UNIVERSITÀ
DEGLI STUDI
DI PADOVA

Sede Amministrativa: Università degli Studi di Padova

Dipartimento di Biologia

SCUOLA DI DOTTORATO DI RICERCA IN BIOSCIENZE E BIOTECNOLOGIE

INDIRIZZO: BIOTECNOLOGIE

CICLO XXVI

Soil microbial communities in environmental-agronomical context: quantitative analysis, metagenomics and signal exchange monitoring

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Abstract

Soil is one of the most challenging environments for microbiologists. The astounding number of microbial species living in soil makes it one of the most diverse and still mysterious environments known.

The study of the soil nitrogen cycle is gaining in importance, as recently a strong land-use intensification and increase in N fertilization led to the rising of threats to environmental and human health. Moreover, major interest is given to the investigation of the structure of soil microbial communities, to their responses in the presence of external stimuli, and to their capacity to organize thanks to the exchange of specific signals dispersed in the environment. In this study, these aspects of soil microbial ecology were taken into account, and microbiology and molecular biology techniques were used to probe the bacterial community function and structure and to study the physical diffusion of signals emitted by certain bacteria.

Real Time PCR analysis of functional genes of the N-cycle in different soils highlighted interesting differences and permitted to select the bacterial *amoA* and *nosZ* genes, for nitrification and denitrification, respectively, as useful indicators of soil health and functionality. Furthermore, a strict relationship between bacteria involved in nitrification and in denitrification was found in all the analysed soil samples, indicating that these microorganisms could be part of the same environmental niche, though being involved in opposite processes.

Through T-RFLP experiments and a metagenomic analysis by amplicon sequencing performed in a 454 system (Roche), the effects of different fertilizers on an agricultural soil microbial community were investigated. In particular, it was possible to determine how bacterial communities changed over time in response to this kind of inputs, and to have a first insight into the relation among some bacterial phyla, which fluctuated in parallel or in opposition.

Concerning microbial cell-to-cell signalling, in this work, efforts were made to define the consequences of the reflecting or adsorbing boundary conditions on the physical diffusion of AHL molecules, and therefore on bacterial intercellular communication, by combining physics and microbiology approaches. It was observed that the properties of the boundaries play a major critical role on the quorum sensing AHL concentration profiles around the cell environment.

Riassunto

Il suolo rappresenta uno degli ambienti più misteriosi e ricchi di biodiversità sulla Terra, e, nella maggior parte dei casi, i microrganismi adattati a vivere in questo ambiente non sono coltivabili e non sono stati ancora caratterizzati.
Questo lavoro di tesi ha lo scopo di analizzare la complessità dell'ecologia microbica del suolo da due punti di vista diversi: da una parte si vuole ottimizzare e utilizzare tecniche di biologia molecolare per esaminare la struttura e la funzione delle comunità microbiche di diversi suoli, e dall'altra parte lo scopo è quello di studiare le forme di comunicazione tra i batteri, e i segnali che essi scambiano tra loro (Quorum Sensing).
Di recente, molti ricercatori si sono dedicati allo studio dei batteri e degli archaea coinvolti nel ciclo dell'azoto, poiché il disequilibrio in questo processo, causato principalmente dall'uso eccessivo di fertilizzanti azotati e da altre pratiche agricole, è una minaccia per la salute dell'uomo, oltre che per l'ambiente. Inoltre, anche lo studio delle comunità microbiche del suolo sta assumendo sempre più importanza, in particolare per quanto riguarda la loro risposta in presenza di stimoli esterni, e la loro capacità di organizzarsi grazie allo scambio di segnali specifici emessi nell'ambiente dagli stessi microrganismi.
L'analisi dei geni funzionali per il ciclo dell'azoto mediante esperimenti di Real Time PCR quantitativa in suoli di diversa provenienza o trattati in modi diversi ha permesso di evidenziare differenze significative e di selezionare i geni *amoA* batterico e *nosZ*, rispettivamente per la nitrificazione e per la denitrificazione, come possibili indicatori della qualità del suolo e della produttività in agricoltura. Questo tipo di analisi ha anche permesso di osservare come ci sia, in tutti i suoli analizzati, una correlazione positiva e statisticamente significativa tra i geni batterici per la nitrificazione e per la denitrificazione. Questo risultato potrebbe indicare che questi due gruppi di microrganismi sono in qualche modo legati, e che probabilmente vivono nella stessa nicchia ecologica.
In questo lavoro, mediante esperimenti di T-RFLP e grazie all'uso della tecnologia di Next generation sequencing 454 (Roche), è stato possibile analizzare la struttura della comunità microbica in suoli trattati con fertilizzanti di diverso tipo, a due tempi di campionamento. Inoltre, sono state ricercate le correlazioni tra i phyla presenti, in modo da avere una prima idea di come questi varino anche in relazione alla variazione dell'abbondanza di altri gruppi batterici. Grazie a questo esame sono state trovate molte correlazioni forti e significative, che indicano come alcuni gruppi batterici siano strettamente in relazione tra loro e si influenzino fortemente.
Per quanto riguarda lo studio dei segnali scambiati tra batteri presenti in una stessa comunità, e quindi del Quorum Sensing e del Diffusion Sensing, sono stati analizzati i

meccanismi fisici di diffusione delle molecole quorum AHL in ambienti circoscritti, ed in particolare sono state definite le conseguenze delle condizioni al contorno, sia nel caso di confini di natura riflettente che di natura assorbente. Queste proprietà sono risultate giocare un ruolo fondamentale nel determinare i profili di concentrazione della molecola segnale AHL.

1 Introduction

The present research aims at a dual combined approach to seize the complexity of applied microbial ecology from both its ends. From one side, probing the bacterial community function and structure; from the other side, studying the signals emitted by the microbes themselves, in order to decode and interpret their on-going communications.

Microbial life in the environment contributes to its ultimate structure and activity, ruling the functioning of the global ecosystem. Almost 97% of the flux of energy and matter occurring on earth passes through the microbial compartment. The genesis of soil itself is driven by microorganisms and these profoundly affect its attitude to host and support plants. Both productivity and health of the cultivated plants as well as the distribution of natural vegetation are conditioned by interactions with ground microbiota. Besides their key role in natural primary productivity, microbes take active part also in recycling and degradation and can be exploited and managed in a number of industrial applications. Due to these qualities, the microbial communities, including thousands of taxa of unknown identities and proportions, constitute systems on which it becomes increasingly important to assess the presence of functionally relevant species and their quantity within the whole community.
However, even the closest and simplest soil or water environments, in terms of microbiodiversity, still represent overly underexplored systems. For this reason, the need of novel monitoring approaches is highly manifested from environmental and agronomical scientists and in many applied biotechnological contexts.

In this thesis work, the major interest is focused on the activities determining the transformation of nitrogen as a major element affecting both fertility and pollution and tightly connected with the cycling of carbon, and on the general structure of soil microbial communities. A number of molecular tools were tested and optimized in order to assess the qualitative and quantitative presence of different sets of genes playing key roles in this biogeochemical cycle, and to determine the main characteristics of the soil microbial community.

As a complementary approach the project aims at “listening” to some specific signals emitted by the microorganisms in the environment as part of their internal communication strategies. In specific terms, the focus is on bacterial Quorum Sensing as a widespread intercellular signalling mechanism.

Quorum Sensing is the regulation of gene expression in response to fluctuations in cell-population density. Quorum Sensing bacteria produce and release chemical signal molecules called autoinducers that increase in external concentration as a function of cell density. The detection of a minimal threshold stimulatory concentration of an extracellular autoinducer by a responding cell leads to an alteration in its gene expression. Gram-positive and Gram-negative bacteria use quorum sensing communication circuits to regulate a diverse array of physiological activities that are important to the establishment and occupancy of their ecological niche. These processes include symbiosis, virulence, competence to DNA uptake, conjugation, antibiotic production, motility, sporulation, and biofilm formation. In general, Gram-negative bacteria use acylated homoserine lactones (AHLs) as autoinducers to communicate, and Gram-positive bacteria use processed oligo-peptides. Cell to cell communication via autoinducers can occur both within and between bacterial species. Furthermore, there is mounting data suggesting that bacterial autoinducers elicit specific responses in the host organisms to which they associate .

In the present project, efforts were made to define the physical diffusion of AHL molecules in defined microcosms, and more in detail to define the consequences of the boundary conditions (signal-reflecting vs. signal-adsorbing) on bacterial intercellular communication by a combined physics and microbiology approach.

2. Environmental biogeochemistry of nutrient cycling in relation to bacterial community diversity and methods for its assessment

2.1 Introduction

2.1.1 The importance of soil microbiology

Soil is one of the most challenging and complex environments for microbiologists. Indeed, it represents the largest reservoir of microbial diversity known, with an astounding number of microorganisms adapted to survive and grow in it. In 2001, it has been estimated that about $4x10^6$ different taxa are contained in a ton of soil (Curtis *et al.*, 2002), while more recent estimates of the number of species vary between 2000 and 8.3 million per gram soil (Schloss and Handelsman, 2006; Gans *et al.*, 2005). Soil represents one of the most immense sources of hidden biodiversity, which could be a great resource of natural products for agriculture, medical and biotechnological applications (Mocali and Benedetti, 2010). Indeed, over the past 50 years, many products derived from microbial secondary metabolites have been used to meet medical, industrial and agricultural needs. Some examples are antibiotics, anticancer drugs, antifungal compounds, immunosuppressive agents, enzyme inhibitors, anti-parasitic agents, herbicides, insecticides and plant growth promoters (Omura, 1992).
Many processes and interactions take place in soil, contributing to a considerable number of ecosystem services (Coleman and Whitman, 2005). Given the importance of soil functions for most aspects of our lives, surprisingly little is known about the subsurface living world. This is because soil is a heterogeneous environment and its biochemistry and its spatial complexity was not yet fully determined. Moreover, there is still much to be investigated for what is concerning microbial life and how it interacts with soil properties and biogeochemical cycles, such as carbon cycle and nitrogen cycle.

