations support diversity of soil microbial communities and may affect the sustainability of agricultural ecosystems (Lupwayi et al. 1998). Changes in community structure and Biolog potential occurred in some soils in response to winter cover crops, although effects were not observed until cover crop incorporation; greater amounts of fungal and protozoan FAME markers were detected in some cover-cropped soils compared to winter fallow soils (Schutter et al., 2004). In another field study, cucumber vitamin C content and yields were lower in the 7-year mono-cropping treatment than in the field in which rotation was performed; diversity index and richness of the microbial community were reduced by mono-cropping (Wu and Wang, 2006).

Soil solarization, alone or in combination with other disease management practices, has been shown to be effective in reducing the inoculum density of many soilborne disease causing organisms (Stapleton and Devay, 1986). Solarization also affects the indigenous microbial activity which ultimately result in soil suppressiveness and/or increased antagonistic activity. This special mulching process, which causes hydrothermal disinfestation and other biological and physical changes to the soil, has also been shown to be beneficial to plant growth and health. Other beneficial effects of solarization include the reduction of competitive thermotolerant populations of resident bacteria and

fungi in favour of less competitive antagonistic bacteria that were introduced into the soil following treatment (Gamliel and Katan, 1991). Rather contrasting results were recently reported regarding the fate of soil bacterial microflora in response to soil solarization of mulched soils. According to recent works (Coates-Beckford et al., 1997; Shukla et al., 2000) soil solarization significantly decreased the fungal population while leaving unaffected soil bacteria. Recent evidence of shifts in the soil microflora as a result of solarization combined with other treatments (e.g. organic amendment) was given by the study of Stevens et al. (2003): the authors stated that the population density of rhizobacteria in plants (tomatoes and sweet potatoes) grown in solarized soils was significantly higher than that in plants grown in non-solarized soils.

1.7 Aims of the work

In the following two chapters, I tried to define the impact of different management practices on the fate of microbial guilds having fundamental role in soil healthiness and functions. As seen in the previous paragraphs, understanding of the microbial destiny, and in turn of the subsequent ecosystem functional modifications, are necessary to drive political decisions about the best practises to apply at the different territorial scales. The final goal is always improving soil quality and conservation.

In particular, in chapter II is reported the effect of minimum tillage, compost and synthetic metalporphirins addiction on several culturable microbial groups, while in the third chapter the outcome of solarization was evaluated with respect to the modifications in bacterial and fungal overall community structure, as indicated by a molecular based approach. In such case, cultivable *Pseudomonas* populations were also studied and relationships with crop productivities addressed.

In the last chapter, soil microbial diversity was experimentally manipulated to ask a central ecological question: is there a relationship between survival of exotic species

and diversity? Answering this question would contribute to the debate on biodiversity and soil functioning, unfortunately still open after decades of theoretical and experimental works.

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-CHAPTER II-

INFLUENCE OF AGRICULTURAL PRACTICES ON ORGANIC MATTER MINERALIZATION MEDIATED BY SOIL MICROBIAL COMMUNITIES

2.1 Introduction

Soil carbon sequestration is an important and immediate sink for removing atmospheric carbon dioxide and slowing global warming. It is noteworthy that the flux of CO2 from the soils is ten times greater than fossil fuel emission (Schlesinger, 1997). Decreasing the CO2 flux from the soils by only a ten percent, we will be able to counteract the CO_2 increasing and the greenhouse effect.

Agricultural practices strongly impact CO_2 emission from the soil. For instance, the loss of soil organic matter is lower when "no-till" agriculture is practiced (Schlesinger, 1997). Piccolo (1996) stated that hydrophobic humic components in soil exerted hydrophobic protection towards easily degradable compounds. He postulated that associations of apolar molecules deriving from plant degradation and microbial activity incorporate more polar molecules, thereby preventing their otherwise rapid microbial degradation and enhancing their persistence in soil. A novel understanding of the structural features of humic substances (HS) supports the self-assembly supramolecular association of relatively small molecules rather than their polymeric nature. An increase in the conformational stability of humus may thus be achieved through promotion of intermolecular covalent bondings between heterogeneous humic molecules by an enzyme-catalyzed oxidative reaction (Piccolo et al., 2000) mediated by synthetic metalporphyrins. However, new molecules added to the soil environment, even apparently harmless, may deeply alter the behavior of microbial populations through complex and unexpected interaction (biotic and/or abiotic). Studies on the breakdown of artificially produced organic chemicals, such as pesticides, suggest that some pesticides are broken down very slowly, even under the most favourable environmental conditions (Ogunseitan and Odeyemi, 1985). Molecular recalcitrance may be linked with several properties of the microflora, including the inability of organisms to produce the necessary enzymes, the impermeability of the micro-organisms to the substrate and the

susceptibility of the organisms' enzymes to inhibition by the pesticide (Johnen and Drew, 1977). Innate characteristics of the chemical could also be responsible for recalcitrance. Aromatic chemicals such as polyphenols are relatively resistant to microbial attack and may protect other chemicals from attack if they are associated with them (Ogunseitan and Odeyemi, 1985). Hence, although synthetic metalporphyrins mimic well-known natural products, a careful evaluation should be faced before releasing such a molecules, especially regarding possible alterations in ecosystem processes.

Humic substances (HS) comprise the major part of stable organic matter in many environments and their formation and decomposition processes regulate the global carbon cycling. Investigations into the mechanisms of humus biotransformation and the responsible organisms are particularly important, since better predictions of the dynamics of soil organic matter could be addressed. HS are resistant to microbial attack due to their high molecular mass and heterogeneous structure, comprising recalcitrant aromatic building blocks (Haider and Martin, 1998; Willmann and Fakoussa, 1997). Structural differences may heavily affect microbial HS degradability, the latter usually expressed as decolorization (bleaching) of liquid medium and/or as change in the molecular-mass distribution between HA and FA (Steffen et al., 2002). ^{14}C-HA mineralization was also reported for monitoring microbial degradation (Haider and Martin, 1988). Humic acids (HA) degradation has been studied in several fungi, e.g. *Phanerochaete chrysosporium*, *Trametes versicolor*, *Nematoloma frowardii* (White – rot fungi), capable to break up HA to give Fulvic acids (FA) and CO_2. It has been shown that those organisms might degrade very efficiently HA (Hurst et al., 1963). Brown – rot fungi, namely *Fomitopsis pinicola* e *Scytalidium lignicola*, may also decolorizing humic extract solutions leading enrichment in smaller molecules from initially bigger HA complexes, as indicated by Gel-Permeation Chromatographic data

(Gramss et al., 1999). *Collybia dryophila*, a so-called soil litter – colonizing fungus, is both able to decompose natural and C_{14}– HA (Steffen et al., 2002). Although fungi have demonstrated a higher activity, HA degradation and/or modification has been found as well in the bacterial world. Kontchou and Blondeau (1991) reported the ability of *Streptomyces viridosporus* to decolorize a liquid medium containing glucose and HA, whereas *Pseudomonas* spp. and *Arthrobacter* spp. have been shown decomposing soil HA to radical analogues (Hurst and Burges, 1967; Nikitin, 1960).

Whereas the most part of the fungi present extracellular HA enzymatic activities, in *Streptomyces* spp. such activities are bind to the cell surface (Kontchou and Blondeau, 1991; Dari et al., 1995). HA metabolism derives from non-specific activity of ligninolytic enzymes, such as laccase and peroxidase (Mn-perossidase and Lignin-Perossidase) toward their substrates, even though others enzymes interact with the former to better support their action.

The present work was performed in order to better inquire upon microbial effects of certain soil management practises known to be responsible in limiting CO_2 release from agricultural soils, such as minimum tillage and compost addiction. Many microbial groups directly implicated in OM mineralization, such as actinobacteria, fungi and cellulosolytic bacteria, as well as microbial groups involved in key bio-geochemical processes (e.g. aerobic free-living N2-fixing bacteria and ammonia-oxidizing bacteria) were enumerated and culturable populations structure discussed. Moreover, we made a first evaluation of synthetic metalporphyrins addiction on the destiny of microbial populations responsible for important processes into the biological component of soils.

2.2 Materials and methods

2.2.1 Field design and sampling

Field experiments took place at three Italian locations strongly differing in pedological, chemical and climatic characteristics: Napoli (NAP), Torino (TOR) and Piacenza (PIA). Two different crops, wheat and maize, were grown in Napoli and Torino, whereas in Piacenza only Maize was planted. Soils were subjected to different treatments such as traditional (TRA) and minimum tillage (MIN), amendments with compost (COM) and synthetic metalporphyrins (POR), in order to evaluate their effects on different soil culturable microorganisms, coming from either bulk and rhizosphere soil.

In each site, a randomized block design with four replicates per block was adopted to evaluate the differences among management systems on maize crops (i.e. TRA, MIN and COM). Hence, four plots were prepared per treatment and sampling site, giving a total of 36 plots. In TRA, tillage (35 cm deep) and nitrogen fertilization (250 kg N $year^{-1}$ given all at once at the beginning of the crop) took place just before sowing (April). MIN differed from the previous treatment only for a 10 cm deep tillage. In COM, compost (20 t/ha) was incorporated into soil by the conventional tillage that took place as for TRA. Moreover, nitrogen and other elements were adjusted in COM to equalize the quantities added to the others treatments. For each plot one bulk soil sample and one rhizosphere sample was taken respectively in October and July 2006

Effects of POR addiction were studied on wheat in Napoli and Torino, whereas in Piacenza on maize. A randomized block design with three replicates was used in such experiment, as compared with a control without POR addiction (NPOR), totally giving others 18 plots. For each wheat plot one bulk soil sample and one rhizosphere sample were taken in September and April, respectively. Bulk and rhizosphere soil from maize were taken at the same periods as for management system evaluation (i.e. October and July).

In all cases, plots were 6 x 5 m wide, even though synthetic metalporphyrins were only applied in a smaller square (1 m^2) within the POR plots because their synthesis was very time-consuming.

Soil (15 cm of top soil) was collected aseptically and stored at 4 °C until use. Root apparatus were energetically shaken to detach soil not tightly adherent and thus stored in sterile bags until use. In all cases analyses did not occur later than 5 day after sampling.

2.2.2 Cultural analyses

Total aerobic bacteria (Tot Ae Bact), fungi, actinobacteria (Actino), aerobic and anaerobic cellulosolytic bacteria (Ae Cell and Anae Cell, respectively), aerobic free-living N_2-fixing (N2-fix) and ammonia-oxidizing bacteria (AOB) were enumerated from all the soil and rhizosphere samples by using specific cultural media.

Analyses were performed after shaking 10 grams of either soil or roots with firmly adhering soil in 90 ml of sterile 0.1% wt/vol Sodium Pyrophosphate plus 10 grams of gravel for 30 minutes. After 15 minutes waiting to allow sedimentation of the largest soil particles, decimal dilutions of the mother suspension were made in Ringer solution 1/4X (OXOID). Both solid and liquid media were utilized depending on the microbial group under study. For solid media, 0.1 ml aliquots of useful dilution were spread onto the plate surface in triplicate and the results were always expressed as CFU g^{-1} of dry soil. For liquid media, 1 ml aliquots were added to the most-probable-number series, using three replicates per dilution. In all cases, plates and tubes were incubated at 20 °C in the dark. Total aerobic bacteria were enumerated by using PCA medium (OXOID) after 3 day incubation. Malt extract agar (Oxoid) plus Chloramphenicol (100 μg/ml) was used to count fungal colony at two and three day from inocula addiction. Starch-casein agar (Okazami and Okami, 1972) supplemented with Cycloheximmide (50

μg/ml) was employed for Actinobacterial colonies counts after one and two weeks of growth. Aerobic and anaerobic cellulosolytic colonies were counted onto as reported by Pochon and Tardieux (1962). Free-living aerobic N_2-fixing bacteria and Ammonia-oxidizers were enumerated using an MPN technique, following the instructions reported by Pochon and Tardieux (1962).

2.2.3 Statistics

Bacterial densities were always $\log_{10}$ transformed. SPSS 15.0 for windows software package was utilized. Data were analyzed using multivariate analysis of variance (MANOVA) and univariate analysis of variance (ANOVA) to explore the effects of origin (i.e. bulk vs. rhizosphere), porphirins, and management practices (TRA, MIN, COM) on the microbial groups under study. To assess relationship between species abundance and environmental variables on the overall set of data, a Pincipal Components Analysis (PCA) was performed by using the software package Canoco 4.5.

2.3 Results

2.3.1 Rhizosphere effect

Samples origin (e.g. bulk vs. wheat or maize rhizosphere) explained much of the difference among the microbial populations under study. As expected, we observed a noticeable rhizosphere effect exerted by the rhizosphere of maize (tab. 1 and fig 1). Such effect was limited to the total aerobic bacteria and fungal population and to a lesser extent to the soil actinobacterial community. On the other hand, ammonia-oxidizing bacteria were found decreasing significantly in comparison to bulk and wheat rhizosphere. Wheat rhizosphere did not show such a strong effect on driving microbial communities in our experiments, except for a decrease in the anaerobic cellulosolytic biomass (Fig. 1).

The rhizosphere effect appeared even more striking after considering only bulk and maize rhizosphere samples, running several one-way ANOVA with sites as supplementary factor for each microbial group (Fig. 2). Site variability was quite marked, since 5 times out of 7 their means were significantly different. Remarkably, anaerobic cellulosolytic and ammonia-oxidizers amounts in bulk soil and maize rhizosphere were highly variable among locality; depending on the site, increased or reduced biomasses in samples from different origin were found. However, a sound rhizosphere effect was assessed in almost all the remaining groups under study.

2.3.2 Synthetic metalporphyrins

Synthetic metalporphyrins amendment did not impact significantly the microbial groups analyzed, as indicated in a MANOVA testing effects of both sites and porphyrins (Tab. 2). Only the sampling site influenced such model, whereas POR addiction and the interaction between these factors did not affect significantly the means of the different bacterial and fungal biomasses (Tab. 2). Thus, we performed a MANOVA splitting the data in bulk and rhizosphere soil and analyzing the effect of the treatment separately (tab. 3). In this case, porphyrins faintly influenced microbial responses. Indeed, univariate ANOVA's for single populations (Fig. 3) clearly proved that only ammonia-oxidizing bacteria (both for bulk and rhizosphere) and actinobacteria (only for rhizosphere) were responsible for those results. Interestingly, ammonia-oxidizers number decreased and increased respectively in bulk and rhizosphere environments.

2.3.3 Management practices

The fate of microbial populations was affected by management practices as applied in our experimental scheme. Overall, only a small effect was assessed analyzing data from all the samples (bulk plus maize rhizosphere) submitted to the soil management

treatments (Fig. 4). Management effects were masked by a differential behavior of bulk and rhizosphere communities. In fact, separating data according their origin, a different outlook was found out. Four out of seven populations were significantly influenced in the bulk soil samples, whereas only two microbial populations were shown to be impacted in the rhizospheric ones (Tab.4 and Fig. 5). The general tendency was a higher biomass as passing from traditional tillage to compost addiction, through minimum tillage. Total aerobic bacteria, actinobacteria, fungi and cellulosolytic bacteria showed larger biomasses in COM respect to TRA and MIN in the bulk soil. In maize rhizosphere, free-living N2-fixing bacteria were particularly enhanced in MIN and to a lesser extent in COM.

2.3.4 Overall effects

A Principal Component analysis (PCA) was applied to the overall set of results obtained from our field experiments to get a general outlook of the major factors influencing data distribution (Fig. 6). The factors bulk - maize rhizosphere were the principal ones influencing distribution within the samples, which were separated along the first principal axis of the PCA biplot, while wheat rhizosphere samples were much more related to the bulk soil (FIG. 6a). A site-related effect was also observed (Fig. 6a): the factors Torino and Napoli affected the samples distribution along the second axis. As expected, among the treatments, only the compost addiction (COM) allowed the clustering of the samples, but to a lesser extent. Fig. 6b shows a species – environmental variables biplot where the strong rhizosphere effect exerted from the maize rhizosphere towards almost all the microbial groups taken in account is evident. A similar trend was shown for COM, but to a lesser extent, whereas traditional tillage (TRA) showed an opposite tendency. The strongest effect associated to the POR treatment in comparison

with the control without porphirine (NO POR) was related to the relatively higher counts of the nitrogen fixing bacteria group found in the former treatment.

2.4 Discussion

In this work, we attempted to better understand microbial responses to different soil management practices performed in different sites under field conditions. Experimental fields were extremely different in pedological and climatic characteristics and microbial populations were strongly influenced in both amount and composition by site and origin of sampling.

Our results showed a consistent rhizosphere effect on maize rhizosphere as compared to bulk soil under the same crop. The size and composition of the rhizosphere microflora is to a large degree plant dependent, a phenomenon known as the 'rhizosphere effect' (Burr and Caesar, 1984) due to the root exudates. The composition of root exudates was shown to vary depending on the plant species as well as on the stage of plant development (Jaeger et al., 1999). Many environmental factors, such as temperature (Rovira, 1959), light (Hodge et al., 1977) and atmospheric CO2 concentration (Cheng and Johnson, 1998) are known to modify the exudation profile. Many microbial populations increased their numbers at all the sites, even thought there were some interesting exceptions. In fact, ammonia-oxidizers population diminished significantly in maize rhizosphere in comparison to bulk; this behaviour was verified in two sites, Napoli and Piacenza, whereas in Torino they did not change in a significant manner. Nitrification, the conversion of ammonium to nitrate via nitrite, is critical to the cycling of nitrogen in terrestrial environments, increasing nitrogen losses through leaching and denitrification of nitrate (Prosser, 1989). This process is carried out in most ecosystems by autotrophic bacteria and is often rate limited by the activities of ammonia-oxidizing bacteria that are responsible for the oxidation of ammonia to nitrite. AOB are notorious

for their slow growth (Alexander, 1982), hence, being the rhizosphere a much richer environment, r-strategists microbes could have had more chance to establish there and AOB could have been loosed in this competition. Alvey and coworkers (2003) found out a large differentiation in the ammonia oxidizers of two soils. According to our study, in that work, the response of the bacterial community structure to plant species was surprising, but ammonia-oxidizing communities were significantly affected only in one site. Our results did not permit to depict any conclusion about cellulosolytic bacteria; while aerobic cellulosolytic were clearly enhanced in maize rhizosphere in two cases out of three, the situation was really variable for the anaerobic ones. Cellulosolytic bacteria have a key role in the decomposition and transformation of organic matter in soil and additional study are needed to understand their activities in the maize rhizosphere as compared to bulk soil.