It is known that soil mineral and organic particles form strong interactions with soil biota through formation and stabilization of microaggregates, micropores and clay-organic matter complexes that were determined to be the dominant structural characteristics of the soil matrix and among the most important parameters that affect microbial composition (Girvan *et al.*, 2003). Moreover, the soil matrix is extremely complex and heterogeneous and microbial communities are spread in a patchy manner, forming the so-called “hot-spot” distribution (Nunan *et al.*, 2002). In this last scientific work, evidences were reported that indicated the presence of a spatial aggregation of bacteria, which was stronger in the topsoil and decreased with depth in the subsoil.

However, extremely high degrees of aggregation were found at very short distances in the deep subsoil.
Franklin and Mills (2003) conducted a survey to examine the spatial organization of microbial community structure in an agricultural field. Their results suggested that microbial communities may have several nested levels of organization and that these mechanisms could be dependent on different soil properties or groups of properties. These observations suggest that the environmental factors regulating the development of the communities in soil may operate at different scales. Bacterial distributions can be highly structured, even within a habitat that appears relatively homogeneous. Therefore, it is possible to find a number of microsites with different conditions and with a different microbial community since within a few millimeters. For example, *in* a work by Parkin (1987), a surprising result indicated that almost all bacterial denitrification in a 98 g sample was situated within a 0.08 g subsample containing plant debris.

Our poor knowledge of soil microbial diversity is also limited by our inability to properly study soil microorganisms, caused both by the immense diversity present in this kind of matrix, as mentioned before, and by the impossibility to culture the majority of soil bacteria and archaea. In fact, it has been assumed that less than 1% of soil prokaryotes are culturable by traditional cultivation and isolation methods (Torsvik *et al.*, 1990). There are several reasons for microbial unculturability under laboratory conditions; for example, extremely high substrate concentrations or the lack of specific nutrients required for growth. Rondon *et al.* (1999), suggested different hypothesis to give an explanation to this peculiar aspect of soil microorganisms. One possibility is that 'unculturable' cells could be microorganisms that are phylogenetically similar or identical to the culturable minority, but which are present in a physiological state that makes them recalcitrant to culturing. This interpretation is plausible as it is known that microorganisms that are culturable have the ability to become viable but nonculturable in adverse conditions (McDougald *et al.*, 1998).
Another hypothesis by Rondon (1999) is that unculturable cells represent novel lineages of bacteria, which are phylogenetically and genetically distinct from the 1% of the members of the community, which is culturable in laboratory.

Recently, a number of culture-independent methods have been developed, in order to overcome this problem. In particular, the improvement of molecular biology techniques and the application of next generation sequencing (NGS) technologies to the study of environmental samples, and specifically to soil samples, give new opportunities to the study and the comprehension of such a complex environment as soil.

2.1.2 Microorganisms and Biogeochemical Cycles

During the first half of the evolution of our planet, which is ~ 4.5 billion years old, important metabolic processes evolved, which were performed exclusively by microbes and that would alter the chemical speciation of virtually all elements on earth. The establishment of these fundamental processes has allowed life to persist on earth, even if it has been subject to extraordinary environmental changes, such as bolide impacts, global glaciations and massive volcanic outgassing (Knoll, 2003). Even if these great perturbations led to the extinctions of a major part of plants and animals, the core biological machines responsible for planetary biogeochemical cycles have survived intact.

The major biogeochemical cycles involve six fundamental elements - H, C, N, O, S, and P – which constitute the building blocks for all biological macromolecules.
The biological fluxes of the first five of these elements are largely mediated by microbiota, which catalyse thermodynamically constrained redox reactions (Figure 2.1.1).
It is not yet known, whether all the niches for all possible redox reactions are occupied by a microbial metabolism. Indeed, some metabolic transformations, and the microorganisms that enable them, before being shown to actually occur, have been predicted to exist only on the bases of thermodynamics. Anyway, not all predicted pathways have been found and demonstrated (Jetten *et al.*, 1998).

Although biogeochemical cycles have usually been studied in isolation, due to physiological and biochemical convenience, these processes have evolved in parallel and each one influences the outcomes of the other. The metabolic pathways evolved by modification of pre-existing metabolisms or by using established ones in reverse, to use available substrates, which were produced as end products of other types of microbial metabolism (Gerlt and Babbitt, 2001; Falkowski *et al.*, 2008).

In this thesis work, the focus is on the nitrogen cycle, which, as just stated, is strictly connected with the other biogeochemical cycles and in particular with the carbon cycle, and is fundamental both for fertility and environmental pollution.

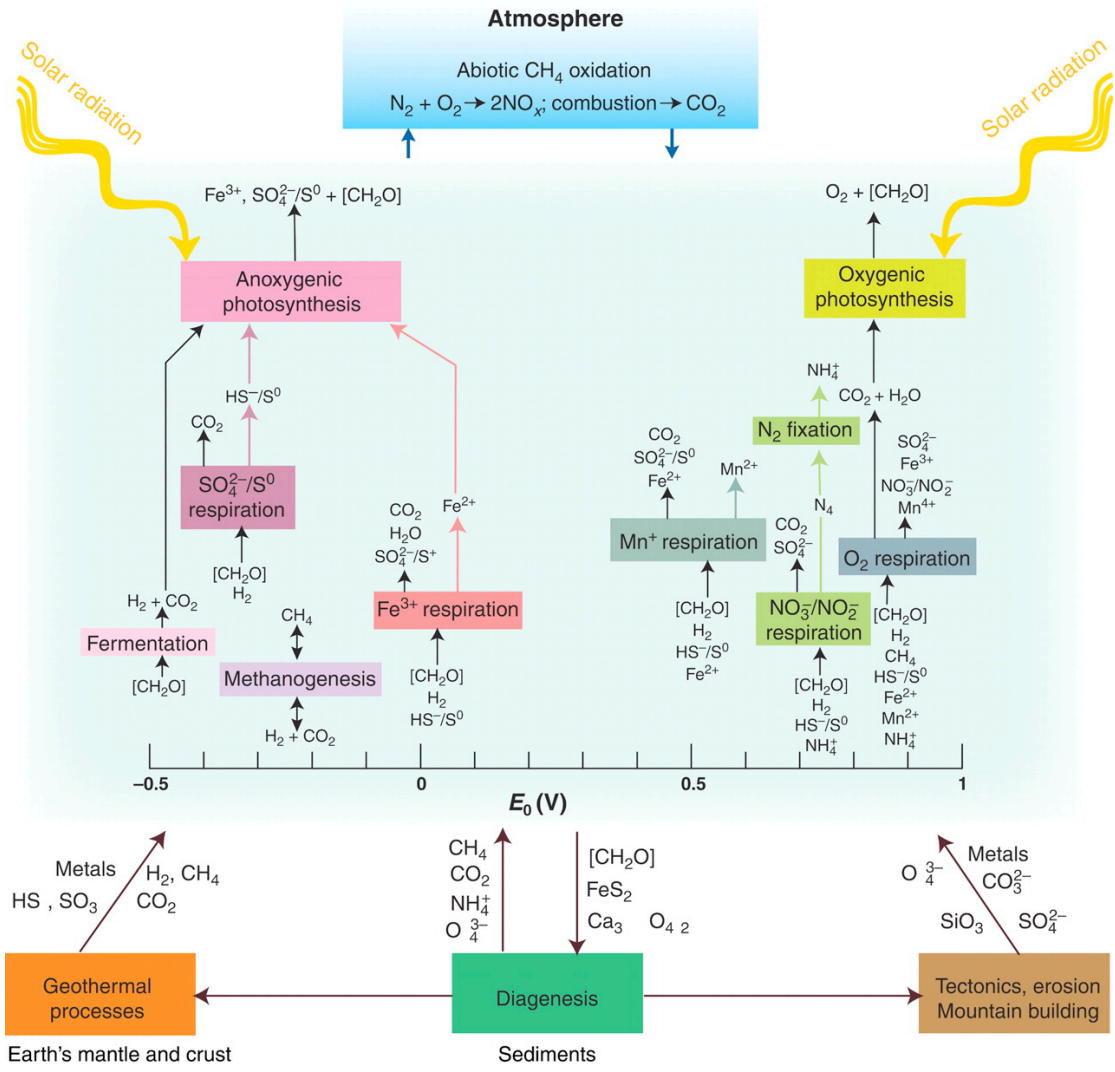

Figure 2.1.1

A generalized biosphere model showing the basic inputs and outputs of energy and materials. Geochemical (abiotic) transformations are represented at the top (atmospheric) and bottom (tectonic and geothermal) compartments, while microbially-driven biochemical processes are represented in the middle biospheric compartment (in blue) and in the sediments. Biological element cycling is not completely closed due to losses through sedimentation of organic carbon and nitrogen, carbonate, metal sulfides, sulfate, and phosphate. Regeneration of available forms of these elements is contingent on geological processes: erosion and geothermal activity. Electron acceptors (oxidants) in the respiratory processes have been arranged from left to right according to increasing capacity to accept electrons. The redox couples (at pH 7) for the reactions are approximate; the exact values depend upon how the individual reactions are coupled (Falkowski *et al.*, 2008).

2.1.3 Nitrogen cycle in soils

Until few years ago, it was assumed that the microbial nitrogen cycle was essentially complete (Strous and Jetten, 2004). The cycle comprehended free-living or symbiotic bacteria able to fix dinitrogen gas providing ammonium for assimilation, nitrifying microorganisms that oxidized ammonium through the formation first of nitrite and than of nitrate (Winogradsky, 1890), and finally denitrifying bacteria that returned nitrogen in the atmosphere by releasing N_2 (Gayon and Dupetit, 1886), closing the cycle.
In the last 10 years, however, new discoveries regarding the soil N-cycle showed that the understanding of this complex process is far from being complete. Some examples of these new findings that changed the view of the scientific community on microbial nitrogen cycle are anaerobic ammonium oxidation (anammox) (Strous *et al.*, 1999), ammonia oxidation by archaea (AOA) (Konneke *et al.*, 2005; Francis *et al.*, 2007), the interaction between these two groups (Lam *et al.*, 2007), nitrate reduction to dinitrogen gas by foraminifera (Risgaard-Petersen *et al.*, 2006), nitrite-oxidizing phototrophs (Griffin *et al.*, 2007), nitrite-dependent anaerobic methane oxidation (N-DAMO) (Raghoebarsing *et al.*, 2006), hyperthermophilic N_2-fixing methane-producing archaea (Mehta and Baross, 2006) and genome sequencing of several N-cycle organisms (Strous *et al.*, 2006; Arp *et al.*, 2007) (Figure 2.1.2).

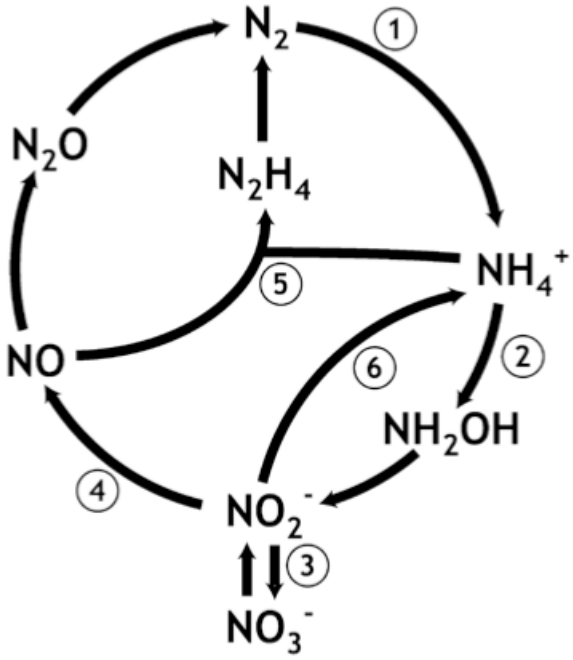

Figure 2.1.2
Reactions of the microbial nitrogen cycle. (1) Dinitrogen gas fixation; (2) aerobic ammonium oxidation by bacteria and archaea; (3) aerobic nitrite oxidation; (4) denitrification; (5) anaerobic ammonium oxidation; and (6) dissimilatory nitrate and nitrite reduction to ammonium (Jetten, 2008).

These discoveries, apart from giving new important insights into the crucial process of nitrogen cycle, indicate that there is an enormous biodiversity and metabolic capability of nitrogen conversions hidden in the microbial world of which we know only very little to date (Jetten, 2008).

The increase combustion of fossils and the strong demand for nitrogen in agriculture and in industry, are major causes of the profound changes induced by humans in the global nitrogen cycle.

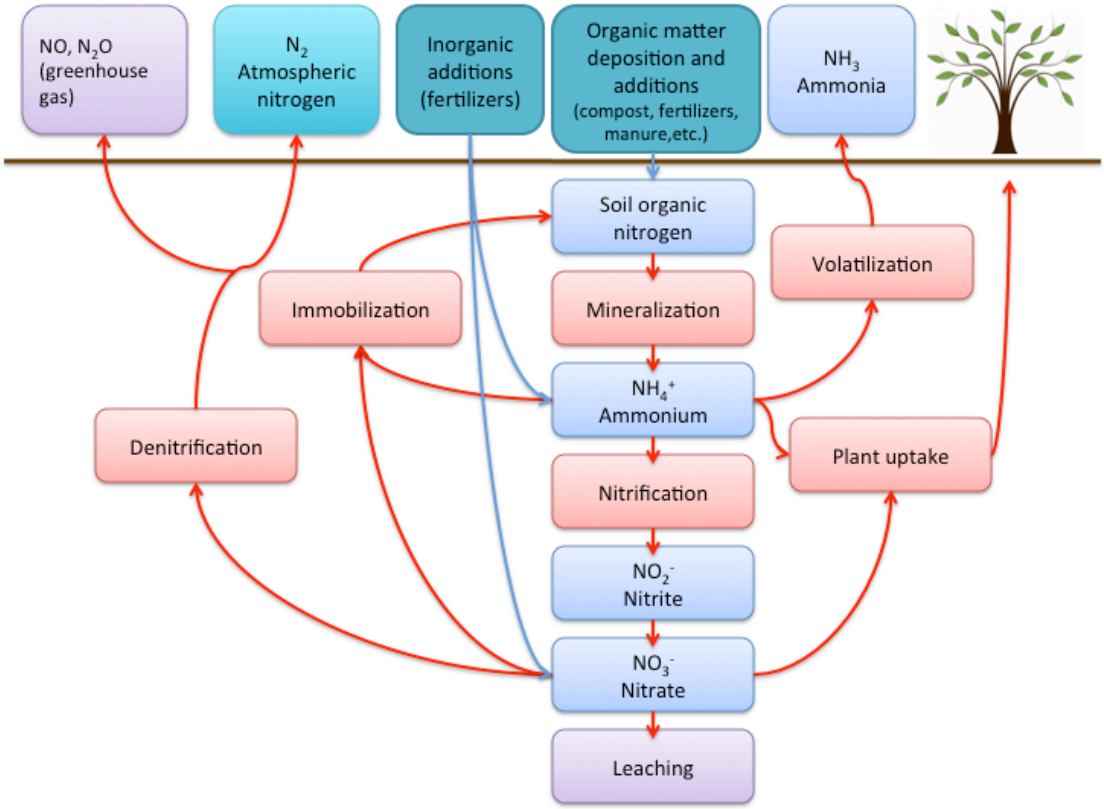

Figure 2.1.3

Schematic representation of the soil nitrogen cycle.
Soil organic nitrogen, which could derive from organic matter deposition or by the addition of compost, fertilizers, manure, is subject to mineralization. Another important source of nitrogen in soil is nitrogen fixation by some specialized microorganisms (e.g. Rhizobia). From mineralization and nitrogen fixation processes, ammonium is formed, which can have different fates: it can be absorbed by plants, it can volatilize and be released in the form of ammonia, it can be subject to immobilization, or to nitrification. This process converts ammonium to nitrite and subsequently to nitrate. This last nitrogen form can be easily absorbed by plants, and thus is an easily available nutrient for them. When nitrates are present at high levels in soils, they can be subject to leaching and cause groundwater pollution and eutrophication. Alternatively, nitrates can be also immobilized via reductive assimilation by biota, or can go through the process of denitrification. The final product of this process is molecular nitrogen (N_2), but in some cases intermediate products can be released in the atmosphere, such as the greenhouse gas nitrous oxide (N_2O) or nitric oxide (NO).

Moreover, the inefficiencies in the use of this fundamental element cause that much anthropogenic nitrogen is lost in the atmosphere, in water, and in land causing a cascade of environmental and human health problems (Galloway *et al.*, 2008). In particular, Duce *et al.* (2008) indicated that the increase of atmospheric nitrogen deposition, in that case in oceans, is expected to continue to grow in the future, and may be a threatening cause of global climate change.
For these reasons, a better understanding of the microbes that are involved in nitrogen transformations is necessary to understand and eventually counteract the negative effects of nitrogen pollution (Jetten, 2008).