The further goal of our research was the finding that synthetic metalporphirins addiction did not dramatically change microbial composition and culturable biomasses either in bulk and rhizosphere soil, even though the field factor was once more prominent. The only exception to this trend was represented by AOB. Soil ammonia oxidation is restricted to a single monophyletic group of betaproteobacteria (Kowalchuk and Stephen, 2001) and presently characterized species belong to only four genera, namely *Nitrobacter, Nitrospira, Nitrococcus* and *Nitrospina* (Teske et al., 1994). Hence, it is likely that even small and temporally-limited changes in biological and/or environmental factors can affect their behaviour. Although such microorganisms are generally considered to be aerobic, they have also been isolated or enriched from low-oxygen environments; instead their carbon source, CO2, is not thought to be limiting to ammonia oxidizer activity and growth, in most cases (Kowalchuk and Stephen, 2001). We observed a decreased ammonia-oxidizers number in bulk soil. Synthetic metalporphirins were shown to diminish CO_2 emission significantly by in-situ oxidative

polymerization of soil OM (Piccolo et al., 2002b). As a result, an increased amount of oxigen could have enhanced AOB populations in bulk soil. Additionally, factors as microbial composition within individual groups of microbes could have been responsible for it; unfortunately our approach culture-dependent based did not permit to confirm this possibility. Conversely, porphirins in rhizosphere led to a significant decrease in AOB. Microbes and roots must compete for nutrients and NH_4^+ is a central N source for plants, as well as for AOB. In maize and wheat seedlings has been shown that when NH_4^+ and NO_3^- are either supplied in the medium, ammonium ions uptake by the primary roots is preferred (Taylor and Bloom, 1998). OM mineralization is thought to provide a large part of the NH_4^+ source for soil inhabiting organisms and the competition versus plant might be crucial for AOB. Moreover, plants tend to acidify the rhizosphere when NH_4^+ serves as the sole N-source and alkalinize the rhizosphere when NO_3^- serves as sole N-source (Marschner, 1995). Thus, pH changes near the roots can also affect responses in AOB population, but chemical analyses should support those hypothesis. Nonetheless, the community patterns of ammonia oxidizer 16S rRNA clones were found to depend on the soil acidity, with *Nitrosospira* cluster 2 being established at low soil pH (Kowalchuk et al., 2000a). Another selective factor was the concentration of ammonium, with *Nitrosospira* cluster 3 dominating in early successional soils with relatively high ammonium concentrations while *Nitrosospira* clusters 2 and 4 dominated in old successional soils with low ammonium concentrations (Bruns et al., 1999; Kowalchuk et al., 2000a; Kowalchuk et al., 2000b). Those finding strongly support our hypotheses but further studies should be addressed to explore the within-group community structure of AOB following porphirins addiction.

In conformity with earlier knowledge, several recent studies have shown that soil management practices, such as crop rotation, tillage, fertilizer, compost, manure, or pesticide applications and irrigation greatly affect soil microbial parameters (Anderson

and Gray, 1990; Omay et al., 1997; Schonfeld et al., 2002; Sparling et al., 1994). Our results emphasize the positive effect exerted by compost addiction towards many microbial populations known to be important in the turn-over of OM, such as actinobacteria, fungi and cellulosolytic bacteria. This was particularly true in bulk soil, whereas in maize rhizosphere the different practices did not show such an obvious effect. Rhizosphere is a more stable and richer environment than bulk soil. In fact, microorganisms depend principally on the supply of organic component excreted by the root apparatus. Even though exudates can strongly vary depending on the soil practices, it is likely that in the bulk the different soil management can enhance much more microbial activities and in turn their biomasses. In the rhizosphere instead microbial populations react indirectly via the plant response and mechanisms of such response are definitely more complex.

Therefore, our results pointed out that plant rhizosphere (as compared to the bulk soil) and sampling site are much stronger factors in shaping microbial community composition than short-term treatments. Nevertheless, compost addiction caused a significant increase in culturable biomasses of many microbial groups known to be positively related to soil health and quality.

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2.6 List of figures and tables

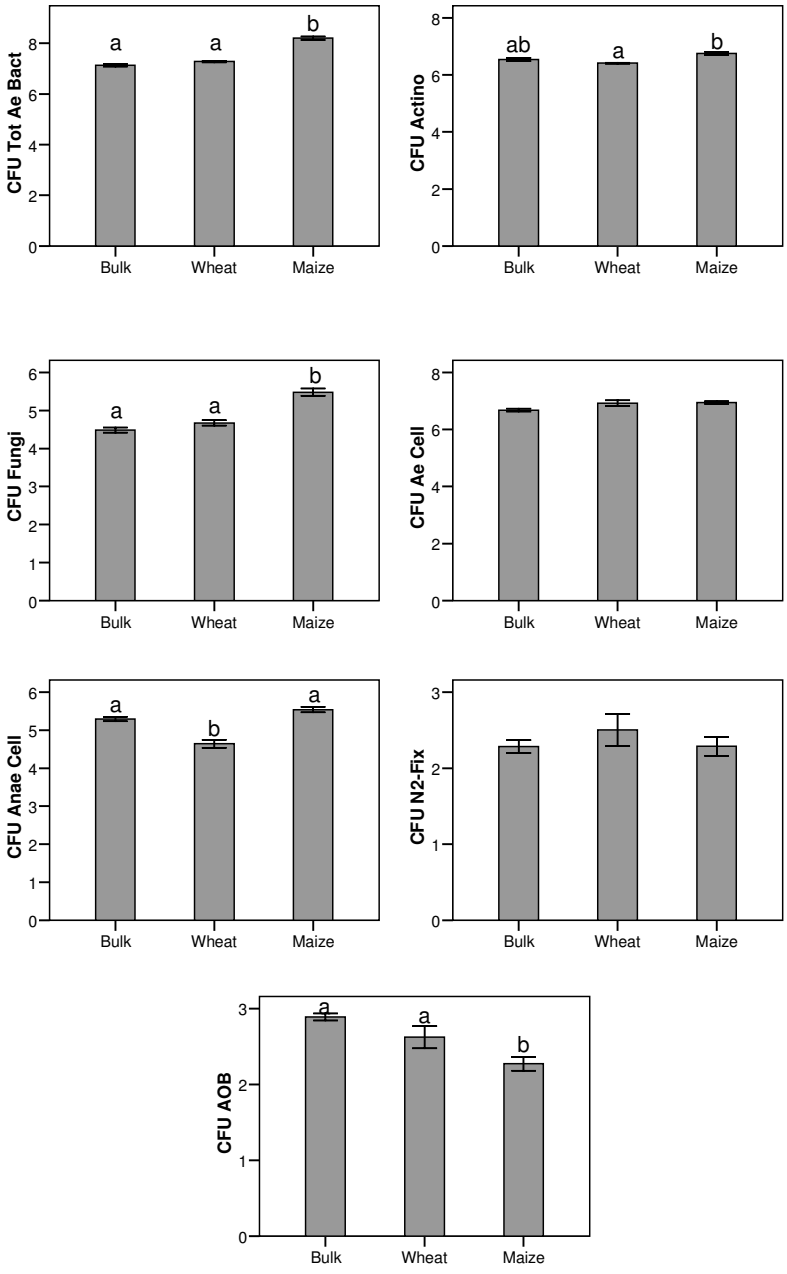

Fig 1. Effect of samples origin on the microbial groups under study. Values on y-axes represent mean $\log_{10}$ CFU g^{-1} dry soil values (± standard errors) with no distinction between sampling sites and treatments. Bulk soil bars, n = 54; Wheat rhizosphere bars, n = 12; Maize rhizosphere bars, n = 42. Results of one-way Analysis of Variance (ANOVA) within each microbial group are reported in tab 1. Significant differences (Tukey's post-hoc test; $P<0.05$) within the same microbial group are indicated by different letters. Abbreviations are reported in the text. Note the different scales on the y-axes.

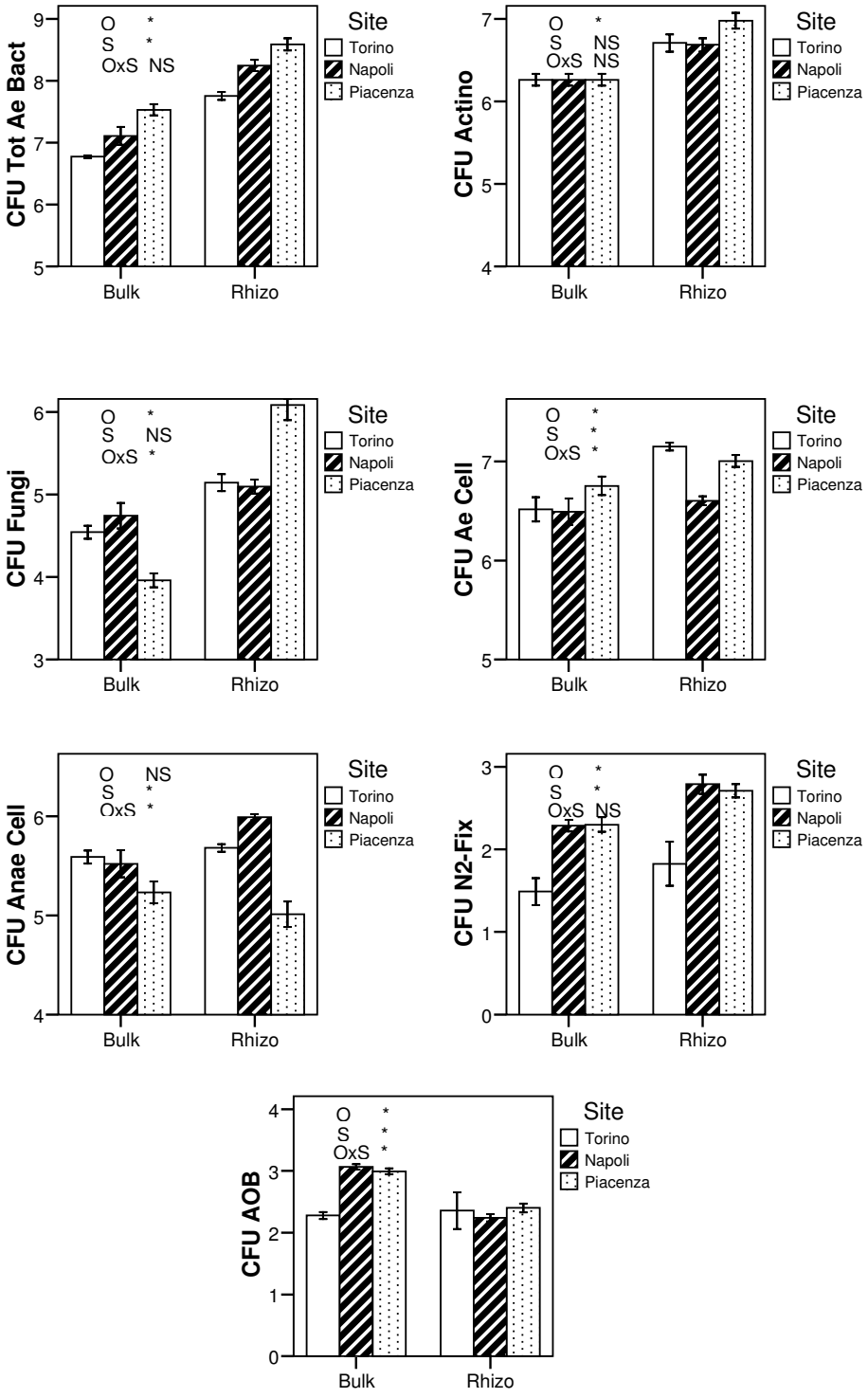

Fig. 2: Culturable microbial biomasses (log CFU g-1 dry soil ± standard error) as affected by origin (i.e. Bulk vs. Maize rhizosphere) and sample site. Data were analyzed using a fully-crossed, two way ANOVA with origin and site as the factors within each group (for each bar n=12). O, origin; S, site; * significant at P<0.05; NS, not significant. Note the different scales on y-axes.

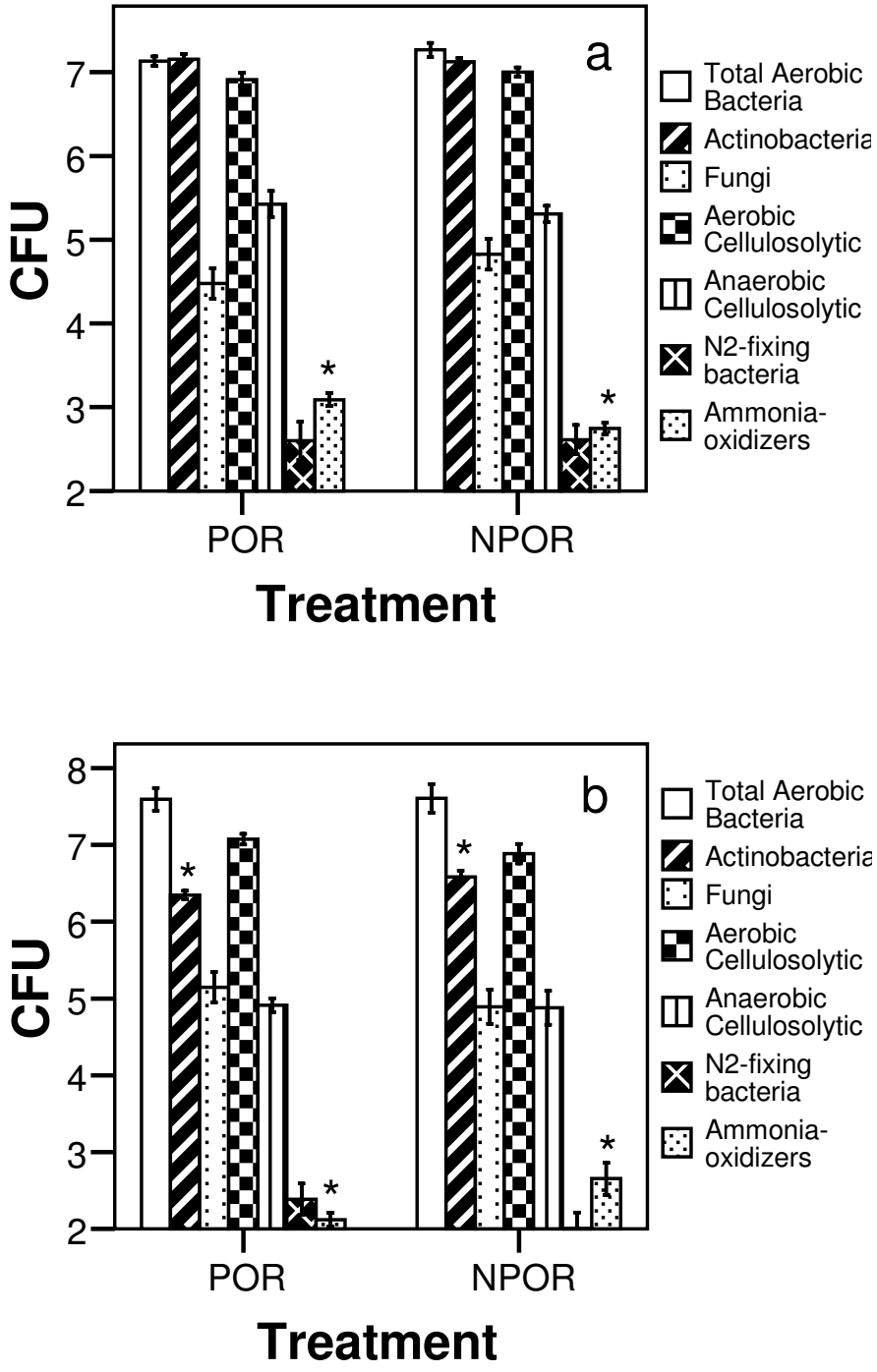

Fig 3: Effect of synthetic metalporphyrins addiction on the biomass (log CFU g^{-1} dry soil) of microbial groups in bulk (a) and rhizosphere (b) soil. Results of one-way Analysis of Variance (ANOVA) within each microbial group (separately for bulk and rhizosphere) are reported in tab 3. Significant differences within the same microbial group are indicated by an asterisk. POR, synthetic metalporphirins addiction; NPOR, control.