2.1.3.1 Nitrogen fixation

Dinitrogen gas fixation is widely recognized as an important process in many ecosystems (Houlton *et al.*, 2008; Hungate *et al.*, 2003), and since many years researchers are interested in this intriguing process (Farkowski, 1997).
The capacity for nitrogen fixation is widespread among Bacteria and Archaea (Zehr *et al.*, 2003). The great diversity of diazotrophs also extends to their physiological characteristics, as N fixation is performed by both chemotrophs and phototrophs and by autotrophs as well as heterotrophs (Malik and Schlegel, 1981; Bürgmann *et al.*, 2003; Young, 1992).
N-fixation is crucial for the maintenance of biological productivity in ecosystems, as nitrogen fixers have the ability to replenish nitrogen that is lost through anaerobic ammonium oxidation and denitrification (Capone and Knapp, 2007). Many studies have focused on nitrogen fixation in oceans, and recent observations show that nitrogen fixation and nitrogen loss in oceans may be tightly coupled (Brandes *et al.*, 2007; Deutsch *et al.*, 2007).
In the last years, several studies have been devoted to the analysis of the microorganisms able to fix nitrogen, and now a large set of genomic data and molecular tools are available to investigate this group of microbes (Zehr *et al.*, 2007).
Apart from free-living nitrogen-fixers present in soils, N_2 fixing bacteria of the genera *Rhizobium, Bradyrhizobium* and others can form nodules on roots of leguminous plants, in which they find favourable conditions, and a relatively anoxic environment, to convert N_2 to ammonia (Figure 2.1.3).
For nitrogen fixation process, the functional gene that is widely used as biomarker is the *nifH* gene that encodes for the iron-containing reductase component of nitrogenase (Zehr and McReynolds, 1989). This gene is assumed to be highly conserved among diverse microorganisms, and for this reason it has been used in several scientific studies

to assess the diversity of bacteria involved in nitrogen fixation, both in terrestrial and in aquatic ecosystems (Jetten, 2008).
An example is found in the paper by Babić and colleagues (2008), which, through a study on the influence of different terrestrial nitrogen-fixing *Sinorhizobium meliloti* strains in the rhizosphere of the leguminous plant alfalfa, demonstrated that the effectiveness of rhizobial inocula is related to the abundance of the *nifH* gene in the late flowering phase of the plant. Other examples of works in which the nitrogen-fixation functional gene *nifH* was taken into account, among the other genes involved in the nitrogen cycle, are the work by Hai *et al.* (2009), where the major aim was to detect the effects of agricultural management, in a moderate ecosystem, on biogeochemical cycles, and the work by Rosch *et al.* (2002), in which the focus was on the biodiversity of denitrifying and nitrogen-fixing bacteria in an acidic forest soil.

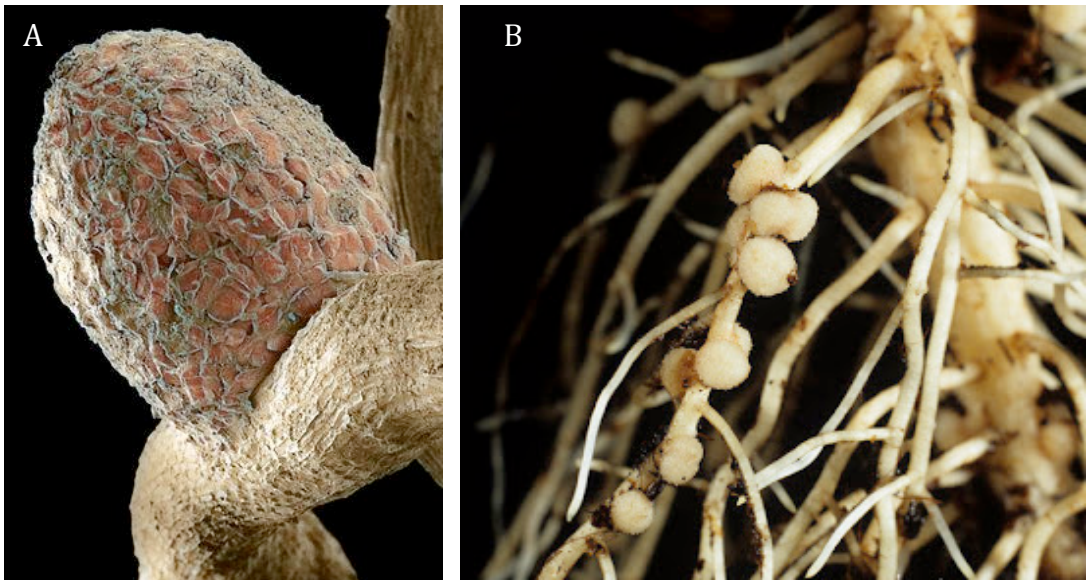

Figure 2.1.3
Rhizobium root nodules. **A**: microscopy image of a *Rhizobium* nodule on a clover root. **B**: *Rhizobium* nodules on bean roots.

2.1.3.2 Nitrification

The importance of soil nitrification, the microbial oxidation of ammonia to nitrite (NO_2^-) and then to nitrate (NO_3^-), has been widely recognized in the last century, as it results in enormous commercial losses of ammonium-based fertilizers, and threatens the environment through leaching of the nitrates in groundwater and eutrophication (Prosser and Nicol, 2012).
Since the isolation of nitrifying microorganisms in the late 19th century, it was believed that all autotrophic ammonia-oxidizing microbes were bacteria (AOB), belonging to the

Beta- and Gamma-subgroups of Proteobacteria or to the phylum Nitrospirae (Purkhold *et al.*, 2000; Kowalchuck and Stephen, 2001; Jetten, 2008). Recently, metagenomic studies performed by Venter *et al.* (2004) and Treusch *et al.* (2005), indicated that Crenarchaea may harbour distantly related *amoA* genes, and subsequently, the isolation and cultivation of Candidatus *Nitrosopumilus maritimus*, a marine archaeal ammonia oxidizer (AOA) (Konneke *et al.*, 2005), suggested a role for AOA in ammonia oxidation, a discovery that radically changed our view on the microbial players involved in this fundamental process. Archaeal ammonia oxidizers are now placed within the phylum Thaumarchaeota (Brochier-Armanet *et al.*, 2008).

The first step of nitrification, leading to the formation of nitrite, is catabolised by the enzyme ammonia monooxygenase. As mentioned before, microorganisms able to perform this step of nitrification are called ammonia-oxidizing bacteria (AOB) and archaea (AOA). The gene for the subunit A of ammonia monooxygenase (*amoA*) has become the standard functional gene for nitrification, and was first discovered in 1981 by Thomas Hollocher. Its sequence from the nitrifying microorganisms *Nitrosomonas europaea* was published in 1993 by McTavish *et al.*, permitting a new start of a series of studies on this field.

The second step of nitrification, the oxidation of nitrite to nitrate, is performed by nitrite-oxidizing bacteria (NOB) (Prosser, 1989).

Nitrification, whether facilitated by bacteria or archaea, can proceed only in the presence of oxygen, or in anoxic environments only by selected species externally supplied with NO_2 (N_2O_4) (Schmidt *et al.*, 2001). The recently discovered process called anammox, performed by anaerobic ammonia-oxidizing bacteria (ANAOB), couples the oxidation of ammonia to the reduction of nitrite to produce dinitrogen in anoxic ecosystems (Strous *et al.*, 2006; Kartal *et al.*, 2007).

As already mentioned, knowledge of nitrification, one of the fundamental processes of the nitrogen cycle, and of the microorganisms involved in it, the AOB and NOB, goes back to more than 100 years ago, and in specific to the work of Winogradsky (1892). The catabolism of AOB involves at first the aerobic oxidation of ammonia to hydroxylamine by the enzyme ammonia monooxygenase (AMO), and subsequently the dehydrogenation of hydroxylamine to nitrite by hydroxylamine oxidoreductase (HAO), which is proposed to relay the four extracted electrons to the ubiquinone pool via two interacting cytochromes, c554 and cM552 (Hooper *et al.*, 2005). The proteins HAO and cytochrome c554 have both been crystallized (Igarashi *et al.*, 1997; Iverson *et al.*, 2001) and, as their structures have been resolved, much is known about their function. In contrast, AMO, wich is a multimeric transmembrane copper-enzyme, has yet to be functionally isolated, crystallized and its structure solved (Klotz and Stein, 2008).

Recently, many works focused on the analysis and quantification of nitrifying microorganisms in different kinds of environments. Through the use of Real Time PCR technology, searching for the presence and copy number of the *amoA* gene, the abundance of bacterial and archaeal ammonia-monooxidizers was inferred in the presence of different conditions.
In general, quantification indicated that AOA were abundant in soils, for example in the works by Leininger *et al.* (2006) and by Nicol *et al.* (2008), and indicated a possible great role of this group of nitrifiers, as suggested by Prosser and Nicol in 2012, at least in some environments.
Prosser and Nicol, in 2012, with an analysis of current data from genomes, cultures, field studies, and microcosms, observed that no single factor can discriminate between AOA and AOB. Thus, further investigation and characterization of a wide range of environments and a greater consideration of small-scale soil heterogeneity are needed in order to have better insights into the issue of niche specialization of ammonia oxidizers.
Recently the genome sequences of several nitrifying bacteria have become available (Arp *et al.*, 2007; Chain *et al.*, 2003; Bollmann *et al.*, 2013). The advances in technology and the enormous increase in molecular data for nitrifying microorganisms has facilitated our understanding of the evolution of the nitrogen cycle and of microorganisms involved in it (Klotz and Stein, 2008).
Furthermore the genome assemblies made possible the design of a whole set of expression and regulation studies.

2.1.3.3 Denitrification

Denitrification is one of the key processes in the biogeochemical nitrogen cycle, and it is the most important source of nitric oxide (NO) and of the greenhouse gas nitrous oxide (N_2O) emissions to the atmosphere (Conrad, 1996; Zumft, 1997). More specifically, soils account for 70% of the total load of N_2O in the atmosphere (Mosier *et al.*, 1998). This greenhouse gas has a 100-year average global warming potential, a value which is 296 times higher than the one calculated for an equal mass of CO_2 (Ehhalt *et al.*, 2001). Moreover, Forster *et al.* (2007) calculated that anthropogenic greenhouse gas emissions and atmospheric concentrations of N_2O have been increasing since preindustrial times at a rate of about 0.26% per year. For these reasons, and to have a more complete knowledge of the soil nitrogen cycle, the study of denitrification process and of its major drivers is considered of great importance. Besides, this process is a cause of loss of nitrogen from agricultural soils, and therefore its importance in agronomical research is also recognized.

Denitrification can be performed by a wide range of microorganism, bacteria, archaea, and eukaryotes (Kraft *et al.*, 2011), and it is a dissimilative redox process, involving the reduction of nitrate, via nitrite and nitric oxide, to nitrous oxide and finally dinitrogen gas, by a respiration process under oxygen limiting conditions (Zumft, 1997). In bacteria, this process is used as an alternative to aerobic respiration, when the conditions are not favoured for this last metabolism. Like in aerobic respiration, the reaction chain is divided between the periplasmic and the cytoplasmic compartments, and it permits the generation of a proton motive force across the bacterial membrane, which is exploited for ATP synthesis (Figure 2.1.4).

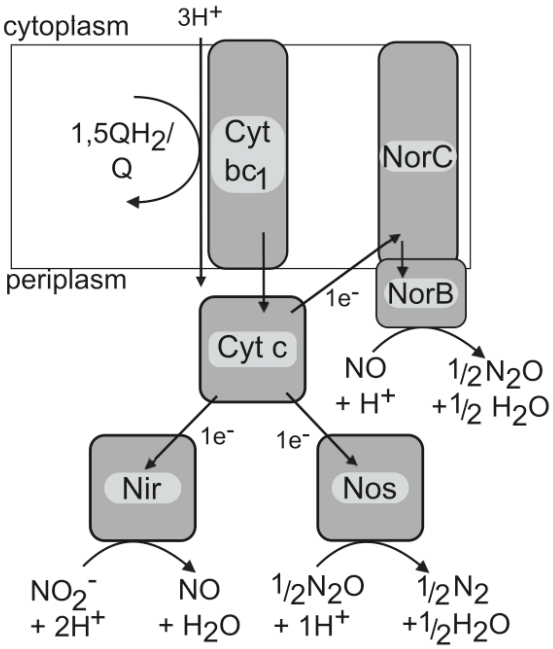

Figure 2.1.4

Respiratory chain in bacterial denitrification.
Cyt bc_1: cytochrome bc_1 complex, Cyt c: cytochrome c, Nir: nitrite reductase, Nor: nitric oxide reductase, Nos: nitrous oxide reductase, Q: co-enzyme Q (Kraft *et al.*, 2011).

Denitrification pathway involves four enzymes, which were studied in gram-negative bacteria. The reduction of nitrate to nitrite, which represents the first step of denitrification, is catalysed by a membrane bound nitrate reductase. A transporter protein allows nitrate to reach the periplasm, where a periplasmic nitrite reductase further reduces nitrite to nitric oxide. The integral membrane protein nitric oxide reductase performs the reduction of this species to nitrous oxide, which is subsequently reduced by the periplasmic nitrous oxide reductase, to obtain dinitrogen gas (Kraft *et al.*, 2011). Several species of denitrifying organisms are involved in the complete reduction

of nitrate to molecular nitrogen, but on the contrary some denitrifiers present only a part of the denitrification genes.
Denitrifiers are more frequent within the Alpha and Beta subclasses of Proteobacteria, even if the Gammaproteobacteria *Pseudomonas* genus is an important denitrifying bacterium (Zumft, 1997; Pastorelli *et al.*, 2011).

Many functional genes of denitrification process were successfully used as biomarkers to study this process in different environments or in the presence of particular external inputs. Pastorelli *et al.* (2011), for instance, evaluated the influence of different soil managements on the diversity and activity of soil denitrifying microorganisms. Considering the genes *nirS* and *nirK*, encoding the two forms of nitrite reductase, *qnorB* for nitric oxide reductase and *nosZ* gene, encoding for nitrous oxide reductase, they observed seasonal shifts in denitrifying community, and a significant variation depending on soil variables such as pH, soil texture and organic carbon.
The *nosZ* gene, together with *nirS* and *nirK*, were taken into consideration in the work by Dandie *et al.* (2011), in order to compare denitrifying communities and activities in an agricultural field and in an adjacent riparian zone. Even if the abundance of denitrifiers did not change in the two environments, the expression of denitrification genes was detected at higher levels in the riparian zone. The same functional genes were studied in soils with different pH, to have an indication of how pH affects denitrification gene pools (Liu *et al.*, 2010). Kleineidam *et al.* (2010), on the other hand, searched for the effects on denitrification of an antibiotic, which is often used in agriculture (Sulfadiazine). They observed that the community composition did not change, despite an alteration in the abundance of the functional genes of denitrification.

Together with denitrification, another process is responsible for the reduction of nitrates, which are not assimilated by plants and microorganisms: dissimilatory reduction of nitrate to ammonium (DNRA, Figure 2.1.5). Two types of dissimilatory nitrate reductase, differing in their locations, were characterized: a membrane-bound (Nar) and a periplasmic (Nap) nitrate reductase (Berks *et al.*, 1995; Moreno-Vivián *et al.*, 1999; Zumft, 1997). The membrane-bound nitrate reductase is present in Proteobacteria, Firmicutes, Actinobacteria, and even archaea, whereas the periplasmic nitrate reductase was detected only in Proteobacteria (Philippot and Højberg, 1999; Richardson *et al.*, 2001). Proteobacteria able to reduce nitrate can harbor Nar or Nap or both (Philippot, 2002; Zumft, 1997). While the physiological role of Nar has been understood, the role of Nap is still unclear, and could differ among bacteria (Richardson *et al.*, 2001; Gonzalez *et al.*, 2006). It was proposed that Nap could support anaerobic metabolism as an alternative to Nar, and that it could facilitate the switch from aerobic

respiration to denitrification or scavenge nitrate in some pathogenic bacteria (Siddiqui *et al.*, 1993; Bedzyk *et al.*, 1999; Potter *et al.*, 2001; Delgado *et al.*, 2003).
Although many biochemical and molecular studies have been performed on dissimilatory nitrate or nitrite reduction to ammonium (DNRA) in enteric bacteria (Simon, 2002) and some molecular tools for the key enzyme calcium-dependent cytochrome c nitrite reductase (*nrfA*) are available (Mohan *et al.*, 2004), we know very little about this process in the environment (Burgin and Hamilton, 2007; Jetten, 2008).

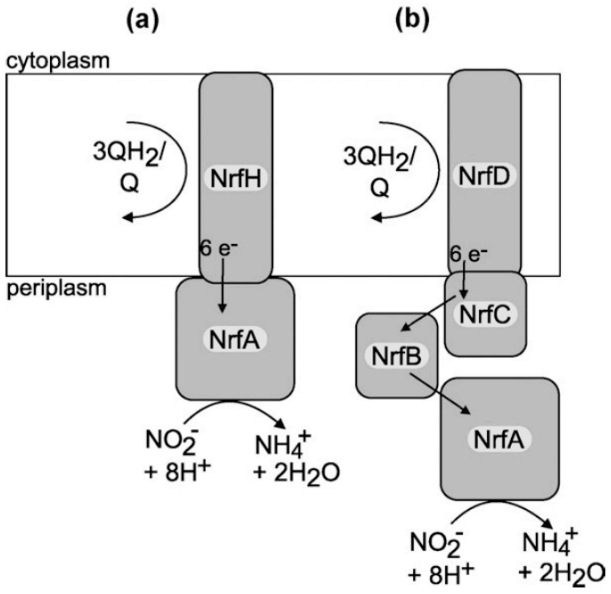

Figure 2.1.5
Respiratory chain in DNRA (dissimilatory nitrate reduction to ammonium). (a) NrfHA complex; (b) NrfABCD complex (Kraft *et al.*, 2011).

2.1.4 The metagenomics of soil: accessing the complexity of microbial communities

2.1.4.1 The complexity of environmental microbial communities

The total number of prokaryotic cells on Earth has been estimated to be about 4–6x10^{30}, and the major part of these have not yet been characterized (Sleator *et al.*, 2008). Our planet is predominated by microorganisms. Whitman *et al.*, (1998) calculated that bacterial life accounts for 350–550 billion tons of biomass, thus representing the dominant form of life. Moreover, as already stated in paragraph 2.1.1 and 2.1.2, microorganisms have a major role in the control of biological and geochemical cycles. The importance of microbial life and metabolism was also highlighted in the work of

Bardgett *et al.* (2008), in particular in their impact on Earth climate and consequently on our ability to predict the consequences of global warming events.

Soil and marine environments represent the major reservoir of microbial diversity in the minority of biomass (Ashby *et al.*, 2007; Elshahed *et al.*, 2008; Pedrós-Alió, 2006). For example, Elshahed *et al*. showed that rare members of the soil bacterial community represented 18.1 to 37.1% of the clones in the dataset, and that the members of this rare biosphere could either belong to novel bacterial lineages, to lineages often detected in other kinds of environments but usually not in soil, or were closely related to more abundant taxa present in the soil sample. Moreover, they observed that a significant portion of the rare biosphere represented evolutionarily distinct lineages at various taxonomic cutoffs. Sogin *et al.* (2006), by analysing the microbial population in the deep sea, demonstrated that also in this environment a relative small number of bacterial groups dominate in abundance, but that thousands of low-abundance populations account for most of the observed phylogenetic diversity.
From these and other scientific works, it can be deduced that microbial communities found in these very rich and puzzling environments are usually characterized by a few dominant taxa and numerous less abundant taxa.