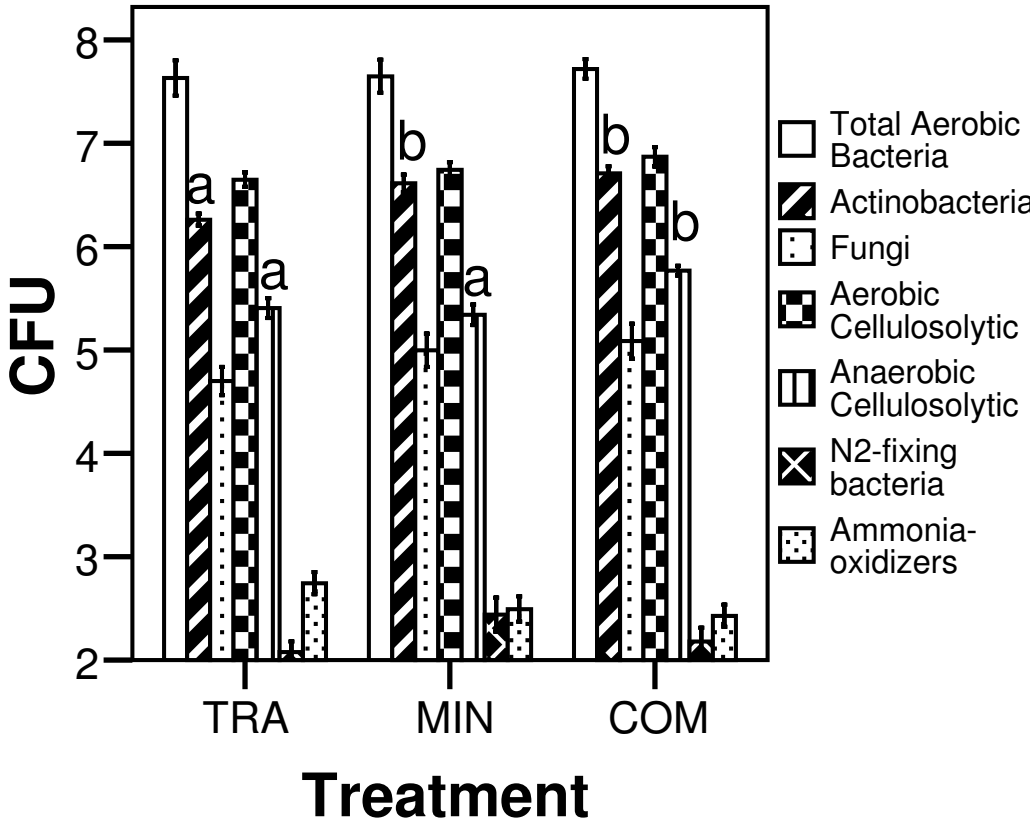

Fig 4: Effect of management practices on the biomass (log CFU g^{-1} dry soil) of microbial groups with no distinction in origin (i.e. bulk vs. rhizosphere) and sample site. One-way Analysis of Variance (ANOVA) was performed within each microbial group with mamagement as the only factor. Significant differences (Tukey's post-hoc test; $P<0.05$) within the same microbial group are indicated by different letters. TRA, Traditional tillage; Min, minimum tillage; COM, compost addiction

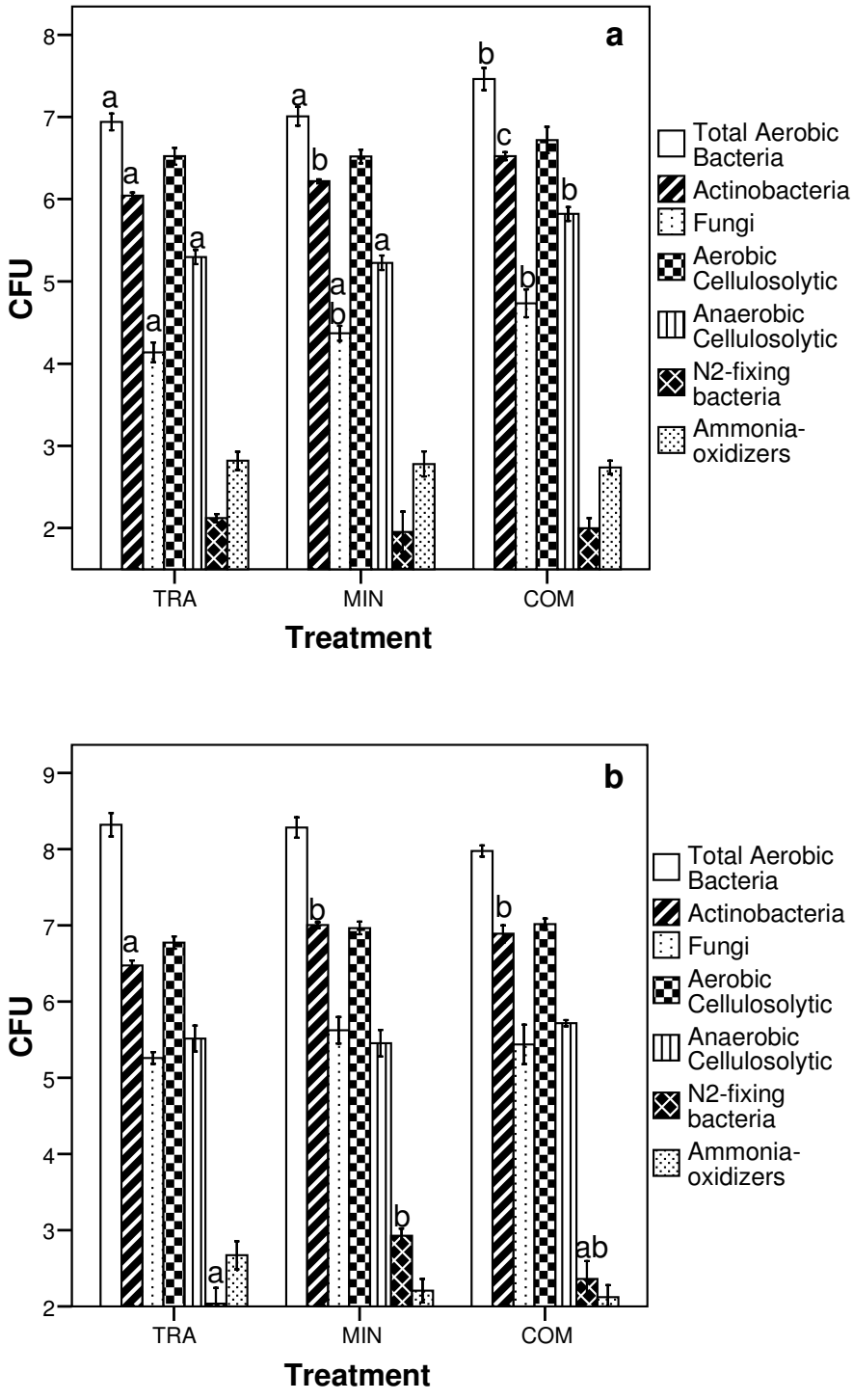

Fig 5: Effect of soil management practices on the biomass (log CFU g^{-1} dry soil) of microbial groups in bulk (a) and rhizosphere (b) soil with no distinction among sites. Results of one-way Analysis of Variance (ANOVA) within each microbial group (separately for bulk and rhizosphere) are reported in tab 4 Significant differences (Tukey's post-hoc test; $P<0.05$) within the same microbial group are indicated by different letters.

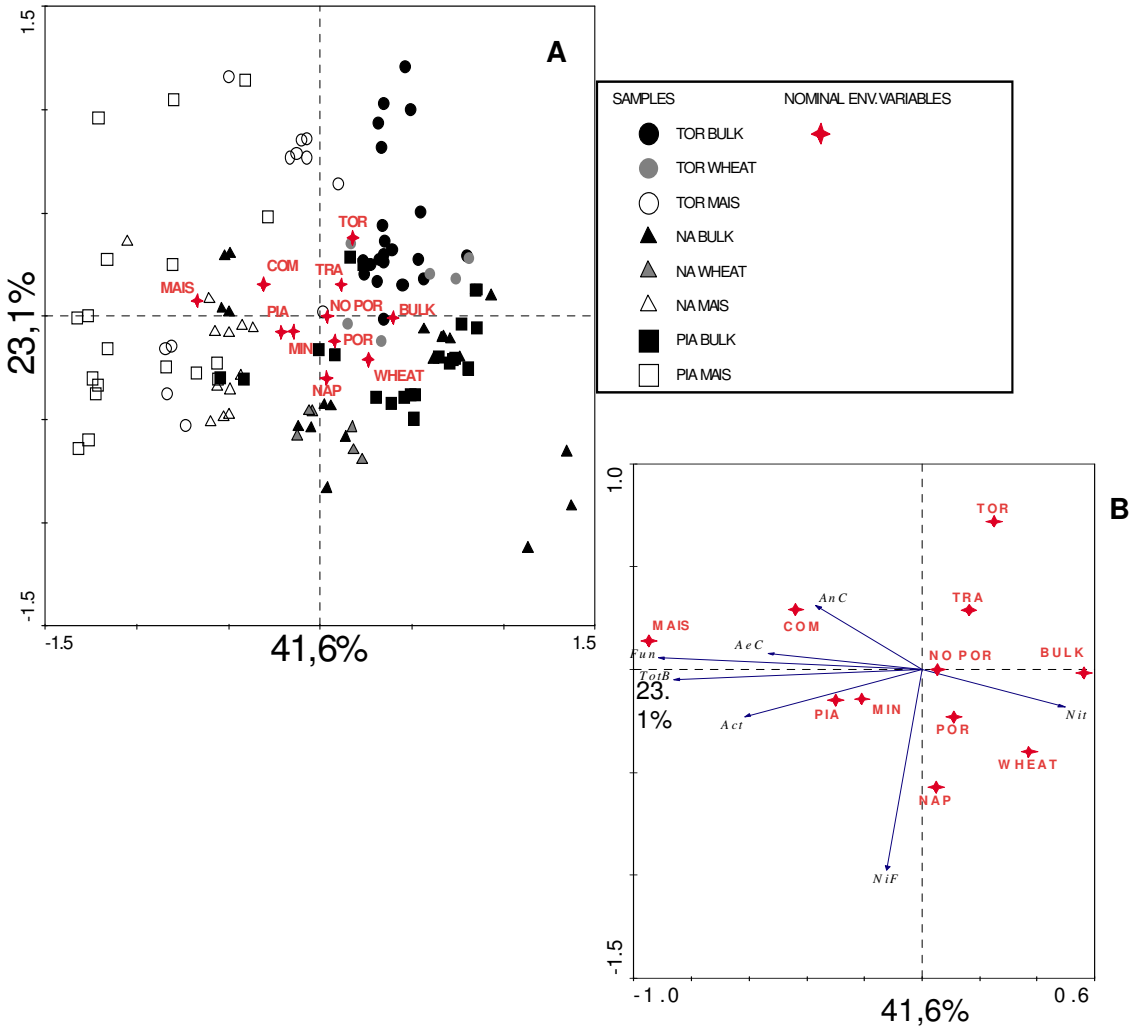

Fig 6: Ordination biplots generated by PCA using cultivable group specific microbial counts of all the soil and rhizosphere samples (n=117) as related to the environmental nominal variables under study. Labels displayed on diagram axes refer to the pecentage variation of species-environment correlations accounted for the respective axis. a: samples-environmental variables biplot. b: species-environmental variables biplot. TotB, Total aerobic bacteria; Act, Actinobacteria; Fun, Fungi; AeC, Aerobic cellulosolytic; AnC, Anaerobic cellulosolytic; NiF, N_2-fixing bacteria; Nit, Ammonia-oxidizers

Tab 1: Multivariate analysis of variance (MANOVA) and univariate analysis of variance (ANOVA) results for the effect of sampling origin (i.e. bulk soil vs. rhizosphere of maize and wheat) on culturable microbial populations.

Multivariate	df (num, den)	Wilks'λ	F-value	P
Origin	14,198	0.17	19.77	0.0001

Univariate

Dependent var	df	Tot Ae Bact	Actino	Fungi	Ae cell	Anae Cell	N-fix	AOB
		F-value	F-value	F-value	F-value	F-value	F-value	F-value
Origin	2	90.22*	4.74*	38.77*	5.75*	21.89*	0.79	15.92*
Error	105							

*For univariate analyses, significant effects at the 0.05 α-level are indicated with an asterisc

Tab 2: Multivariate analysis of variance (MANOVA) and univariatere analysis of variance (ANOVA) results for the effect of Porphyrins addiction and sampling site on culturable microbial populations.

Multivariate

	df (num, den)	Wilks'λ	F-value	P
Porphyrins	7,24	0.603	2.26	0.065
Site	14,48	0.015	24.92	0.0001
Porph x Site	14,48	0.513	1.36	0.211

Univariate

Dependent var	df	Tot Ae Bact	Actino	Fungi	Ae cell	Anae Cell	N-fix	AOB
		F-value	F-value	F-value	F-value	F-value	F-value	F-value
Porphyrins	1	0.48	0.60	0.06	0.33	0.24	2.54	0.33
Site	2	17.78*	1.25	2.96	0.029	3.37*	28.47*	2.23
Porph x Site	2	0.58	0.32	2.27	2.66	1.57	0.50	0.98
Error	30							

*For univariate analyses, significant effects at the 0.05 α-level are indicated with an asterisc

Tab 3: Multivariate analysis of variance (MANOVA) and univariate analysis of variance (ANOVA) results for the effect of Porphyrins addiction on culturable microbial populations (after splitting data between bulk and rhizosphere soil)

Multivariate bulk	df (num, den)	Wilks'λ	F-value	P
Porphyrins	7,10	0.179	6.56	0.0004

Univariate

Dependent var	df	Tot Ae Bact	Actino	Fungi	Ae cell	Anae Cell	N-fix	AOB
		F-value	F-value	F-value	F-value	F-value	F-value	F-value
Porphyrins	1	1.73	0.13	1.85	0.78	0.41	0.002	11.33*
Error	16							

Multivariate rhizosphere	df (num, den)	Wilks'λ	F-value	P
Porphyrins	7,10	0.072	18.36	0.0001

Univariate

Dependent var	df	Tot Ae Bact	Actino	Fungi	Ae cell	Anae Cell	N-fix	AOB
		F-value	F-value	F-value	F-value	F-value	F-value	F-value
Porphyrins	1	0.002	5.91*	0.74	1.69	0.02	1.67	5.6*
Error	16							

*For univariate analyses, significant effects at the 0.05 α-level are indicated with an asterisc

Tab 4: Multivariate analysis of variance (MANOVA) and univariate analysis of variance (ANOVA) results for the effect of soil management system on culturable microbial populations (splitting data between bulk and rhizosphere soil)

Multivariate bulk	df (num, den)	Wilks'λ	F-value	P
Management	14,54	0.13	6.75	0.0001

Univariate

Dependent var	df	Tot Ae Bact	Actino	Fungi	Ae cell	Anae Cell	N-fix	AOB
		F-value	F-value	F-value	F-value	F-value	F-value	F-value
Management	2	5.81*	39.88*	5.28*	0.93	14.12*	0.29	0.11
Error	33							

Multivariate rhizosphere	df (num, den)	Wilks'λ	F-value	P
Management	14,54	0.24	4.05	0.0001

Univariate

Dependent var	df	Tot Ae Bact	Actino	Fungi	Ae cell	Anae Cell	N-fix	AOB
		F-value	F-value	F-value	F-value	F-value	F-value	F-value
Management	2	2.31	13.2*	0.96	2.65	0.92	5.78*	3.21
Error	33							

*For univariate analyses, significant effects at the 0.05 α-level are indicated with an asterisc

-CHAPTER III-

SOIL SOLARIZATION WITH BIODEGRADABLE MATERIALS AND ITS IMPACT ON SOIL MICROBIAL COMMUNITIES

3.1 Introduction

The increasing concern about the impact of mineral fertilizers, fungicides and herbicides on the environment and human health requires the development of alternative agronomic techniques that may reduce the use of these products. This need is furthermore emphasized by the occurrence in pests of resistance to fungicides, the breakdown of host resistance by natural populations (McDonald and Linde, 2002), and the phasing out of methyl bromide in 2005 for its negative impact on the ozone layer (Martin, 2003). Among the alternative strategies, soil solarization (SS), which is a method used to increase soil temperature by using transparent plastic sheets over the soil to retain the sun radiation energy, seems one of the most promising techniques to control soilborne plant pathogens and weeds (Katan et al., 1976; Stapleton, 2000). In solarized soils, control of pests is imputable to multiple mechanisms which primarily involve thermal inactivation of plant pathogens, because of soil temperature increases under plastic films (Katan et al., 1976), or weakening of the pathogen propagules that become more susceptible to competition or antagonistic activity of the native soil microflora (Stapleton, 2000). Saprophytic microorganisms, including several antagonists, are usually more tolerant to heat than plant pathogens (Stapleton, 2000). SS has been proved to be effective in controlling populations of many important soilborne fungal pathogens such as *Verticillium dahliae*, the causal agent of vascular diseases of many plants (Pinkerton, 2000), certain *Fusarium* spp. that cause *Fusarium* root-rot and wilt in several crops (Bourbos, 1997; Tamietti and Valentino, 2006), and *Rhizoctonia solani* and *S. minor* that cause lettuce drop (Sinigaglia et al., 2001). In addition, like other soil-disinfestation techniques, SS often promotes plant growth by disease independent mechanisms such as the improvement of soil structure (Chen et al., 1991), release of mineral nutrients (Chen et al., 1991; Grünzweig et al., 1999) and stimulation of plant growth promoting rhizobacteria (PGPR) (Gamliel and Stapleton, 1993). It is

well known that SS with plastic films profoundly affects some soil chemical and microbiological parameters. For example, an increase of the NH4-N and NO3-N concentration in the top 15 cm of soil has been reported in several studies (Stapleton and DeVay, 1995; Grünzweig et al., 1999; Gelsomino et al., 2006. The concentration of soluble mineral nutrients, including calcium, magnesium, phosphorus, potassium, and others, increased sometimes, but frequently the results were not consistent (Chen et al., 1991; Grünzweig et al., 1999).

Although it is well recognized that SS affects a broad range of soil microrganisms, rather contrasting results were reported regarding the fate of soil microflora in response to SS. Both positive and negative effects on total bacterial and fungal populations have been found (Khaleeque et al., 1999; Barbour et al., 2002; Sharma et al., 2002). Solarization may increase many groups of bacteria like fluorescent *Pseudomonas* and *Bacillus* spp. in the bulk soil or rizosphere (Gamliel and Stapleton, 1993). However, recent studies, by the use of denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S ribosomal RNA (rRNA) gene coding fragments from soil-extracted DNA (Muyzer and Smalla, 1998), provided useful information on the effect of SS on the structure and diversity of soil microbial communities (Schönfeld et al., 2003; Gelsomino and Cacco, 2006). PCR – DGGE is a powerful method for assessing the structure of microbial communities in environmental samples (Muyzer and Smalla, 1998), without cultivation steps on cultural media. Following amplification, DGGE separates the products and allows the detection of a larger microbial community diversity compared to cultivation methods (Winding et al., 2005). The utilization of this approach for studying microbial ecology revealed the existence of a vast and previously unknown bacterial diversity (Felske et al., 1998). However, to be visible as a band on the gel, a species should represent at least 1% of the soil microbial community (Casamayor et al., 2000).

In addition to SS, alternative techniques to methyl bromide are based on the use of soil fumigants with a wide spectrum of actions such as Metham Sodium or Dazomet (Martin, 2003), the application of calcium cyanamide which has herbicidal and fungicidal properties (Bourbos, 1997), or soil amendments with organic matter (OM) (Hoitink and Boehem, 1999). OM can control soilborne pathogens through several mechanisms such as the release of fungitoxic compounds, generation of fungistasis (Lockwood, 1977), or selective stimulation of soil microbes which are antagonists to pathogens (Hoitink and Boehm, 1999).

Despite its potential, an important limitation to the diffusion of the SS technique is the serious drawback regarding the disposal of used traditional plastic materials: plastic waste management, such as on-farm burning or land filling, has environmental and monetary costs for the farmers. A possible solution to this problem is the use of biodegradable plastics (Al-Kayssi and Al-Karaghouli, 2002), which gradually degrade when plowed-down due to the action of soil microorganisms. The use of biodegradable solarizing materials would eliminate the monetary costs for the farmer and reduce the environmental impact. Although there are some comparative studies between SS with biodegradable and plastic films (Russo et al., 2005), most of the research done deals with the effect of biodegradable materials on soil temperature. Little attention has been paid to their effects on crop productivity and on soil chemical and microbiological parameters.