As already stated, this thesis work focuses on the challenging world of soil.
Soil is a complex habitat for microorganisms. This matrix comprises mineral particles of different sizes, shapes and chemical characteristics, and usually presents organic compounds in different stages of decomposition.
The formation of clay–organic matter complexes and the stabilization of clay, sand and silt particles through the formation of aggregates are the dominant structural characteristics of the soil matrix (Daniel, 2005). These aggregates can have a dimension from about 2 mm or more (macro aggregates) to fractions of a micrometer for bacteria and colloidal particles (Paul and Clark, 1998).
Microorganisms find microhabitats in such a complex environment, and in particular they often adhere or adsorb onto soil particles such as sand grains or clay-organic matter complexes. Microhabitats for soil microorganisms include the surfaces of the soil aggregates, and the complex pore spaces between and inside the aggregates (Daniel, 2005; Hassink *et al.*, 1993; Foster, 1988). This patchy structure of soil, and in consequence of soil microbial community, can cause surprising situations, as the already cited result described by Parkin in 1987, who observed a concentration of denitrifying bacteria within a 0.08 g soil subsample, from a sample of about 100 g.
The composition of soil microbial communities is subject to frequent fluctuations, as the survival and metabolism of its components are strongly influenced by the availability of water and nutrients, which is normally subject to dramatic cycling changes (Daniel,

2005). Other environmental factors can cause alterations in the microbial composition of soil, such as pH, availability of oxygen, and temperature.
The patterns of species distribution and diversity in soil environment are not yet completely understood for microorganisms.
To adequately document the microbial diversity and the corresponding gene pool, the scale of soil surveys must be large.

The discipline of metagenomics, defined as the culture-independent genomic analysis of all the microorganisms in a particular environmental niche (Handelsman *et al.*, 1998), evolved as an effort to discover more about the microbial diversity of natural environments such as soil, marine water and the gastrointestinal tracts of vertebrates and invertebrates. Metagenomics, which is improving with new discoveries and with the development of new technologies, implies the direct isolation of DNA from a defined habitat, followed by the construction of a DNA library and by sequencing.
The first extensive large-scale environmental sequencing-metagenomic project was carried out by the J. Craig Venter Institute in 2004, and involved the sequencing of fragments of DNA derived from the entire microbial population of the nutrient-limited Sargasso Sea, in the Atlantic Ocean (Venter *et al.*, 2004).

2.1.4.2 Application of next generation sequencing (NGS) technologies in environmental research

Advances in molecular biology techniques, and in particular in next-generation sequencing (NGS) technologies (Figure 2.1.6), led to a revolution in biological sciences, and permitted the start of new analyses of environmental samples.
Access to massive amounts of sequencing data, and the consequential improvement of throughput and read length, permit to have better insights into complex microbial communities and bacterial diversity, at reasonable costs.
The traditional method for DNA sequencing (Sanger *et al.*, 1977), though being the most efficient method for the development of large DNA barcode reference libraries, cannot be used for complex environmental samples, as it only permits the sequencing of individual specimens. Indeed, environmental samples, such as soil DNA extracts, contain mixtures of DNA from hundreds or thousands of individuals.
Next-generation sequencing platforms have made possible to recover DNA sequence data directly from these kinks of samples (for example, from the deep see in the work by Sogin *et al.,* 2006).
With NGS experiments, several hundred thousands to tens of millions of sequencing reads can be potentially generated, and thanks to this high throughput capacity, entire

genomes of specific species can be sequenced (genome sequencing), expression profiles can be inferred (RNAseq or transcription sequencing), or pools of PCR-amplified molecules can be analysed (amplicon sequencing). In all these cases, sequences are generated without the need of a conventional, vector-based cloning procedure.

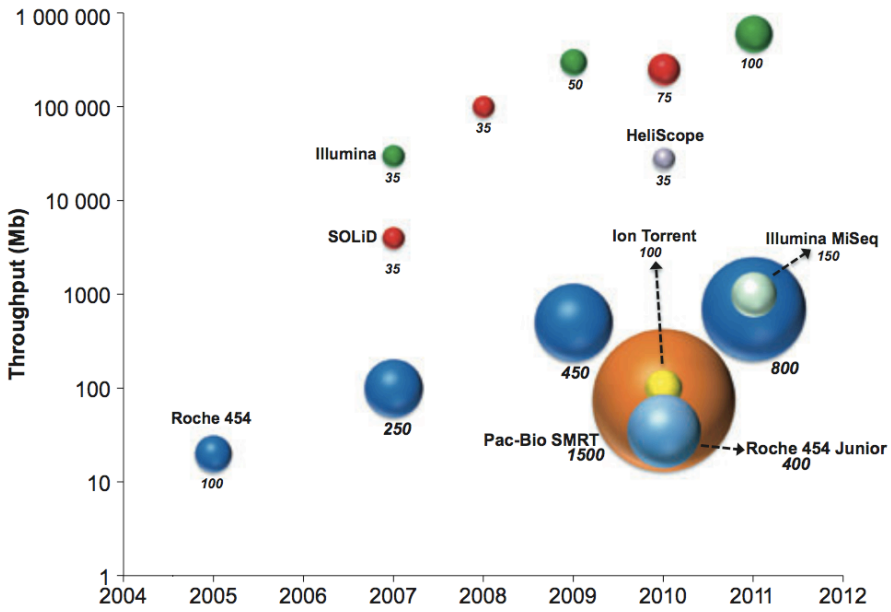

Figure 2.1.6

Historical development of next-generation sequencing technologies. The diameter of each bubble represents the sequencing read length of the platform in base pairs (bp). Colours correspond to individual platforms (Shokralla *et al.*, 2012).

16S ribosomal RNA (16S rRNA) gene amplicon sequencing is commonly used for bacterial identification in environmental samples (e.g. Sogin *et al.*, 2006; Flanagan *et al.*, 2007; Ramirez *et al.*, 2010; Pilloni *et al.*, 2012; Fierer *et al.*, 2012). Recently, multiplex sequencing has emerged as a strategy that permits the sequencing of many different samples at a time. This method involves the use of a unique sample-specific identifier, called "barcode" sequence, which permits to sort the reads into sample libraries via detection of the appropriate barcode. Although this method is crucial for this type of experiment, it produces amplification biases. This problem has been analysed by Berry *et al.* (2011), who proposed the use of a two-step PCR approach to avoid these biases, to increase reproducibility, and to recover higher genetic diversity in pyrosequencing libraries.

2.1.4.3 Roche 454 sequencing platform

Roche 454 genome sequencer, which was introduced in 2005 and was the first NGS technology to become commercially available, has been in the recent past the most commonly used NGS platform for the analysis of environmental DNA for ecological applications.
The 454 technology involves real-time sequencing-by-synthesis pyrosequencing.
The major advantages of the 454 pyrosequencing platform are its long read length and its relatively short run time. Longer sequences generated through 454 provide higher flexibility in terms of accurate annotation of reads in ecological applications involving nonmodel organisms (Shokralla *et al.*, 2012). For this reason, this sequencing platform is often used in the study of environmental samples, as stated above, and in particular of soil samples (e.g. Lauber *et al.*, 2009; Ramirez *et al.*, 2010; Fierer *et al.*, 2012; Rodrigues *et al.*, 2012; Yarwood *et al.*, 2013).

2.1.4.4 Metagenomic analyses of soil environments

Several studies were recently published, in which the main focus was on the comprehensive analysis of bacterial diversity from the complex environmental samples. Figure 2.1.7 shows a comprehensive analysis of microbial diversity in different kinds of environments, summing the work by Tamames *et al.* (2010).
In this study, 16S rRNA sequences from more than 3000 sampling experiments were re-analysed, and the environmental distribution of microbial taxa was inferred. It is visible from the image (Figure 2.1.7) that the majority of the taxonomical families were detected in the soil environment.

A number of scientific works concentrated on the study of soil microbial communities, and in specific on the analysis of the differences among soils coming from geographically distant areas, and/or subject to different environmental conditions. In particular, major efforts concentrate on the study of agricultural soil, and on the effect of nitrogen additions on microbial communities. For example, an analysis reported by Fierer *et al.* (2012) on soil microbial communities across nitrogen gradients showed that copiotrophic taxa, such as Proteobacteria and Bacteroidetes, increased in their relative abundance in the presence of high levels of N, while for oligotrophic taxa, and in particular Acidobacteria, opposite trends were observed. These effects on community composition were significant, even though there were no differences on bacterial diversity caused by the changes in N levels.