The aim of this study was to investigate the impact of SS with biodegradable materials and plastic films, organic amendments and soil disinfestation with fumigants on crop productivity, soil chemical parameters and microbial community structure and diversity.

3.2 Materials and methods

3.2.1 Solarizing materials

SS was carried out by applying the following materials to soil: a) polyethylene plastic sheets POLYSOLAR (PLS), b) starch based biodegradable film MaterBi (MB), and c) polysaccharides mixture based (1.5%) biodegradable spray material PSS (PSS). MB is a biodegradable transparent film (thickness 30 μm) produced from a starch base by NOVAMONT (S.p.a. Novara, Italy). PSS is a biodegradable spray material obtained from a mixture of polysaccharides at a concentration of 1.5% and with the addition of fibres of cellulose for mechanical reinforcement produced by P.S.I. (Polysaccharide Industries AB, Sweden).

3.2.2 Field experiments

Experiments were carried out in Southern Italy (Salerno) during the 2005 cropping season in two sites with different soil types. The first was a clay soil (sand 45%, silt 21.5 %, clay 33.5%, pH 8.21, organic matter 0.85%, total N 0.81 g/kg, C/N 6.1, total CaCO3 189 g/kg, available phosphorus (P2O5) 6.2 mg/kg, exchangeable potassium 0.46 meq/100 g, exchangeable magnesium 1.89 meq/100 g, exchangeable calcium 27.6 meq/100 g, exchangeable sodium 0.35 meq/100 g, EC 0.096 dS/m); the second was a sandy soil (sand 83.9%, silt 1.9 %, clay 14.2%, pH 8.41, organic matter 0.55%, total N 0.65 g/kg, C/N 5, total CaCO3 140 g/kg, available phosphorus (P2O5) 48.1 mg/kg, exchangeable potassium 0.53 meq/100 g, exchangeable magnesium 0.96 meq/100 g, exchangeable calcium 15.5 meq/100 g, exchangeable sodium 0.17 meq/100 g, EC 0.188 dS/m). Experimental plots consisted of three adjacent areas measuring 21 x 12 m, and treatments were arranged in the block in a randomized design with three replications. Plots (3 x 3 m) were separated in beds by 1.0 m buffer areas. Seven soil treatments were made: (i) SS with PLS, (ii) SS with MB, (iii) SS with PSS, (iv) soil amended with calcium cyanamide at a rate of 300 kg/ha (CNN), (v) soil amendment with *Medicago*

sativa straw (MS), (vi) soil disinfestation with covered Dazomet at a rate of 50 gm2 (DZ), and (vii) control as bare soil. All treatments were applied to soil without pathogen inoculum and soil artificially inoculated with *F. oxysporum* f.sp. *lycopersici* (FOL) and *S. minor* (SM). FOL is the causal agent of the wilt disease of tomato and SM is the causal agent of soft rot of a wide range of hosts, including lettuce. For the artificial inoculum, common millet seeds, placed in 2-l flasks and imbibed with a Czapeck (OXOID) solution (1/10), were inoculated with FOL or SM previously cultured on PDA (Potato Dextrose Agar, DIFCO). Flasks were incubated for 21 days at 21°C. The resulting FOL or SM millet inoculum was air-dried for three days and added at a rate of 50 g/m2 to the field plots seven days before soil treatments. This inoculation method proved to be effective in previous greenhouse experiments (data not shown). To avoid thermal stress to the fungal inoculum, the material was applied in the afternoon after 5:00 p.m. and manually incorporated by rake into the first 20 cm of soil. In the control, not inoculated common millet was added to plots. Before the application of solarizing materials, soil was milled (first 20 cm), levelled and subsequently brought to water field capacity through irrigation by aspersion. The SS with plastic films was carried out by mulching soils with PLS (thickness 50 μm) in the period June–August for 63 days. MB was applied as for PLS, while PSS, at the dose of 2 l/m2, was sprayed with a gun connected to a compressor with an internal-combustion engine. During solarization soil temperatures were recorded at 2 and 20 cm deep in the untreated control and solarized plots by using thermocouples connected to a digital thermometer. Soil temperature was measured hourly during the whole day cycle six times during the solarization period (3, 14, 29 July and 3, 18 and 24 August). At the end of the solarization period, the solarizing materials were removed.

Soil amendments were carried out with air-dried MS straw (C/N ratio of 20) at rate of 500 g/m2 equivalent to 12.5 g/m2 of N. The straw was manually spread over the plots,

and then incorporated into the soil by rototilling. The application of CNN was done with the same procedure of MS at rate of 30 g/m2 equivalent to 6 g/m2 of N. Finally, Dazomet, at a rate of 50 g/m2, was incorporated by rototilling in the first 20 cm and soil covered with polyethylene sheets. MS, CNN and DZ have been applied at the beginning of the period of solarization.

At the end of the solarization period, 30 day-old seedlings of tomato (*Lycopersicon esculentum* cv. San Marzano) and lettuce (*Lactuga sativa* cv. Cambria) were planted on each plot. After 40 and 80 days of growth for tomato and lettuce, respectively, plants were harvested (n=30 per plot) and their fresh weight measured.

3.2.3 Effects on soil chemical parameters

Immediately after the end of solarization, from each plot three composite soil samples each consisting of four different soil cores pooled together were randomly collected from the upper 20-cm layer. After air-drying (3 days) soil samples were sieved (mesh size 2 mm) and stored at 4 °C. Soil was analyzed for total N, ammonium, nitrate, available phosphorus (Olsen method) and organic matter content. For all chemical analyses the official methods of the Italian National Society of Soil Science were used (Violante, 2000).

3.2.4 Effects on soil microbial community structure

Microbial activity was assessed with the Fluorescein Diacetate method (FDA) (Workneh et al., 1993). Bacterial and fungal monitoring was done by using a multi-technique approach that combines both conventional cultivation-based methods and ribosomal RNA gene-based molecular analysis of soil community DNA (Liesack et al., 1997).

3.2.4.1 Fate of *Pseudomonas* population

Pseudomonas Agar Base (OXOID) combined with *Pseudomonas* CFC supplement was chosen as medium for *Pseudomonas* enumeration. Ten grams of soil were transferred to a 250 ml flask with 90 ml of sterile distilled water containing 0.025% W/V of Na4P2O7 to facilitate microbial release from the soil. Flasks were shaken for 30 min at 200 r.p.m. and then stored for 30 min to allow the sedimentation of soil particles. Aliquots of supernatants were serially diluted in the Ringer solution 1/4X (OXOID) and each dilution was spread on the plate surface in triplicate. Plates were incubated at 20 °C for 24 – 48 hours and colonies counted under UV – light. The results were expressed as CFU/g of dry soil.

3.2.4.2 Bacterial and fungal community structure

DNA extraction was performed from 0.5 g of each soil by using the Fast DNA Spin kit for soil according to the manufacturer's instructions (BIO 101, Vista, CA, U.S.A.). The amount of DNA extracted from each soil was standardized by gel electrophoresis to obtain 10 ng of DNA template in each PCR mixture. Bacterial 16S rRNA gene fragments were amplified with primers 341f-GC and 534r which generated amplicons of about 194 bp (Muyzer et al., 1993). Amplifications were performed in a MyCycler thermocycler (Bio – Rad, Hercules CA 94547 USA) by using a touchdown temperature scheme as follows: 5 min at 94 °C, then 10 cycles of 231 1 min at 94 °C, 1 min from 65 °C to 55 °C (touchdown of 1 °C per cycle), and 3 min at 72 °C. Then, 25 additional cycles, each of 1 min at 94 °C, 1 min at 55°C and 3 min at 72 °C were carried out. Finally, a time extension of 30 min at 72 °C was performed for eliminating artefact in DGGE profiles (Janse et al., 2004). Each 50 µl mixture contained 1X PCR Buffer (Invitrogen; La Jolla, USA), 1.25 mM MgCl2, 250 µM of each deoxynucleoside triphosphate, 0.1 µmol of each primer, 5 µg of bovine serum albumine and 5 U of *Taq*

polymerase (Invitrogen; La Jolla, USA). Fungal 28S rRNA gene fragments were amplified with primers 403-f and 662-r (Sigler and Turco, 2002). Amplifications were performed by using a touchdown temperature scheme as follows: 5 min at 94 °C, 10 cycles of 30 sec at 94 °C, 1 min from 60 °C to 50 °C (touchdown of 1 °C per cycle), and 2 min at 72 °C. Twenty additional cycles, each of 30 sec at 94 °C, 1 min at 50 °C and 2 min at 72 °C were carried out. Finally, a time extension of 7 min at 72 °C was performed. Each 50 µl mixture contained 1X PCR Buffer (Invitrogen; La Jolla, USA), 1.25 mM MgCl2, 250 µM of each deoxynucleoside triphosphate, 0.2 µmol of each primer, and 5 U of *Taq* polymerase (Invitrogen; La Jolla, USA). DGGE analysis were performed by using a DCode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Acrylamide gels (8% W/V) were prepared by means of a Model 475 Gradient Delivery System (Bio-Rad Laboratories) by using a denaturing gradient ranging from 30 to 60% (100% denaturant solution contained 7M urea and 40% deionized formamide). DGGE was performed with 1X Tris Acetate EDTA buffer at 60 °C and a constant voltage of 200 V for 4 hours. After staining with ethidium bromide gels were observed by using an UV transilluminator. Banding patterns were photographed by using the Gel Doc 2000 documentation system (Bio-Rad Laboratories, Hercules, CA, USA).

3.2.5 Data analysis

Data were analysed statistically using analysis of variance (ANOVA). Two-way ANOVA was used to test the effects of soil type and soil treatments on crop productivity and soil chemical and microbiological parameters. The relationships between soil chemical and microbiological parameters and between these two types of parameters and crop growth were estimated using regression analysis. Significance was evaluated in all cases at $P<0.05$.

Banding patterns of eubacterial and fungal DGGE were analyzed by Quantity One Image Analysis Software (Bio-Rad Laboratories, Hercules, CA, USA). After applying a rolling disc background subtraction (setting 8) and a sensitivity setting of 10, the software performed the analysis of each lane: band was detected if it accounted for greater than 0.5 % of the total lane intensity. The program also provided the total band number and identification of bands occupying common position between the lane. The clustering of the patterns was performed by the Unweighted Pair Group method with Mathematical Average (UPMGA; Dice coefficient of similarity). Band richness (BR) from DGGE profile was used as quantitative assessment of both bacterial and fungal species richness.

3.3 Results

3.3.1 Soil temperature during solarization

The temperature of soils covered with solarizing materials was always higher than that of bare soils. The highest values were recorded with the PLS and the biodegradable film MB (Fig. 1). The biodegradable spray PSS increased soil temperature, but was less effective compared to PLS and MB (Fig. 1). The maximum temperatures in bare control, and covered soil with PSS, PLS and MB were respectively 39.2, 52.2, 62.8 and 70.5 at 2 cm and 33.0, 33.8, 41.1 and 38.6 at 20 cm of depth. PSS material showed only limited evidence of biodegradation during solarization, which does not affect its solarizing capacity (data not shown). Differently, solarization with MB lasted only 20-25 days in both soil types because, after such period, the material was completely torn in correspondence of the points in which it was buried.

3.3.2 Effects on crop productivity

SM and FOL inoculum did not affect plant growth (ANOVA, P=0.46; data not shown). Soil treatments significantly influenced tomato growth in both soil types (Fig. 2a; ANOVA, P<0.01 in both cases), but the interaction between soil type and treatments was not significant. Tomato growth was increased by the MS amendment and fumigation with DZ, and by PLS solarization only in the sandy soil (Fig. 2ab). Soil treatments significantly affected lettuce growth in both soil types (Fig. 2b; ANOVA, P<0.01 in both cases), and the interaction between soil type and treatments was significant (ANOVA, P<0.05). Lettuce growth was significantly higher in the clay compared to the sandy soil (Paired t-Test: P<0.01) and it was increased by PLS and MB solarization and DZ fumigation in the clay soil, but only by DZ in the sandy soil (Fig. 2b).

3.3.3 Effects on soil chemical parameters

All the soil chemical parameters analysed were significantly affected by the treatments in both soil types (One-way ANOVA: P<0.05; Table 1), with the exception of nitrate nitrogen. Total nitrogen was increased by the amendment with MS in both soils and by the application of CNN and DZ in clay soil and it was weakly decreased by solarization with PSS and MB in sandy soil (Table 1). Ammoniacal nitrogen was increased by the application of MS, CNN and solarization with MB in the clay soil. In the sandy soil the most evident increase was due to the amendment with MS and the application of CNN and DZ, while a less marked increase was recorded after the solarization with MB and PLS. The soil concentration of nitrate nitrogen was always very low, without any differences among treatments (data not shown).

Available phosphorus in clay soil was reduced, compared to control, by solarization with PLS and MB and to a lesser extent by the application of CNN. In sandy soil available phosphorus was slightly increased only by the amendment with MS (Table 1).

Organic matter was significantly increased only by the soil amendment with MS in both soil types (Table1). In addition, a weak but not significant decrease was recorded after the application of PLS, MB and CNN in both soils, with a clearer effect in the sandy soil.

3.3.4 Effects on soil microbial populations

FDA activity was not different between the two soil types (t-Test: P=0.09), and only the amendment with MS significantly increased this parameter in both soil types (Fig. 3). Since preliminary tests exhibited no significant influence of SM and FOL inoculum on the recovery of *Pseudomonas* spp. and on the diversity of DGGE banding patterns (results not shown), all the analysis were performed exclusively on soils without pathogen inoculum.

3.3.4.1 *Pseudomonas* enumeration

Population size of fluorescent *Pseudomonas* (PF) was significantly higher in the sandy soil (t-Test: P<0.01). MS amendment and soil fumigation with DZ strongly increased the PF in both soil types compared to control (Fig. 4). SS affected PF in a contrasting way: in the clay soil MB decreased the PF number, while in the sandy soil PF was decreased by PSS and increased by PLS. Finally, the application of CNN reduced the PF in both soils compared to control (Fig. 4).

3.3.4.2 DGGE analysis

Denaturing gradient gel electrophoresis patterns of DNA from sandy and clay soils showed an elevate number of bacterial and fungal amplicons (Fig. 6). Band richness (BR) was significantly higher for fungi compared to bacteria, in both soils (t-Test: P<0.001 in both cases). BR of bacteria from sandy and clay soils were not different (t-

Test: P=0.43). All treatments decreased BR of bacteria from the sandy soil compared to control, with the exception of the CNN application (Table 2). BR of bacteria from the clay soils showed a significant decrease with MB solarization and DZ sterilization, and a decrease with MS amendment (Table 2). Since cluster analysis revealed high similarities between replicates of bacterial patterns (95% similarity, data not shown), we showed only one replicate out of the three analyzed (Fig. 7a). The composition of the soil bacterial community was differently influenced by the treatments in each soil type and cluster analysis clearly separated sandy and clay soils, with the only exception of the MB treatments (cluster 3). In fact, MB solarization enabled the clustering of the sandy and clay soil samples together, overcoming the soil effect (Cluster 3; Fig. 7a). Samples of DZ, CNN, PSS and PLS grouped together with a similarity around 82% (cluster 1), while all the clay soil treatments except MB and CNN clustered at 72% (cluster 2). MS amendment in the sandy soil and CNN application in the clay soil showed low similarities to any other treatment of each soil group.

Complex fungal community structure was found by analyzing DGGE profiles (Fig 6). Fungal BR of sandy soil was significantly greater than that of clay soil (t-Test: P<0.05). As observed for bacterial BR, all soil treatments reduced fungal BR compared to the control and DZ and MS treatments exhibited the greatest reduction (Table 2). No statistically significant differences were recorded for fungal BR in the clay soils. Fungal cluster analysis (Fig. 7b) clearly distinguished the two soil categories (10% similarity). Similarity values observed among the sandy soil samples were higher than those observed among the clay soil cluster (about 45% vs. 35%). However, replicates of each soil treatment were highly dissimilar and did not allow any separation among the treatment effects. Only replicates of DZ fumigation on sandy soils were grouped with a similarity around 60% (Fig. 7b).

3.3.5 Relation among soil variables and crop growth

Tomato growth was positively related to the population size of PF in both soil types (Fig. 5). Moreover, tomato growth was positively related to total and ammoniacal nitrogen in sandy soil (Pearson coefficient = 0.84 and 0.92, respectively; $P<0.01$ in both cases) and to OM in clay soil (Pearson coefficient = 0.98; $P<0.01$). Tomato growth was unrelated to bacterial BR in both soil types, while a significant negative relationship was found with fungal BR in the sandy soil (Pearson coefficient = -0.82; $P=0.022$). Lettuce growth was unrelated to all the chemical parameters monitored (data not shown), with the exception of a negative relationship with fungal BR (Pearson coefficient = -0.87; $P<0.01$) and a positive relationship with PF population in the sandy soil (Fig. 5). In addition, the incidence of lettuce drop was unrelated to all measured soil variables, including the FDA activity and fungal and bacterial BR, both in the sandy and clay soils (data not shown).

Among the soil variables, in the clay soil, only the ammoniacal nitrogen was positively related to the FDA activity, and fungal BR was positively related with bacterial BR (Table 3). In the sandy soil several parameters were significantly related (Table 3): a positive relation was found between OM and FDA activity and available phosphorus, and between these latter variables. Total nitrogen was positively related to the ammoniacal nitrogen and FDA activity. Finally, fungal BR was negatively related to PF and bacterial BR was negatively related to P2O5, OM and FDA activity.

3.4 Discussion

3.4.1 Effects on crop productivity

Many studies provided evidence that SS with plastic films increases crop yields and allows the control of many soilborne pests and weeds (Stapleton and De Vay, 1995; Stapleton, 2000). In the present study, SS with plastic films, and to a lesser extent with

biodegradable materials, was only partially effective in increasing crop yields and the effects were limited in comparison to DZ fumigation and amendment with MS. Previous studies related the positive effect of SS on crop yields to increases in $NH4^+$–N or $NO3^-$-N (Chen et al., 1991; Patrício et al., 2006). According to these authors, the increase in $NH4^+$–N is usually greater in soils with higher amounts of organic matter, because temperature enhancement increases soil organic N mineralization. Therefore, the limited increase of $NH4^+$-N in both soil types recorded in our study could depend on to the very low amount of OM in both soil types (< 0.7%). $NO3^+$–N concentration in soils was always very low in both solarized and non-solarized plots (Table 1). Decreases in nitrifying bacteria due to SS has been reported and related to the accumulation of $NH4^+$–N in soil (Chen et al., 1991). However, in the present work, $NH4^+$–N accumulated with all treatments suggesting a very low bacterial nitrifying activity also in the untreated soil. The increased growth observed in solarized soils may be associated to the increase in PF populations (Gamliel and Stapleton, 1993). Our results showed that SS has a variable but limited effect on PF in comparison to other soil treatments. A negative effect of PSS and MB on PF was recorded in the sandy and clay soil, respectively, while a positive effect of PLS was found in the sandy soil. The observed variable effect of SS on PF is in agreement with previous results that some bacteria of the fluorescent group decrease their population because are highly sensitive to SS. However, PF are able to rapidly recolonize the soil after SS (DeVay and Katan, 1991; Stapleton and De Vay, 1984). Amendment with MS significantly increased FDA activity, PF population size, OM level and $NH4^+$–N concentration in both soil types. MS amendment has a contrasting effect on crop yields, with a strong positive effect on tomato and no effect on lettuce. Our data suggest that the positive effect of MS on tomato growth may be due to the increase of PF rather than to the higher availability of mineral nitrogen. DZ fumigation greatly improved the yield of both tomato and lettuce

especially that of the latter species in the sandy soil, but had a limited influence on chemical parameters with only a slight positive effect on $NH4^+$–N concentration. The positive effect of DZ on plant growth is well known and often has been related to elimination of soilborne pathogens (Martin, 2003). However, our experimental results suggest that the yield increase may depend on a change in the microbial community and, specifically, on the increase of PF population. Alternative explanations may be related to the reduction in the populations of minor plant pathogens as well as to enhancements of other beneficial microorganisms. In this context, it should be pointed out that crop yield was positively related to PF population size in both soil types. PF are known as plant growth promoting rhizobacteria and they can improve plant mineral nutrition, release stimulatory compounds and act as biocontrol agent towards soilborne pathogens (Smith and Goodman, 1999; Lugtenberg et al., 2001). Growth of both tomato and lettuce were negatively related to fungal richness in the sandy soil while no significant correlations were found with bacterial richness. These results do not support the hypothesis that microbial richness is directly and positively related to soil ecosystem function and fertility (Coleman and Whitman, 2005).

3.4.2 Effects on soil microbiological and chemical parameters

PF populations strongly increased in both soils with MS amendment and DZ fumigation. In the first case, the effect could be due to the increased availability of carbon and nutrients, especially nitrogen, because of the low C/N ratio of *Medicago sativa* straw that could support PF growth. This is also in agreement with the large increase of FDA activity, a measure of the amount of enzymatic activity (protease, lipase, non-specific esterase) that is related to soil organic matter decomposition (Nannipieri et al., 2003). However, in a previous study, Mazzola et al. (2001) found an increase of PF populations by amending with seed meal of *Brassica napus* at low

dosages but a dramatic decline, below the level of detection, when higher rates were applied. This study and our results suggest that the PF response to organic amendments is dependent on the type of OM and their application rate. DZ fumigation increased population size of PF of several orders of magnitude in both soil types. Elliot and Desjardin (2001) showed that 70 days after fumigation with DZ and other fumigants, the population sizes for all of the bacterial groups studied were greater than those of the non fumigated soil, with a significant increase for PF. The same results have been found by using different soils (Miller et al., 1997; Toyota et al. 1999). This effect could be explained hypothesing that part of the microbial population is killed and used like pabulum by PF. However, it should be noted that since DZ fumigation decreased fungal and bacterial richness in both soil types, PF are able to recovery more rapidly than other bacteria and fungi. In contrast with the impact of MS and DZ on PF, the application of calcium cyanamide, in both soils, reduced the population of PF. This evidence suggests that this compound has a specific negative effect on the populations studied, since the treatment did not affect bacterial and fungal richness.

DGGE analysis is a powerful and reliable method to compare the effect of different treatments on microbial community structure, although DGGE band pattern represents only the most abundant species in soil (Muyzer and Smalla, 1998). In our study, soil type was the major determinant of the composition and structure of the bacterial and fungal communities because it, more than soil treatments, dictated the clustering into groups (Fig. 7). Costa et al. (2006a) stated that the sampling site is one of the main factor affecting the relative abundance and distribution of PCR-DGGE ribotypes. DGGE bacterial patterns among soil replicates were very similar, while fungal replicates were not related at all: cluster analysis was not able to differentiate among the treatments, as reported in other studies (Klamer et al., 2002; Costa et al., 2006b). In this context, a large number of comparative studies have been performed to improve

knowledge about both sample size (Ellingsøe and Johnsen, 2002; Ranjard et al., 2003) and methods of DNA extraction from soils (Zhou et al., 1996; Martin – Laurent et al., 2001). According to Ranjard et al. (2003), assessing soil microbial community structure by the use of molecular techniques requires a satisfactory sampling strategy that takes into account the high microbial diversity and the heterogeneous distribution of microorganisms in the soil matrix. These authors stated that the sampling strategy should be different according to the objectives: large soil samples (≥ 1 g) for fungal community structures, while smaller soil samples (≥ 0.125 g) are sufficient for bacterial ones. By contrast, Ellingsøe and Johnsen (2002) found, by using a DGGE approach, that only bacterial community structures of large sample size (1 and 10 g) were very similar among replicates. The impact of SS by traditional plastic on soil microbial communities has been previously studied (Culman et al., 2006). Gelsomino and Cacco (2006) stated that SS was the main factor inducing strong time-dependent population shifts in eubacterial community structure. However, to our knowledge, this is the first report on the effect of solarization performed with biodegradable materials on microbial population analyzed by DGGE. SS with both biodegradable and plastic films generally decreased fungal and bacterial band richness, with a more pronounced effect on bacteria. Among the solarizing treatments, MB showed the most negative effect on bacterial diversity in both soils and on fungal diversity in the sandy soil (Table 2). MB treatments clustered together with the bacterial clay soils cluster. It is important to note that this was the only case in our study where the effect of the treatment overcomes the soil influence on the community structure. This could be related to the very strong, although temporally limited, soil heating observed with MB. This hypothesis is indirectly supported by the limited effect on microbial richness of SS with PSS, which is the less effective material in soil heating. However, it is also possible to suppose a

specific development of microbial populations capable of degrading the MB constituents (mainly starch).

In our work, a single MS amendment significantly affected bacterial and fungal DGGE profiles, but the effect was strictly dependent on the soil type. Specifically, MS slightly increased bacterial richness in the clay soil, while a strong negative effect on both bacteria and fungi was recorded in the sandy soil. This fact was confirmed by the bacterial cluster analysis (Fig. 7a), which showed a low similarity among MS treated samples and all the other treatments. These microbial richness reductions appear surprising because organic amendments commonly are responsible for an increase of the microbial biomass and richness (Sun et al., 2004). The loss of diversity following MS amendment could be due to the sharp increase of a few dominant microbial species, such as PF (Fig. 4), that may rapidly exclude other species by competition. The impact of organic amendment on soil microbial community depends on its nature and amount applied, soil type and time elapsed from the treatment. Some studies found that organically managed soils had a higher diversity of bacteria than conventionally managed soils (Drinkwater et al., 1995; Sun et al., 2004). On the other hand, some authors found no differences in bacterial diversity (Lawlor et al., 2000) or in fungal communities (Franke-Snyder et al., 2001) between the two types of managements. Moreover, Gelsomino and Cacco (2006) reported a limited effect of organic matter amendment, despite of an huge dosage applied, on soil microbial community compared to SS.

Traditional methods have been previously used to study the effects of fungicides on soil microrganisms (Shukla and Mishra, 1996). In our work, DZ fumigation reduced both bacterial and microbial richness, with the most negative effect on fungi in the sandy soil. DZ replicates in the sandy soil cluster was the only one that clustered together in the PCR-DGGE analysis performed with fungal primers, thus confirming the strong

effect of the fumigation on the fungal community structure in this soil. The limited loss of diversity in the clay soil after DZ fumigation could be due to the formation of clay – humic complexes which may partially absorb the fungicide thus reducing its negative effect on microbial populations. For example, Sigler and Turco (2002) analyzed the impact of the fungicide chlorthalonil on bacterial and fungal soil populations, by using a DGGE molecular approach, and found that after a single application, the community changes were less pronounced in soils with higher organic matter contents.

Soil organic C content was expected to diminish after solarization because of the heat induced breakdown of soil organic resources and the enhanced microbial activity after heating. However, we found that total soil OM was not significantly changed by SS in agreement with previous studies (Chen et al., 1991; Stapleton et al., 1985; Gelsomino et al. 2006) that report a lack of significant differences of organic C amounts between non solarized and solarized bare soils. It is known that soil organic matter is not a very variable soil characteristic and, unless large amendments are made as in our amended plots (Table 1), several years are generally required to detect significant changes resulting from different soil management regimes (Mikha and Rice, 2004). Additionally, in our study sites the OM level was already very low (<0.7%), suggesting that OM is present in a stabilized and recalcitrant form that is not susceptible to a rapid decomposition after soil heating by solarization.

The results of this research show the potential of using the biodegradable solarizing materials in place of plastic films and their effect on chemical parameter and microbial community structure. SS was shown to have only a limited effect on the bacterial and fungal communities, with a tendency to reduce species richness in both the soil analyzed and a concomitant restricted effect on crop yields, both considering plastic and biodegradable materials. Further study is required to improve the positive effect of SS on the crops, even through the enhancement of the soil microbial beneficial activities.

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3.7 List of figures and tables

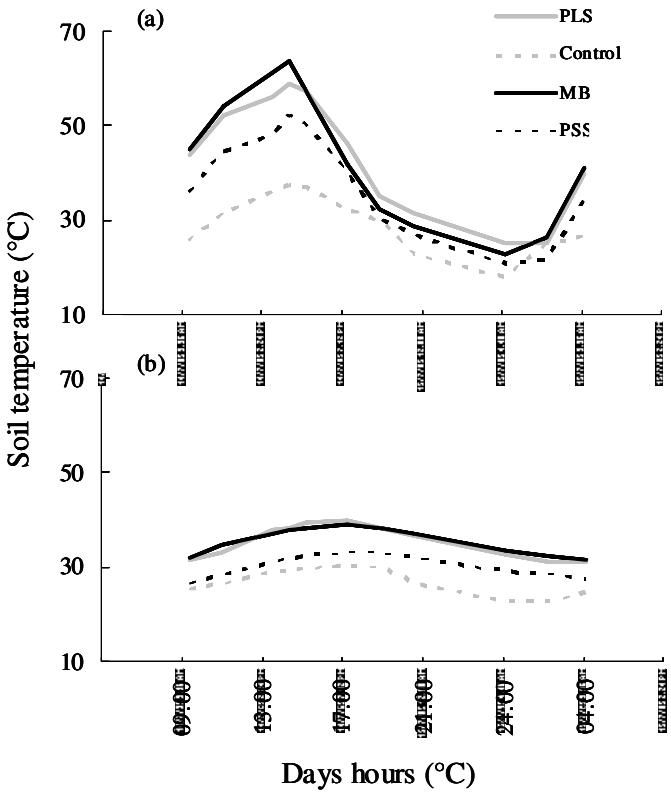

Fig. 1. Soil temperature variations measured at 2 (a) and 20 cm depth (b) during a summer day (14-07) with different solarizing materials. The same dynamics has been recorded in the other days (data not shown).

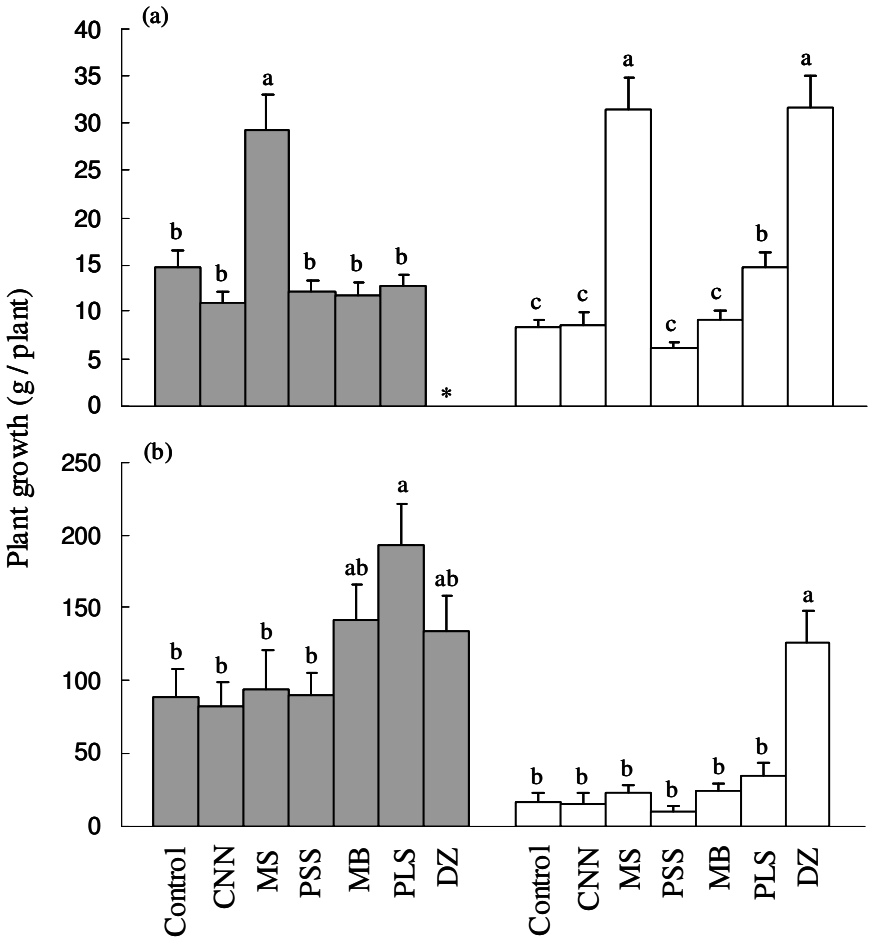

Fig. 2. Effect of soil treatments on growth of tomato (a) and lettuce (b) in the clay (grey bars) and sandy soil (open bas). Different letters indicate significant differences (comparison only within soil type; Duncan test, P<0,05). Data are averages (+1SE) of three replicates. Data of tomato growth in the clay soil treated with DZ (*) was lost for technical problem.

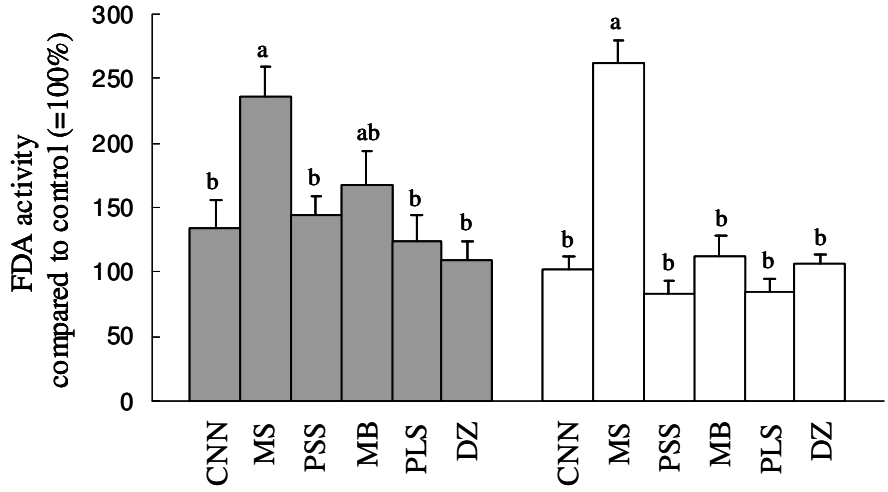

Fig. 3. Effect of soil treatments on soil enzymatic activity (FDA) compared to control (=100%) in the clay (grey bars) and sandy soil (open bars). Different letters indicate significant differences (comparison only within soil type; Duncan test, $P<0.05$). Data are averages (+1SE) of three replicates.

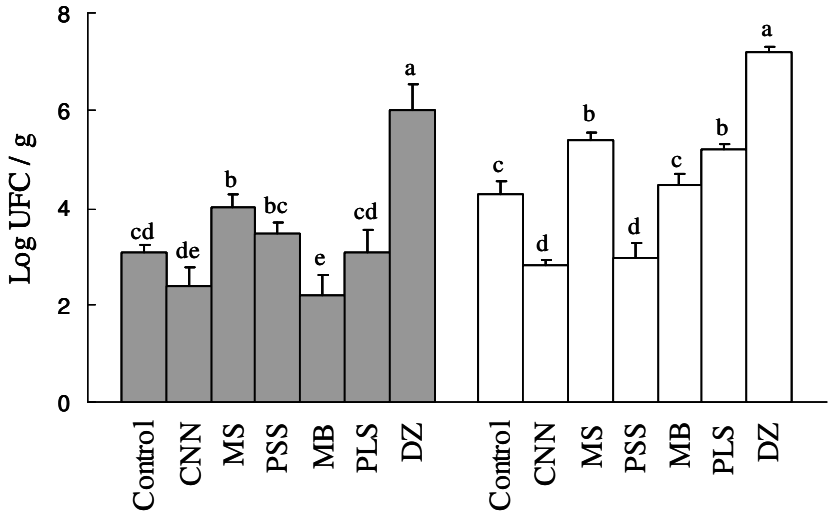

Fig. 4. Effect of soil treatments on the population size of *Pseudomonas fluorescens* in the clay (grey bars) and sandy soil (open bars). Different letters indicate significant differences (comparison only within soil type; Duncan test, $P<0.05$). Data are averages (+1SE) of three replicates.