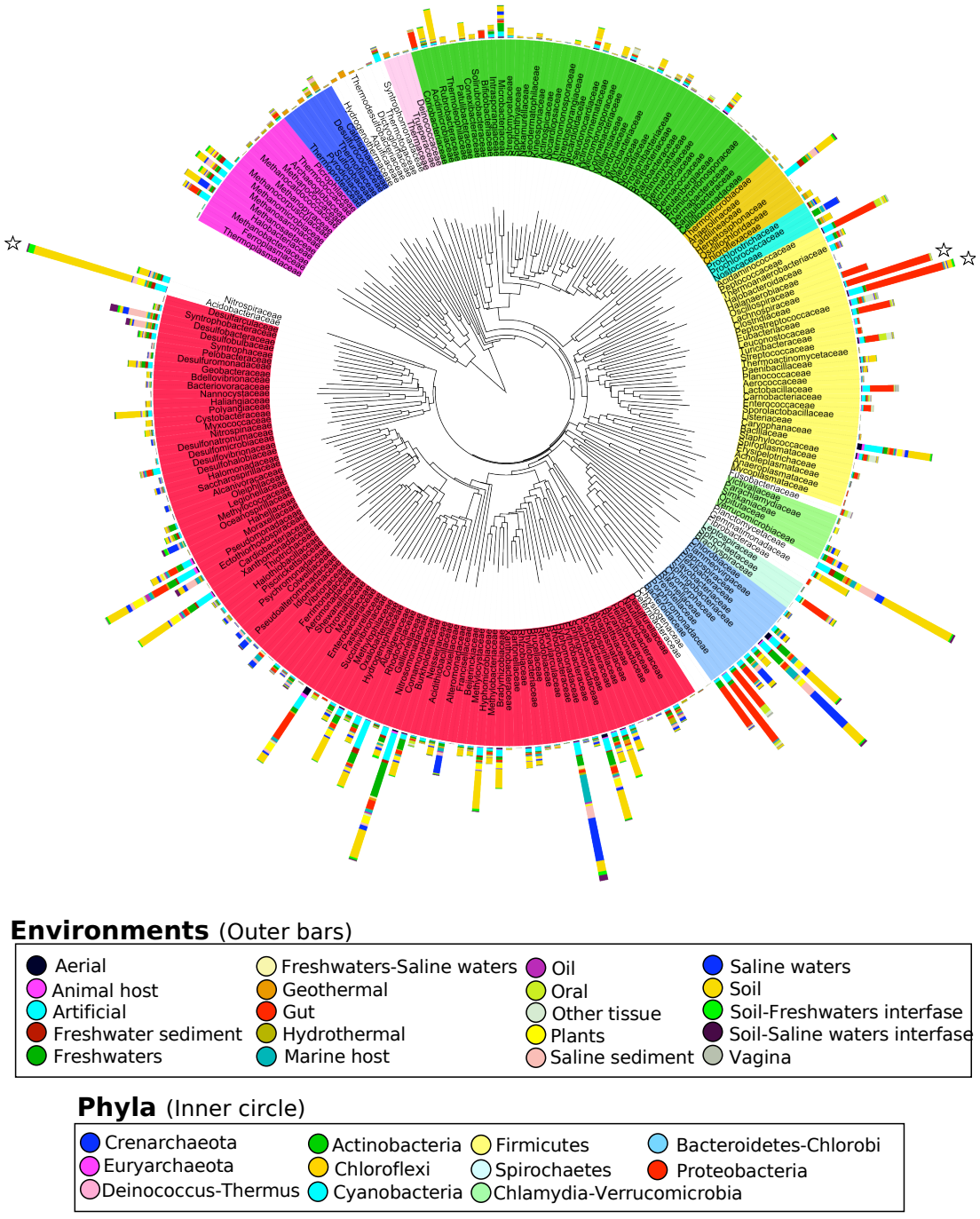

Figure 2.1.7

Distribution of individual taxonomic families in the different environment types. Families are coloured by their corresponding phyla, and only families with more than 10 observations have been considered. The bars in the outer circle indicate the number of times that each family has been observed in a sample from a particular environment. The bars marked with stars have been reduced to one third of their original size, for clarity purposes (Tamames *et al.*, 2010).

The papers by Nemergut *et al.* (2008) and by Campbell *et al.* (2010) describe the effects of fertilization in tundra soil. In both works, Acidobacteria, Actinobacteria and Proteobacteria were the dominating phyla detected in soils. Campbell revealed a significant difference in the two differently managed soils under investigation for what is concerning the types and abundance of OTUs belonging to the phyla Bacteroidetes, Cyanobacteria and Thermotogae.
Nemergut, on the other hand, reported a shift in bacterial community composition, with increase in the relative abundance of sequences related to Bacteroidetes and Gemmatimonadetes, and a decrease in the relative abundance of Verrucomicrobia, in the presence of fertilization. Moreover, this paper reported that Acidobacteria sequences from the fertilized soil were different from those found in the control soil. This particular response was not observed for Actinobacteria and Alphaproteobacteria.
Another study which considered the effects of nitrogen fertilization on soil microbial communities was published by Ramirez *et al.* in 2010. As in the work by Fierer *et al.* (2012), increases in copiotrophic bacterial groups, and in particular of Bacteroidetes and Betaproteobacteria, and decreases in the abundance of oligotrophic groups, such as Acidobacteria, were observed with the increase of N fertilization. Also bacteria belonging to the phyla Cyanobacteria and Nitrospirae were less abundant in fertilized soils.
These works are examples of the increasing interest of the scientific community in the analysis of the structure of soil microbial communities. In particular, the importance of this kind of studies was widely recognized in the fields of agronomical and environmental sciences, with the aim of optimizing soil management, and in particular fertilization practices, not only considering crop production, but also taking into account the responses of microorganisms to these external outputs. Indeed, as reported in this introduction, microorganisms play a fundamental role in soil health and productivity, in the majority of the biogeochemical cycles, and strongly influence environmental pollution.

2.1.5 Project outline

The major aims of this first part of the work were to define and optimize molecular techniques for the analysis of soil microbial activities and soil microbial communities.
Different protocols for the extraction of microbial DNA from various soil samples were tested and compared, to better comprehend how this first and fundamental step could influence the subsequent analysis.
For what is concerning microbial activities in soils, the focus was on the nitrogen cycle, as this process substantially influences both agricultural yield and plant health, and

pollution. The molecular technology selected to detect the presence of microorganisms involved in this process in soil was quantitative Real Time PCR targeting the functional genes of the nitrogen cycle, considering in detail nitrification, denitrification and nitrogen fixation. Once different experimental conditions were tested, this technique was used to analyse the equilibrium of this biogeochemical cycle in different soils.

The analysis of the structure of soil microbial communities was performed on some specific soil samples, subject to different fertilization conditions, in order to detect specific responses of various bacterial groups in the presence of organic, composite or inorganic fertilizers. A first investigation of the major changes in the communities was done through Terminal-Restriction Fragment Length Polymorphism experiments for all the soil samples, while a deep analysis was performed for a selection of the samples through 454 sequencing (Roche). This next generation sequencing technique permitted to detect significant differences in the effects of peculiar fertilization practices on the structure of soil bacterial communities, both at high and low taxonomical level, to observe recurrent trends in the fluctuations of the abundance of specific groups of bacteria and therefore to deduce possible interactions among them.

2.2 Material and Methods

2.2.1 Nucleic acids extraction from soil

2.2.1.1 Soil DNA extraction and purification with PowerSoil DNA Isolation Kit

DNA was extracted from 0.25 g soil using the PowerSoil DNA Isolation Kit (Mobio Laboratories, Carlsbad, CA, USA), according to the manufacturer's instructions. As suggested by the same, as an additional step to improve cell lysis, after the addition of the lysis-solution C1 to the PowerBead Tubes, a 10 minutes incubation at 70°C was performed. Three independent isolations were performed for each sample.
This extraction method was used for the analysis of nitrogen cycle genes in soils coming from different environments (see paragraph 2.3.3) and for PCR tests from soil DNA (see paragraph 2.3.1).
Purified DNA was stored at -20°C.

2.2.1.2 Soil DNA extraction and purification using the workstation Biosprint 96 (Qiagen)

In this thesis work, a new automatic DNA extraction method from soil was tested, and the required optimization was performed. The workstation BioSprint 96 (Qiagen, Valencia, CA, USA) and a modified protocol of the BioSprint 96 DNA Blood Kit (Qiagen) were used to perform automatic DNA extraction from soil samples.
In a 2 ml Eppendorf tube, 750 ml of Washing Buffer (121 mM guanidine isothiocyanate, 181 mM sodium phosphate dibasic Na_2HPO_4) were added to 0.25 g soil. The sample was then subject to a bead-beating step in the TissueLyser system (Qiagen) for 2 minutes at maximum oscillation frequency. In this way, soil samples were homogenized and a first cell lysis was performed. Subsequently, a low speed centrifugation (LSC) at 3000 rpm for 5 minutes was performed, in order to separate cells from soil particles. The supernatant from this step was used as sample for the standard procedure described by the manufacturer for the BioSprint 96 DNA Blood Kit. All the subsequent steps were performed in eight 96 well plates, that were then positioned in the rotating working station BioSprint 96. First, 200 µl of the supernatant from soil samples were mixed with 20 µl Proteinase K and with AL buffer (containing SDS, EDTA and chaotropic salts) and incubated at 70°C for 10 minutes to obtain cell lysis. After incubation, in the same wells, 200 µl isopropanol and 30 µl MagAttract Suspension G (containing magnetic beads covered with silica) were added to the lysate. In the remaining 96-well plates, washing

buffers were added, and in the last one the elution buffer for the DNA, as suggested by the manufacturer. Purified DNA was stored at -20°C.
MagAttract magnetic-particle technology combines the silica-based DNA purification with the handling of magnetic particles. DNA binds to the silica surface of the magnetic particles in the presence of a chaotropic salt. DNA bound to the particles is then washed, improving the purity of DNA. In the last step, DNA is eluted. All these steps are performed automatically by the working station.