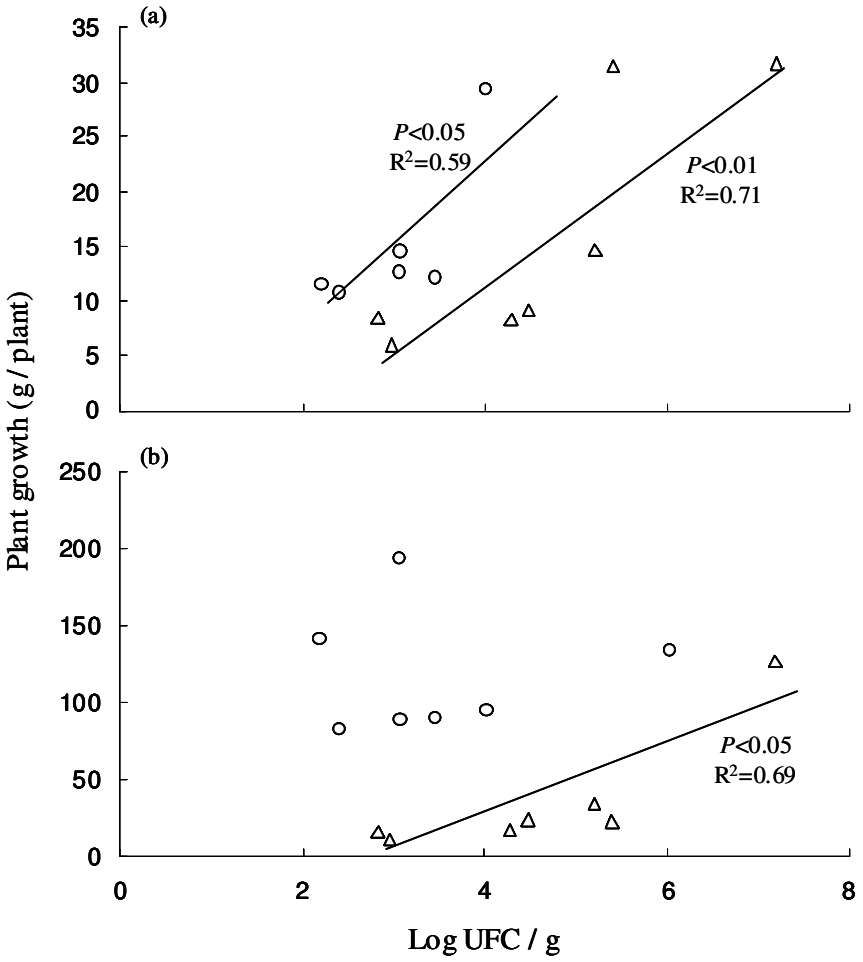

Fig. 5. Regression analysis for crops productivity (a=tomato, b=lettuce) and the soil population size of *Pseudomonas fluorescens* in the clay (circles) and sandy soil (triangles). The levels of statistical significant are indicated on each graph.

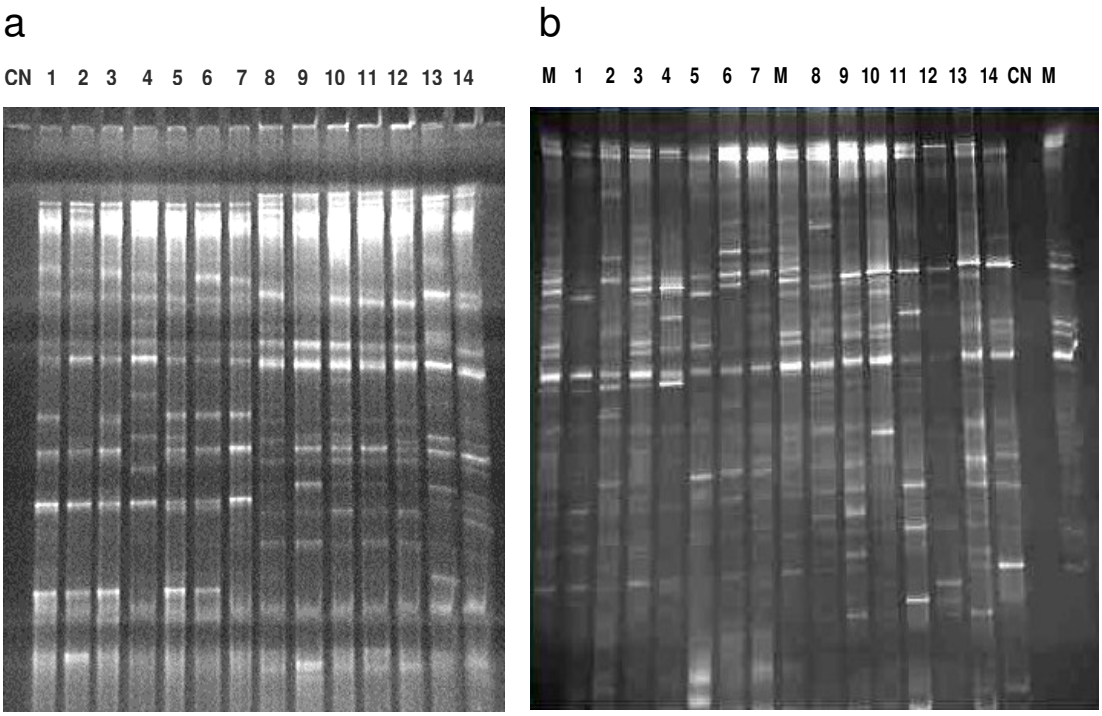

Fig 6: DGGE patterns comparison of 16S rRNA (a) and 28S rRNA (b) amplified genes of clay (lane 1 to 7) and sandy (lanes 8 to 14) soils. Treatments were as follow: lanes 1 and 8, control soils; lanes 2 and 9, DZ treated soils; lanes 3 and 10, PLS treated soils; lanes 4 and 11, CNN treated soils; lanes 5 and 12, PSS treated soils; lanes 6 and 13, MS treated soils; lanes 7 and 14, MB treated soils. CN, negative control; M, markers.

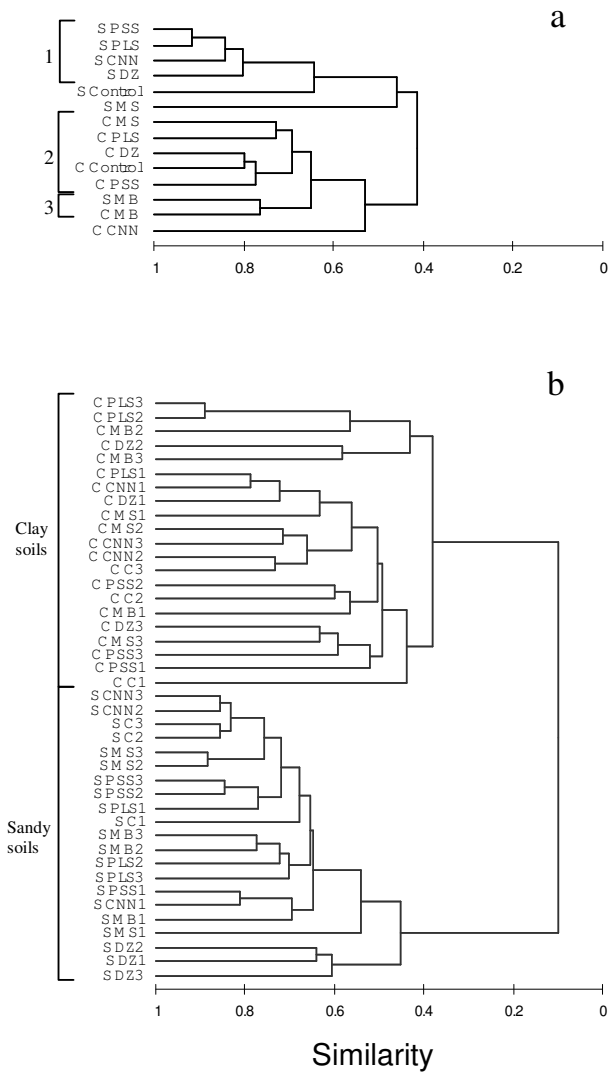

Fig 7: Cluster analysis (UPGMA, Dice coefficient) of bacterial (a) and fungal (b) banding pattern of clay (capital letter C before the sign of the treatment) and sandy (capital letter S before the sign of the treatment) soils. Signs of the treatments were described in the text.

Table 1. Effect of soil treatments on total nitrogen, ammoniacal nitrogen, available phosphorus and organic matter in the clay and sandy soil. Different letters indicate significant differences (comparison only within soil type; Duncan test, $P<0.05$). Data are averages (±1SE) of four replicates.

	Clay soil				Sandy soil			
	Total N (g/kg)	$N\text{-}NH_4$ (mg/kg)	P_2O_5 (mg/kg)	Organic matter (g/kg)	Total N (g/kg)	$N\text{-}NH_4$ (mg/kg)	P_2O_5 (mg/kg)	Organic matter (g/kg)
Control	0.70±0.02b	35.4±1.15b	40.0±6.51a	7.05±1.03b	0.65±0.02b	31.5±1.01c	35.7±2.08b	6.46±0.47b
CNN	0.84±0.08a	61.6±1.63a	23.8±6.11b	6.89±0.71b	0.66±0.04b	38.8±3.75b	32.7±6.31b	6.11±0.62b
MS	0.90±0.11a	60.8±6.69a	38.5±7.10a	8.86±0.53a	0.78±0.04a	47.1±3.01a	48.4±6.91a	8.41±1.49a
PSS	0.69±0.06b	38.8±1.05b	40.2±5.47a	6.89±0.61b	0.57±0.02c	28.4±1.04c	33.3±3.68b	5.88±0.38b
MB	0.75±0.08b	65.2±2.47a	10.5±1.48c	6.98±0.15b	0.56±0.02c	33.0±2.40b	33.7±3.69b	6.26±0.85b
PLS	0.74±0.04b	40.9±0.68b	22.3±3.55b	6.76±0.46b	0.61±0.12b	38.1±2.63b	38.0±7.35b	6.18±0.39b
DZ	0.88±0.05a	39.4±1.15b	30.9±7.30a	6.99±0.89b	0.71±0.14ab	46.8±9.56a	34.1±2.08b	6.12±0.74b

Table 2. Means of band number values for bacterial and fungal PCR-DGGE in response to different treatment in the clay and sandy soil. In the columns, different letters indicate significant difference (Duncan test. P< 0.05)

	Bacteria		Fungi	
	Sandy soil[a]	Clay soil[a]	Sandy soil[a]	Clay soil[a]
Control	14.7 ± 0.3c	12.0 ± 0.6bc	27,00 ± 1,2c	19,00 ± 3,6
CNN	15.3 ± 0.3c	13.0 ± 0.6cd	25,00 ± 3,1bc	20,33 ± 1,5
MS	8.7 ± 0.3a	14.0 ± 0.6d	19,67 ± 2,0b	19,00± 0,0
PSS	13.0 ± 0.0b	13.0 ± 0.0cd	23,33 ± 2,7bc	17,33 ± 4,5
MB	12.7 ± 0.3b	10.3 ± 0.3a	21,67 ± 3.0bc	11,67 ± 2,3
PLS	12.7 ± 0.9b	11.0 ± 0.0ab	23,33 ± 0.7bc	17,00 ± 1,5
DZ	13.0 ± 0.0b	10.3 ± 0.3a	13,00 ± 1,0a	15,33 ± 3,5
Means	12.9 ± 0.5[b]	12.0 ± 0.3[b]	21.9 ± 1,2[c]	17.1 ± 1.1[c]

[a]: Means of three replicates ± standard errors
[b]: Means are not significant (t test, P > 0.05)
[c]: Means are statistically significant (t test, P < 0.01)

Table 3. Cross-correlation matrix between soil chemical and microbiological parameters measured in the clay and sandy soil (PF = *Pseudomonas fluorescens*; BR_F = fungal band richness; BR_B = bacterial band richness). Values represent the correlation coefficient, in bold significant correlation at $P<0.05$.

	Clay soil								Sandy soil							
	Tot N	$N\text{-}NH_4$	P_2O_5	OM	FDA	PF	BR_F	BR_B	Tot N	$N\text{-}NH_4$	P_2O_5	OM	FDA	PF	BR_F	BR_B
Tot N	-	0.44	-0.01	0.58	0.41	0.57	0.17	0.19	-	**0.86**	0.69	**0.76**	**0.75**	0.41	-0.46	-0.51
$N\text{-}NH_4$	-	-	0.55	0.39	**0.84**	-0.47	-0.16	0.20	-	-	0.55	0.56	0.58	0.63	-0.71	-0.51
P_2O_5	-	-	-	0.38	-0.27	0.49	0.63	0.61	-	-	-	**0.95**	**0.90**	0.24	0.14	**-0.87**
OM	-	-	-	-	0.70	0.22	0.26	0.60	-	-	-	-	**0.97**	0.15	-0.14	**-0.82**
FDA	-	-	-	-	-	-0.26	-0.20	0.32	-	-	-	-	-	0.18	-0.24	**-0.85**
PF	-	-	-	-	-	-	-0.01	-0.15	-	-	-	-	-	-	**-0.87**	-0.48
BR_F	-	-	-	-	-	-	-	**0.77**	-	-	-	-	-	-	-	0.43
BR_B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

-CHAPTER IV-

MICROBIAL DIVERSITY DETERMINES THE INVASIVENESS OF SOIL – IMPLICATIONS FOR THE SURVIVAL OF *ESCHERICHIA COLI* O157:H7

4.1 Introduction

Human activities can change the environment at different scales and thus affect ecosystem properties. In particular, effects on the composition of naturally-occurring communities influence ecosystem properties and functioning, and the type and magnitude of the effects can be diverse (Hooper et al., 2005). Among the key properties of natural ecosystems, resistance towards invasion by alien species is a major environmental concern (Vitousek et al, 1996). Theoretical (Case, 1990; Levine e D'Antonio, 1999) and experimental studies, the latter conducted in controlled systems (McGrady-Steed et al., 1997; Kennedy et al 2002; Levine 2000), have provided support for the hypothesis that biologically more diverse communities are less susceptible to invasion than less diverse communities. However, at a regional scale, the opposite might be true, as assemblages of diverse organisms were shown to be invaded more easily than simpler natural communities (Levine and D'Antonio, 1999); most likely, this may have come about from extrinsic factors, such as resource heterogeneity, that covary with the diversity of native and invading species at large scales (Kennedy et al., 2002).

Whereas these and other studies involved plant communities, the effect of the diversity in natural ecosystems on microbial invaders has remained underexplored. In soil, microbial communities are the key components that determine the (complex) nature of the system (ref) and thus resistance to invasion. The number of bacterial species in soil is predicted to be enormous, which is likely caused by their easy speciation and difficult extinction (Dykhuizen, 1997). The concomitant large functional redundancy present in soil microbial communities is often thought to override effects of diversity on specific ecosystem functions (Franklin and Mills, 2006), invasibility being one of them. A traditional dictum has it that the microbiostasis conferred on the soil ecosystem by the microbiota dictates the invasibility of the system. That is, natural soil systems

commonly contain "filled" ecological niches and are therefore recalcitrant to colonization. How microbiostasis relates to the diversity and community structure of resident communities is still a largely unanswered question. In this respect, a recent study revealed an inverse relationship between the microbial complexity in the rhizosphere of wheat and the invasibility of the system by *Pseudomonas aeruginosa* (Matos et al, 2005). In another recent study in microcosms (Irikiin et al, 2006), the chances of *Ralstonia solanacearum* biovar 2 to cause disease in tomato - and thus to presumably establish - were also found to be inversely related to the complexity of the bacterial communities present. However, both studies involved the rhizosphere, which represents a very particular environment in soil. Very recently, van Elsas et al. (2007) reported a presumed similar effect of changed microbial diversity in soil on *Escherichia coli* O157:H7 survival. This organism is a severe pathogen of humans and animals, and it can infect humans from, e.g., food grown in manured soils (Franz, 2005) or in soils treated with contaminated waters. Following invasion, the organism can cause death in elderly and/or immunocompromised people (Ritchie et al, 2003). A major issue that arises is the survival of this pathogen in the environment, e.g. soil (Jiang et al, 2002).

In this study, we addressed the basic issue of survival of an organism considered to be alien to soil, an avirulent *Escherichia coli* O157:H7 derivative, in the same physical habitat, soil, in the face of changed structures of the resident microbial communities. We also specifically investigated whether protozoa and/or other meso- and macrofaunal organisms exert a major role against invaders. To address these questions, two approaches were used, (1) construction of randomly-established communities of progressively-increasing complexity (on the basis of cultured isolates), and (2) dilution-to-extinction based assembly of microbial communities of progressively decreasing complexity.

In the first approach, we assessed the fate of *Escherichia coli* O157:H7 by measuring its survival (EcSurv) in soil containing a series of constructed communities with progressively increasing numbers of bacterial isolates obtained from a species-rich grassland, Wildekamp (W) grassland soil (Garbeva, 2005). A microcosm experiment was set up with this presterilized soil by gamma irradiation. Artificial bacterial communities were assembled in the microcosms that consisted of zero, 5, 20 and 100 randomly-picked bacterial strains added in equal amounts (cell numbers) to the soil. The microcosms were left to stabilize for 30 days at 20 °C, after which the *E. coli* invader was added. Using a time course, bacterial richness and diversity were then evaluated by PCR-DGGE analyses of 16S rRNA genes, while total and culturable bacterial biomass were respectively determined by DTAF microscopic cells count and plating on agar medium (see Methods).

In the second approach, a so-called dilution-to-extinction technique was used. Soil dilutions containing microbial communities of decreasing complexity were prepared by serial dilutions of a suspension obtained from the W grassland soil. Such suspensions were subsequently used to inoculate the previously-sterilized (gamma irradiation) W soil. We also included the natural (unirradiated) W soil, as well as the sterile soil. Three treatments were used, in which equal volumes of 10-, 10^3- and 10^6-fold dilutions were added to the sterile soil in microcosms. We included an additional treatment, in which the 10^{-1} dilution was filtered through nylon membranes to obtain communities free of protozoa (denoted 10-F). This way, effects of protozoan predation were distinguished from those of competition and/or antagonism exerted by the non-protozoan microflora. After a 30-day stabilization period, *E. coli* O157:H7 was introduced at a density of 10^8 cells g^{-1} and microcosms were incubated at 20 C and constant moisture (controlled by regular weighing). *Escherichia coli* survival (EcSurv),

total and culturable bacterial biomass, as well as overall bacterial, fungal and group-specific richness and diversity indices were evaluated across the 60-day experiment.

4.2 Material and methods

4.2.1 Microcosms

We used a sandy soil obtained from the upper 10 cm of an experimental field (Wildekamp) located in Wageningen, the Netherlands, and covered by permanent grassland. The sampled soil contained water at field capacity. Part of the soil was sterilized by using gamma irradiation (50 kGray). Microcosms consisted of 250 ml-Becker filled with 50 g of sterile soil in which the moisture content was set at 75% of WHC by addition of sterile water. Control microcosms consisted of natural soil, as well as sterile soil without addition of inoculant cells (sterile). In total, 135 microcosms were constructed and 9 treatments addressed. Three replicates were used per treatment per time, and microcosms were destructively sampled at each time point (3, 7, 14, 30 and 60 days).

4.2.2 Experimental design

In a first experiment, sterile soil was inoculated with 5, 20 and 100 bacterial strains isolated from the same soil. Since Actinobacteria are dominant soil inhabitants, in each of these treatments 20% of the total stains always encompassed actinobacterial morphs, as identified on the basis of the typical actinobacterial colony morphology. Isolation was performed after shaking 10 grams of soil in 90 ml of sterile 0.1% PirNa plus 10 grams of gravel for 30 minutes. Decimal dilutions of the mother suspension were spread onto R2A medium (Biotech laboratories Ltd) and colonies appearing at different time points and showing different morphologies were randomly purified and stored until their utilization in the mixtures. At that time, material from individual fresh colonies

was dissolved in sterile water to obtain an absorbance at 600 nm (Abs 600) of 1. Assuming that an Abs 600 of 1 corresponded at 10^9 cells ml^{-1}, we set up inocula for each treatment by adding different amount of every bacterial suspension to obtain mixture with the same amount of bacteria but different richness of strains (5, 20 100 strains). As expected, around $5*10^8 – 1*10^9$ bacteria for gram of microcosm soil were reached (checked by plating on R2A as seen before) before *E. coli* inoculation in each microcosm by following this procedure.

In the second experiment, a dilution-extinction technique was adopted. Specifically, microcosms containing sterile soil were inoculated with a volume of different suspensions obtained by serially diluting (1:10) the soil in sterile water. Inocula came from 10, 10^3 and 10^6 – fold soil dilutions. 5 ml of each dilution were added to 50 grams of soil, carefully mixed avoiding contamination and covered to prevent water evaporation. When necessary, water was reintegrated to keep the soil humidity around 75% WHC. 10^{-1} dilution was even inoculated previous vacuum-filtered through membranes with serially smaller pore size (5, 3, 2 and 1 μm) to exclude protozoa and other soil fauna, than obtaining 2 treatment, 10 filtered (10 F)and 10 not filtered (10). Natural treatment did not receive inocula but soil humidity was regulated at 75% WHC by adding sterile water.

In both the microcosm experiments, after waiting for a month to permit establishment of the microbial community at comparable level across all the treatments, an invader, namely a genetically-marked *Escherichia coli* O157:H7 derivative strain Tn5 *luxCDAEB* (Ritchie et al., 2003) was inoculated into the soil microcosms at around 10^8 CFU g-1.

4.2.3 Microcosms microbial monitoring

After *E. coli* addition, we monitored the microcosms for a 2 months period, during which all analyses (cultivation-dependent and –independent) were performed. Microcosm soil was analyzed to assess the total CFU counts and microscopic cell counts (DTAF staining) and *E. coli* number by using a selective medium at all the sampled time. For total culturable bacterial biomass enumeration and *E. coli* survival evaluation, soil was manipulated as above mentioned; thus, decimal dilutions were respectively spread onto R2A plates and Trypticase Soy Agar (TSA) plates plus antibiotics (see Van Elsas et al., 2007). On the other hand, total fungal and bacterial species richness (number of amplicons) and diversity (Shannon index), as well as specific bacterial groups, were evaluated by PCR-DGGE analysis (at 3, 30 and 60 days from *E. coli* inoculum). In our group specific DGGE approach we considered those microbial guilds likely responsible of antagonism toward others microrganisms, as *Pseudomonas* sp., *Bacillus* sp. and *Actinobacteria*. In all the cases DNA was extracted from soil by using Soil DNA isolation kit (Mo Bio laboratories, Inc.). PCR amplifications and DGGE separation procedures were applied as indicated in Costa et al. (2006a) for total bacteria, fungi and *Actinobacteria*, as in Costa et al. (2006b) for *Pseudomonas* spp. and as in Garbeva et al. (2003) for *Bacillus* spp. Patterns obtained were compared across the treatments by using Gel compare version x and diversity measures, such us band richness, Shannon index and evenness were calculated. Species richness (Sr) was evaluated counting the total number of amplicons present in each DGGE pattern, whereas species diversity (Sd) was determined as Shannon index of diversity. For the calculation, band positions and intensities were determined and compared within each pattern in a DGGE gel using GelCompare 4.0 software (Applied Biosystems).

4.2.4 Statistical analyses

Bacterial densities were always $\log_{10}$ transformed. SPSS 15.0 for windows software package was utilized in all the cases. Time series data were first analyzed using repeated-measures ANOVA (rmANOVA) to explore the effects of time, treatments, and time*treatments interaction on the variable from time to time under study. When effects of both time and time*treatments were not significant at $p<0.05$, one-way ANOVA results were reported, specifically mentioning the time points used. We used simple linear regression to determine if Sr, Sd, bacterial culturable biomass or global richness index (GRI) were significant predictors of *E. coli* survival in our experimental systems. Non-linear logarithmic function was fitted to the data to model *E. coli* extinction respect to bacterial species richness (first microcosm experiment).

4.3 Results

4.3.1 Random-established communities approach

Relevant differences were found in Sr and Sd values among the constructed communities containing zero, 5, 20 and 100 bacterial isolates. Increasing bacterial number resulted in increased Sr (ANOVA $F_{[3,56]} = 572.94$; $p = 0.0001$) and Sd (ANOVA $F_{[3,56]} = 89.84$; $p = 0.0001$) . Moreover, the number of culturable bacteria was dissimilar across treatments at 60 day, as monitored by plating on R2A medium (ANOVA $F_{[3,8]} = 142.27$, $p = 0.0001$), giving higher final bacterial numbers with progressively increasing community complexities. As expected, almost all of the bacteria in our microcosms were part of the culturable biomass, since the ratio culturable/total bacteria ranged between 85% and 95%, the highest values being shown by the sterile soil. Effects of treatments on survival of the invader were remarkable. First, the invader survived optimally in sterile soil after a 60 day-period (Fig. 1) and this survival was significantly better than that in any of the other microcosms (Tukey's post-hoc pairwise comparison $p<0.05$; ANOVA $F_{[3,8]} = 102.24$, $p = 0.0001$). Furthermore, the *E. coli* invader survived

comparatively better in microcosms inoculated with 5 strains than in those with 20 and 100 strains, as shown by the same statistical test. Sr did explain only partially decreased survival (Fig. 2). The fitted logarithmic nonlinear pattern predicts a limit in respect of effect of the bacterial diversity (e.g. increasing Sr will not result in greatly-increased death of the invader) which is likely linked to the use of an extremely simplified system. A significant relationship was also found between *E. coli* survival and the bacterial culturable biomass (linear regression, $Y = 11.017 – 0.394X$; $F_{[1,58]} = 37.1$; $p = 0.0001$; $R^2 = 0.39$), which was consistent with the inverse relationship between Sr and culturable biomass, and the presumed progressively-enhanced level of microbiostatic interactions operational in the microcosms with progressively-increasing community complexity. The control (sterile) microcosm that had received the *E. coli* O157:H7 inoculant consistently only yielded colonies typical for this invader on two media (R2A and the specific medium used to enumerate *E. coli*, i.e., TSA + selective antibiotics).

4.3.2 Dilution to extinction approach

The dilution-to-extinction experiment, including natural soil as the comparator, produced communities in which the richness (Sr) of the dominant bacterial types was statistically different across the treatments (Fig. 3), as monitored by 16S rRNA gene based bacterial PCR-DGGE (ANOVA $F_{[4,40]} = 69.56$; $p = 0.0001$) over the entire experimental period. As expected, natural soil showed the highest Sr, whereas the 10^6 soil showed the most reduced richness. The mean bacterial Sr of the 10 and 10-F treatments did not significantly differ, and hence we concluded that filtration did not significantly impact the bacterial community structures. The fungal PCR-DGGE profiles showed a reduction in Sr (ANOVA $F_{[4,40]} = 15.67$; $p = 0.0001$) with increasing dilution similar to the bacterial ones, but the 10-F sample contained a lower number of bands than the 10 sample, probably resulting from the filtration retaining part of the

fungal biomass, i.e. mycelia (Fig. 3). The calculated Shannon diversity (Sd) indices revealed a pattern similar to the Sr ones.

To gain information on the global change in diversity in the differentially-treated microcosms, we constructed a so-called global richness index (GRI) by normalizing each Sr value in relation to the maximum value observed in each microbial group determined by DGGE. These values were summed and than divided by 5, i.e. the number of microbial groups assessed by PCR-DGGE. A repeated-measures ANOVA evaluating the effects of the treatments on the GRI along three time points (3, 30 and 60 days) revealed an effect of treatment ($F_{[4,10]} = 36.62$, $p = 0.0001$), but no effects of time and interaction between time and treatment. This indicates that global richness was different among treatments but did not change along time, and there was no effect of the interaction treatment*time. Natural and 10 soils exhibited similar high richness, and 10^6 soil showed the poorest one (Tukey's post-hoc test, $p < 0.05$; Fig. 4), whereas in this case treatments 10-F and 10^3 clustered together.

Total culturable bacterial biomass was quite stable over time in the majority of treated microcosms(Fig. 5a). In some microcosms, this biomass was slightly different (ANOVA $F_{[4, 70]} = 211.66$; $P = 0.0001$). Thus, treatments 10 , 10-F and 10-3 all showed the same bacterial CFU counts, the lowest biomass being obtained in the natural soil treatment (Tukey's post-hoc test, $p < 0.05$). The latter, at the lowest colony numbers, expectedly contained the highest morphological (morph) diversity on plates; we counted as much as 30 different morphs from this treatment, versus a maximum of 20 from any of the other treatments.

Differences in *E. coli* O157:H7 CFU numbers became evident as soon as 14 days after introduction and these differences increased towards the end of the 60-day experimental period (Fig. 5b). The effect of the microbial diversity established in the different microcosms on the *E. coli* O157:H7 population size varied strongly over the time. The

results of univariate repeated-measures ANOVA (testing effects of dilution on *E. coli* O157:H7 survival over the entire experimental period) are shown in Table 1. There was a significant effect of both time and treatment, as well as the interaction between time and treatment, on the survival of the invader (Table1A). Therefore, microbial diversity, as established by the erosive dilution-to-extinction procedure, effectively controlled the rate at which the population size of the invader decreased. Persistence of the invader over time was also impacted by filtration (Table 1B, comparing the 10 and 10-F treatments). A one-way ANOVA performed for the 60-day samples confirmed the previous analyses, showing highly significant differences among the mean *E. coli* O157:H7 CFU numbers across treatments ($F_{[4,10]} = 112.27$; $P = 0.0001$). Moreover, pairwise comparisons significantly separated all the means ($P < 0.05$; Tukey's HSD), except for the natural soil and 10-F treatments, which clustered together (see Fig. 5b).

Interestingly, GRI was found to predict the invader's survival very well, since 3, 30 and 60 days following introduction, a significant negative relationship was observed between survival rate and GRI (Fig. 6). Moreover, significant linear inverse relationships between Ecsurv and Sr at 60 days were found for almost all microbial groups under study. (Fig. 7). In particular, next to total bacteria (Fig. 7a), actinobacterial (Fig 7e) Sr was strongly inversely correlated to *E. coli* O157:H7 survival in our experimental system. To a lesser extent, Sr of Pseudomonas (Fig. 7c) and Fungi (Fig. 7b) were also negatively related to the survival, while *Bacillus* spp. (Fig. 7d) richness did not show any relationship with EcSurv. A similar negative effect on survival was obtained when the Shannon diversity indices were used (instead than richness) in each one of the regressions.

4.4 Discussion

In most soils, the diversity of the microbiota is extremely high (Dykhuizen, 1997; Torsvik et al, 1990; Gans et al, 2005), certainly way beyond the levels of complexity used in our first experiment. Compelling evidence has shown that competition, antagonism and predation are the key activities in soil that would control invader fate. We surmised that, in our extremely simple soil systems, the balance between resident species and the invader was more strongly determined by specific antagonistic activities from residents. Thus, the diversity of the resident bacteria, all of culturable nature, most likely drove the invasibility of our simple systems. On the other hand, potential effects of the silent majority, i.e. the non-culturable soil microbial fractions, were not addressed.

As the first approach addressed the effects of bacterial diversity at the culturable level, not taking the full microbial diversity in soil into account, we decided to use a dilution-to-extinction approach. Such approaches are increasingly being used in Microbial Ecology (Wertz et al., 2006; 2007; Matos et al., 2005; Cook et al., 2006). We successfully obtained a gradient of microcosm communities with decreasing complexity (as indicated by GRI scores), as initial soil dilution increased from 10 to 10^6. Moreover, we found out a strong negative relationship between the overall species richness and death of *E. coli* invader at 3, 30 and 60 day from the introduction. We demonstrated for the first time that microbial diversity at the level of bacteria and fungi, indicated by overall species richness, affects the recalcitrance of a soil system to invasion. This effect is consistent with the notion of a relationship between diversity and suppressiveness against invaders, and can be explained by the occurrence, in soil with higher species richness, of a larger number of diverse organisms which collectively occupy a larger range of niches that overlap with the niche available for the invading species. Furthermore, given the effect of actinobacterial diversity, our data indicate a central role

of Actinobacteria in making soil ecosystems resist invaders. Such microorganisms are known as important producers of a range of secondary metabolites (Berdy, 1989; Ikeda et al., 2003) that collectively may be responsible for a large part of the microbiostatic status of soil. On the other hand, the CFU numbers obtained from the different treatments did not show any relationship with *E. coli* strain T survival. These CFU numbers also poorly predicted the overall microbial changes.

Additionally, we evaluated effects of filtration on survivability to clarify the role exerted by protozoan feeding on the microbial community. Overall, filtration partially removed the ability of the soil microbiota to counteract the invader, likely because of the reduction of GRI and/or absence of protozoan predators, or both. In fact, the presence of protozoa was assessed using a microtiter plate assay (Darbyshire, 1974). Only the natural and the 10 treatments showed the presence of protozoa. The activity of protozoan predators can be responsible for the progressive death of the *E. coli* invader and for differences between the 10 and 10 -F treatments. This explained also the differences between the two protozoa-containing treatments. Protozoan grazing probably affects bacterial communities through a wide array of mechanisms, such as selective feeding, differences in the susceptibilities of bacteria to predation and indirect effect on the condition for growth of bacterial populations (Rønn et al., 2002). Rather similar flagellate species were shown to exert different effects on the community structures, as assessed by molecular methods. Different compositions of the protozoan communities could explain the different survival of the invader between the 10 and natural soil treatments. In fact, although Enterobacteriaceae are known to be a very good food source to produce high protozoan growth yields (Weekers et al., 1993), feeding activity can be selective, e.g. for prey cell size (Hahn and Hofle, 2001), cell surface properties (Monger et al.,1999), motility (Boenigk et al., 2001) and chemical composition (Verity, 1991).

From the data presented, we conclude that microbial diversity, at the level of overall species richness, can be crucial for the survival of an invading *Escherichia coli* O157:H7. The inverse relationship between the invader survivability and diversity supports the central hypothesis in biodiversity – invasibility studies (Tillman, 1997) that at progressively lowered diversities of resident communities, an increasing number of niches will become available that support local establishment of an invader. The resultant communities, of similar overall biomass, but lowered diversities, may have had diminished functional redundancy, resulting to the lowered niche occupancy. Nevertheless, these results confirmed the difficulties encountered in random assembling processes of artificial communities, frequently being useless to study relationship diversity-functioning. In such simple communities, functional diversity and functional composition seem to drive ecosystem processes more than species diversity (Tilman et al., 1997).

Taken together, our data pointed out that a loss of microbial diversity may dramatically influence ecosystem services such as resistance to invasion by alien microbial species, of soil ecosystems. It is of particular concern when we consider human pathogens, since their survival can be strongly limited in the hostile soil environment. This problem is likely exacerbated in extremely poor situations such as found in heavily polluted environments, in which the biological component can be severely compromised.

4.5 Acknowledgments

This work was performed entirely during an 11-month stay at the RUG University, Microbial Ecology Department, responsible Professor Jan Dirk van Elsas. I would like to thank Dirk for giving me the opportunity to work in his laboratories. It was very pleasant to know such a brilliant scientist and nice friend.

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4.6 Bibliography

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4.7 List of figures and tables

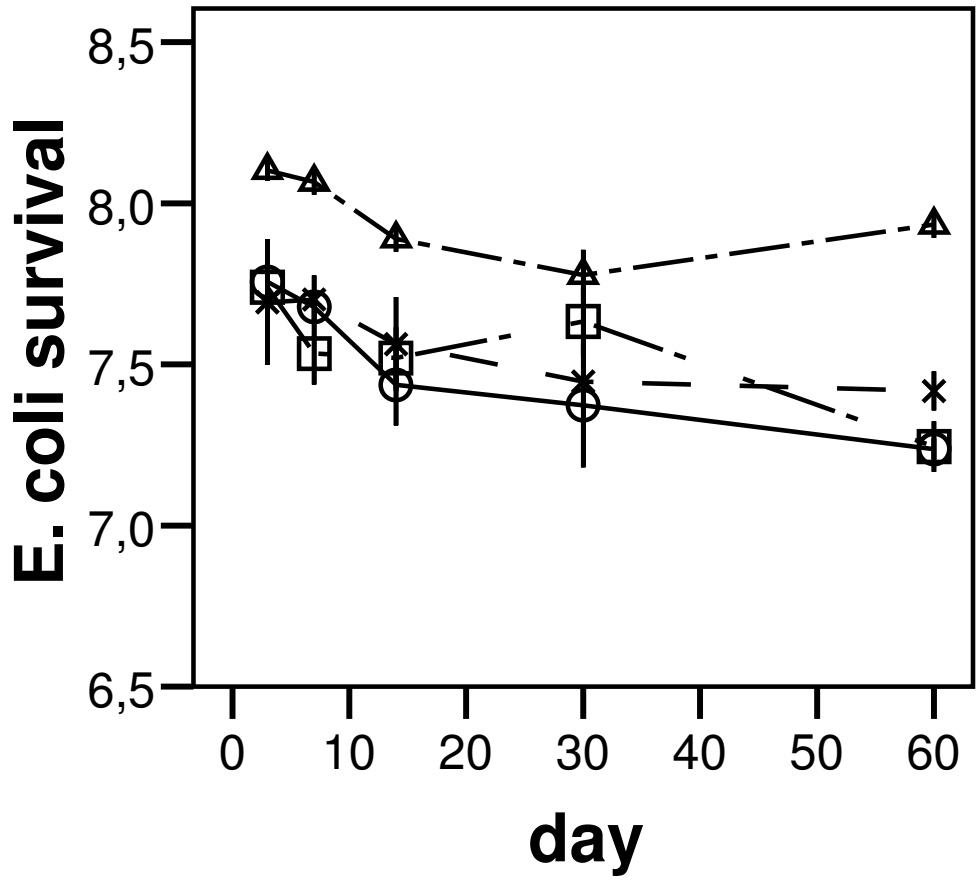

Figure 1 *Escherichia coli* O157:H7 survival (log CFU g^{-1} dry soil) along 60 day period in sterile (Triangles), 5 strains (crosses), 20 strains (squares), 100 strains (circles) inoculated soil. Each symbol represents a mean value of three replicates. Bars represent standard deviations

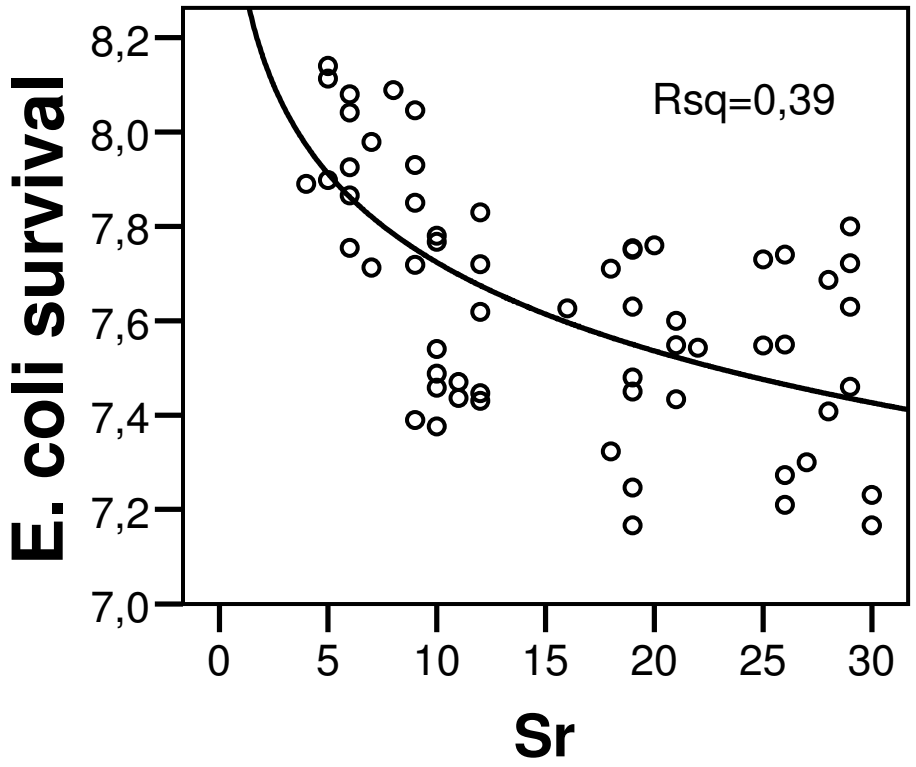

Figure 2 Relationship between *E. coli* survival (log CFU g^{-1} soil) and bacterial Sr (Species richness evaluated by PRC-DGGE; for details see the text) realized along a two-month period. The best-fit equation for a logarithmic decrease is: Ecsurv = -0.27 * Ln (Sr) + 8.35; ANOVA $F_{[1,58]} = 36.70$; $p = 0.0001$

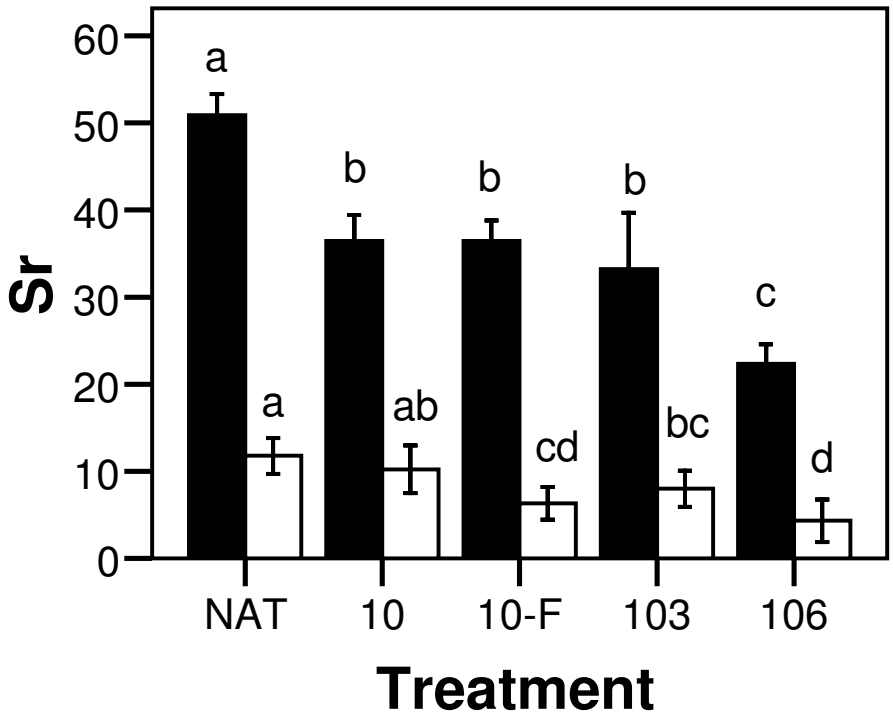

Figure 3 DGGE species richness (Sr; number of fragments) among the treatments evaluated by using universal 16S rRNA bacterial (filled bars) and 18S rRNA fungal (empty bars) PCR-DGGE patterns. Bars represent mean values (± standard deviations) of three replicates from three sampled experimental points (3, 30 and 60 days). NAT, natural soil; 10-fold (10), 10-fold filtrated (10-F), 10^3-fold (103) and 106-fold (10^6) soil dilutions treatments.

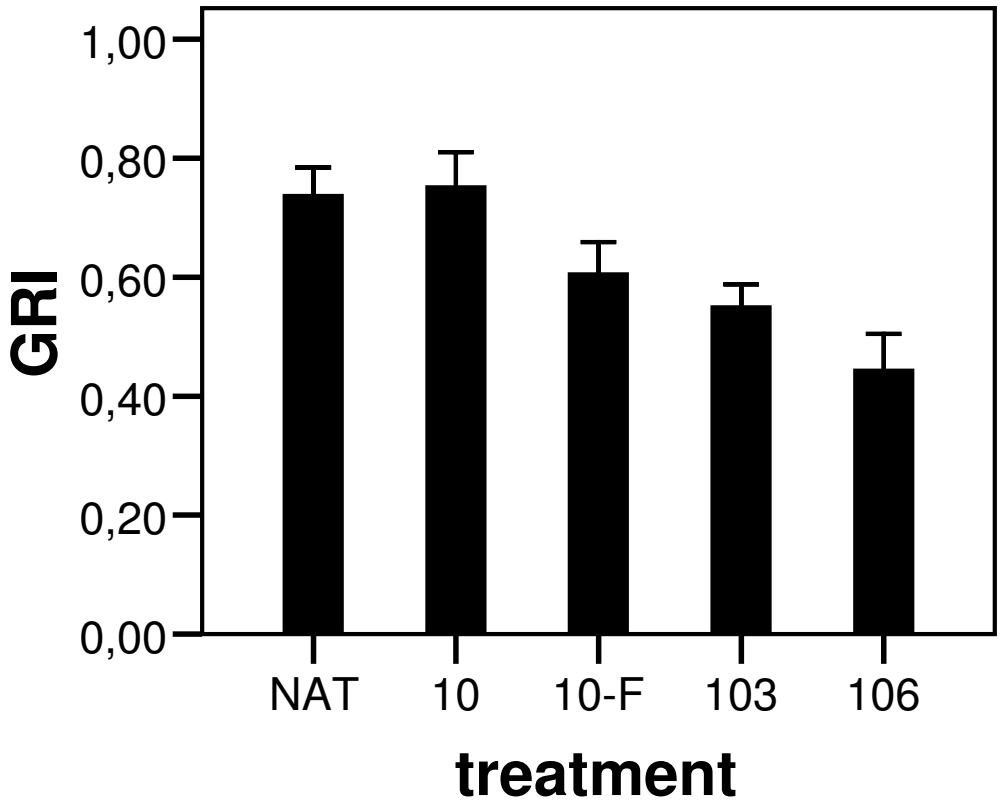

Figure 4 Global richness index (GRI) as impacted by treatments. GRI was derived normalizing each Sr value in relation to the maximum value observed in each microbial group determined by DGGE. These values were summed and than divided by 5, i.e. the number of microbial groups assessed by PCR-DGGE. Treatments initials are explained in fig. 3.

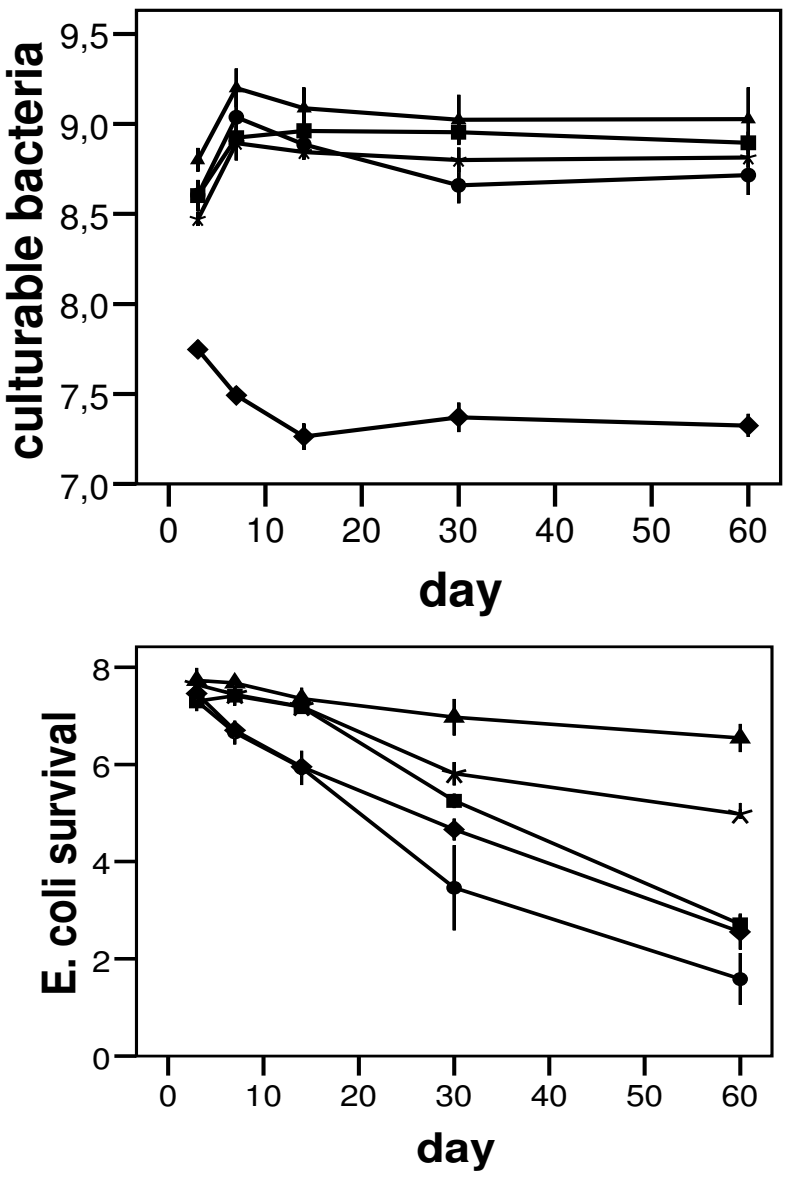

Figure 5 Total culturable bacteria (**a**) and *Escherichia coli* O157:H7 population dynamics (**b**) over time within each biodiversity treatment. On the y-axes are reported the log CFU g^{-1} dry soil. Points are means of three replicates (± standard errors). 10^6-fold (Triangles), 10^3-fold (stars), 10-fold filtrated (squares), 10-fold (circles) soil dilution treatments; natural soil (diamonds).

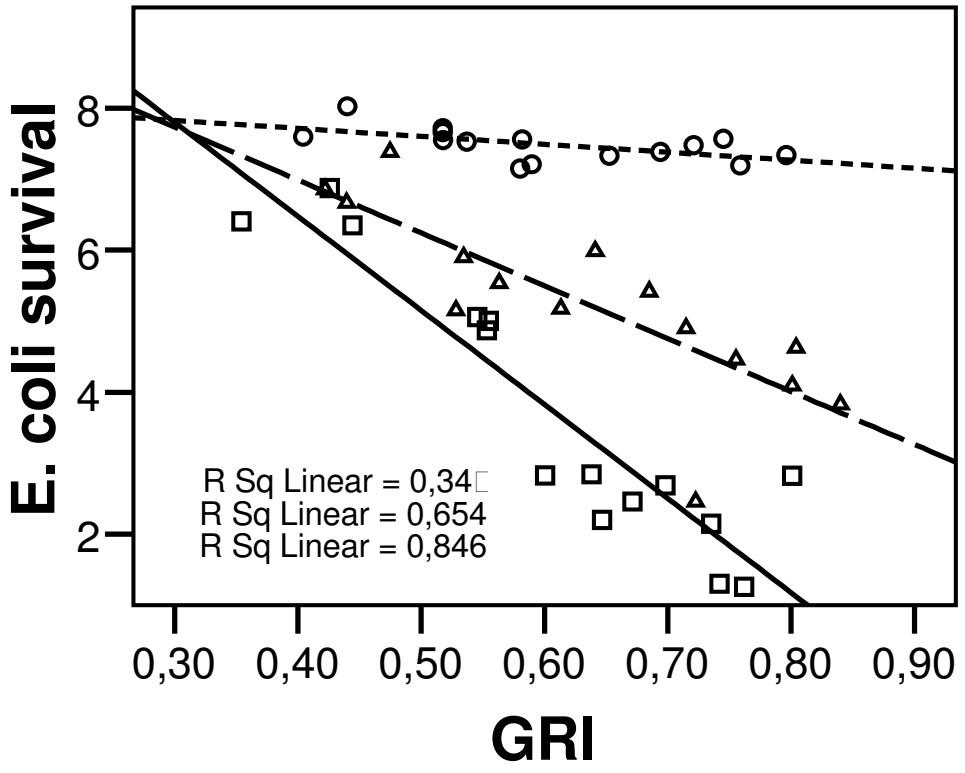

Figure 6 Effect of time from the inoculum and GRI (global richness index) on the survival of *E. coli* O157:H7 (log CFU g^{-1} dry soil). Lines represent the best fit linear regressions for each time point: 3 day (circles); 30 day (triangles); 60 day (squares) samples.

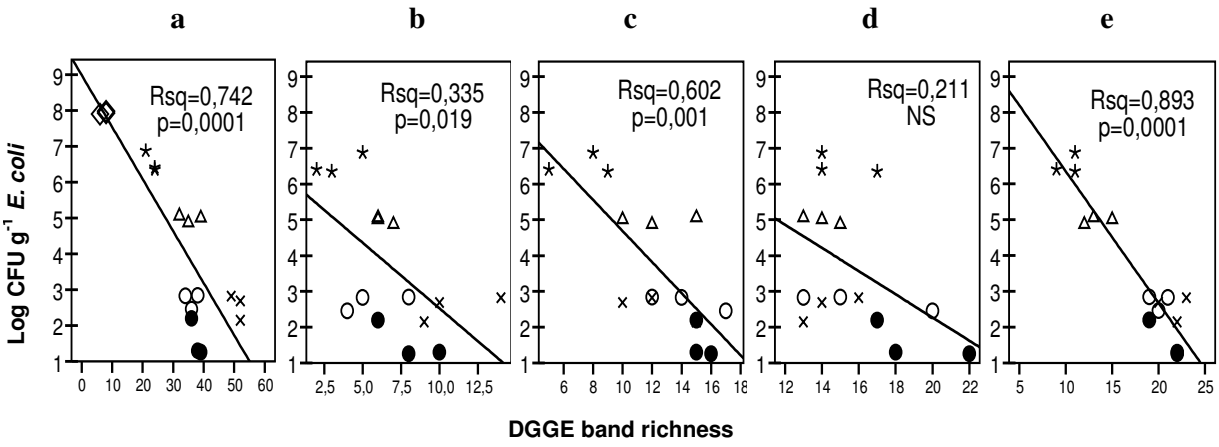

Figure 7 Relationship between group-specific PCR-DGGE bands richness and *E. coli* invasibility after 60 days. Natural soil (crosses); 10-fold (solid circles); 10-fold filtrated (empty circles); 10^3-fold (triangles); 10^6-fold (asterisks) soil dilution treatments. **a** Bacteria; **b** Fungi; **c** *Pseudomonas*; **d** *Bacillus*; **e** Actinobacteria. Lines represent the best fit linear regressions.

Table 1 Univariate repeated measures ANOVAs separately testing for effects of the treatments (**A**) and filtration (**B**) on *E. coli* survival along all the experimental period (5 time points). In **A**, all the treatments were considered. In B, only data from 10 and 10-F treatments were used to evaluate the filtration effects.

Tests	Effects	Df*	F	Sig.
	A) Dilution			
Tests of Between-Subjects Effects	treatment	4	193.70	< 0.0001
	Error	10		
Tests of Within-Subjects Effects	time	4	424.79	< 0.0001
	time * treatment	16	21.36	< 0.0001
	Error(time)	40		
	B) Filtration			
Tests of Between-Subjects Effects	filtration	1.00	114.51	0.0004
	Error	4.00		
Tests of Within-Subjects Effects	time	4.00	180.64	< 0.0001
	time * filtration	4.00	4.16	0.0170
	Error(time)	16.00		

*Degrees of freedom