2.2.1.3 DNA-RNA coextraction from soil

Nucleic acids were extracted from 0.45 g soil. Soil was added to a Lysing Matrix E tube (MP biomedicals, Santa Ana, CA, USA), with 750 µl 120 mM $NaPO_4$ buffer (pH 8) and 250 µl TNS (500 mM Tris-HCl pH 8.0, 100 mM NaCl, 10% SDS wt/vol). To perform cell lysis, the tube was shaken 30 s at 5.5 m/s in a bead beater (Precellys Bead Beater, Bertin Technologies, Montigny-le-Bretonneux, France). Soil and cell debris were pelleted by centrifugation 20 min at 13200 rpm at 4°C, and the supernatant was transferred to a fresh RNase-free 2 ml tube. Nucleic acids were extracted and purified with Phenol/Chloroform/Isoamylalcohol (25:24:1, pH 8, vol/vol) and subsequently with Chloroform/Isoamylalcohol (24:1, vol/vol). The resulting aqueous phase was mixed with 2 volumes of PEG solution (30% (wt/vol) polyethylene glycol 6000 in 1.6 M NaCl), incubated 2 h at 4°C and centrifuged 15 min at 13200 rpm at 4°C. The nucleic acid pellet was washed with 70% ethanol, air dried and resuspended in 50 µl DEPC-water.
All steps were performed on ice.
DNA-RNA samples were stored at -80 °C.

2.2.2 RNA purification and cDNA synthesis

A part of the DNA-RNA sample was digested with RQ1 RNase-free DNase (Promega, Madison, WI, USA), to obtain RNA, following manufacturer's instructions. After digestion, RNA was purified with the phenol-chloroform extraction protocol, and subsequently quantified spectrometrically.
cDNA synthesis was performed using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) as per manufacturer's instructions, adding to each reaction 1 U of Recombinant RNasin Ribonuclease Inhibitor (Promega).

2.2.3 Quantification of nucleic acids concentration

After nucleic acids extraction with one of the described methods, the concentration of the extracts was calculated with either the Quant-iT™ PicoGreen® dsDNA Assay kit (Invitrogen, Eugene, Oregon, USA) and a Spectrofluorimeter (SpectraFluor, TECAN, Männedorf, Switzerland) or spectrophotometrically on a Nanodrop (Thermo Scientific, Tewksbury, MA, USA).

2.2.3.1 Quantification of DNA concentration using PicoGreen® dsDNA quantitation assay

PicoGreen® reagent (Invitrogen) is a fluorescent dye that binds specifically to double strand DNA, increasing its emission of fluorescence of about 200 times (Sandaa *et al*., 1998).
For the quantification, 3 µl of soil DNA samples were diluted in 350 µl TE buffer (diluted from 200x solution: 200 mM Tris-HCl, 20 mM EDTA, pH 7.5), and a standard DNA solution (provided in the kit) was diluted at the concentrations of 500 ng/µl, 100 ng/µl and 10 ng/µl to permit the construction of a standard curve for the exact quantification. 100 µl PicoGreen (diluted to 1X concentration in TE buffer) were dispensed in a black 96 well plate together with 100 µl of the samples and the standard DNA (three replicates for each sample). The fluorencence emitted by the dye in each well was detected by a spectrofluorimeter (SpectraFluor, TECAN). The excitation and the emission wavelengths were of 485 nm and 535 nm, respectively. Thanks to the presence of standard DNA diluted at known concentrations, it was possible to calculate the exact DNA concentration of each sample.

2.2.3.2 Spectrophotometric quantification of nucleic acids' concentration using NanoDrop

Nucleic acids quantification was performed using NanoDrop (Thermo Scientific) after the co-extraction with the phenol-chloroform protocol (paragraph 2.2.1.3), a method that permitted the obtainment of well-purified samples, or, as a comparison, using PicoGreen quantification method (see Results, paragraph 2.3.1.2).
1 µl of undiluted nucleic-acids sample was spectrophotometrically quantified, and the purity ratios A_{260}/A_{280} and A_{260}/A_{230} were automatically calculated.
This quantification method could cause imprecise measurements in the presence of soil DNA samples, in which traces of humic acids are still present. Indeed, humic acids interfere with nucleic acid spectrophotometric quantification since they exhibit

absorbance both at 260 nm and 230 nm (Jackson *et al.*, 1997; Rajendhran and Gunasekaran, 2008).

2.2.4 PCR amplification

The polymerase chain reaction, commonly known as PCR, is a technique used in molecular biology that allows the amplification of a single or few copies of a portion of DNA generating up to millions of copies. The use of specific primers permits the DNA polymerase to amplify only the desired DNA fragment.
In this work, two different enzymes were used in PCR reactions. EuroTaq DNA polymerase (EuroClone, Pero, Milano, Italy) and GoTaq DNA polymerase (Promega).
The reaction mixtures and the thermal cycling programs for the PCR amplification varied based on the enzyme and on the primer couple used in the reaction.
In general, the reaction mixtures for PCR amplifications performed with the two different enzymes were the following:

EuroTaq PCR reaction mixture:	10x PCR reaction buffer	2.5 µl
	$MgCl_2$	1.25 mM
	BSA	0-0.2 mg/ml
	dNTP mix	200 µM
	Primer 5’	0.2-1 µM
	Primer 3’	0.2-1 µM
	Template DNA	1 µl
	EuroTaq	0.5 U
	MilliQ Water	up to 25 µl

EuroTaq reaction buffer contained 160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl (pH 8.8), 0.1% Tween-20. This reaction buffer did not contain Magnesium, which was supplied separately in a 50 mM solution.

GoTaq PCR reaction mixture:	5x amplification buffer	5 µl
	BSA	0-0.2 mg/ml
	dNTP mix	200 µM
	Primer 5’	0.2-1 µM
	Primer 3’	0.2-1 µM
	Template DNA	1 µl
	GoTaq	0.5 U
	MilliQ Water	up to 25 µl

GoTaq reaction buffer contained 7.5 mM Magnesium.

With both the enzymes, in some cases the reaction was performed in a final volume of 50 µl, maintaining the concentrations of the single reagents.

Thermal cycling program:

	Temperature	Time	Cycle No.
Initial denaturation	94° C	10 min	1x
Denaturation Annealing Extension	94° C 50-65° C 72° C	30-60 sec 30-60 sec 30 sec-1.5 min	25-40x
Final extension	72° C	10 min	1x
Cooling	4-15° C		

The annealing temperature depends on the primer sequence, and it is usually 5 to 10 degrees lower than the Tm of the primer. The time of the extension step is dependent on the length of the fragment to be amplified: the longer the fragment, the longer the time needed to copy it.
The PCR reactions were performed in the PCR system I-Cycler (BIO-RAD, Cressier, Switzerland).

2.2.5 Primers

All primers were supplied by Eurofins MWG Operon (Ebersberg, Germany) or by Biomers (Ulm, Germany) and dissolved in MilliQ sterile water to a final concentration of 100 pmol/µl. For PCR amplification, primers were further diluted to specific concentrations. Primers were stored at -20° C.
In the following table, the primers used for this thesis work are reported.

Primer Name	Sequence 5' -> 3'	Gene	References
63F	CAGGCCTAACACATGCAAGTC	16S rRNA	Marchesi *et al.*, 1998
1389R	ACGGGCGGTGTGTACAAG	16S rRNA	Osborn *et al.*, 2000
1404R	GGGCGGWGTGTACAAGGC	16S rRNA	Selenska-Pobell, 2002
Ba27F	CGTATCGCCTCCCTCGCGCCA	16S rRNA	Lane, 1991
Ba519R	CTATGCGCCTTGCCAGCCCGC	16S rRNA	Lane, 1991
Arch-amoAF	STAATGGTCTGGCTTAGACG	Archaeal	Francis *et al.*, 2005
Arch-amoAR	GCGGCCATCCATCTGTATGT	Archaeal	Francis *et al.*, 2005
19F	ATGGTCTGGCTWAGACG	Archaeal *amoA*	Leininger, *et al.*, 2006
CrenamoA616r	GCCATCCABCKRTANGTCCA	Archaeal *amoA*	Leininger S., pers. Comm.
amoA1F	GGGGTTTCTACTGGTGGT	Bacterial *amoA*	Rotthauwe *et al.*, 1997
amoA2R	CCCCTCKGSAAAGCCTTCTTC	Bacterial *amoA*	Rotthauwe *et al.*, 1997
NirS1F	CCTAYTGGCCGCCRCART	*nirS*	Braker *et al.*, 1998
nirS-q-R	TCCMAGCCRCCRTCRTGCAG	*nirS*	Mosier and Francis, 2010
nirS cd3af	GTSAACGTSAAGGARACSGG	*nirS*	Michotey *et al.*, 2000
nirSR3cd	GASTTCGGRTGSGTCTTGA	*nirS*	Throbäck *et al.*, 2004
nosZF	CGYTGTTCMTCGACAGCCAG	*nosZ*	Roesch *et al.*, 2002
nosZR	CATGTGCAGNGCRTGGCAGAA	*nosZ*	Roesch *et al.*, 2002
nirK876F	ATYGGCGGVCAYGGCGA	*nirK*	Henry *et al.*, 2004
nirK1040	RGCCTCGATCAGRTTRTGGTT	*nirK*	Henry *et al.*, 2004
nirK 5R	GCCTCGATCAGRTTRTGG	*nirK*	Braker *et al.*, 1998
nifHF	AAAGGYGGWATCGGYAARTCCACCAC	*nifH*	Roesch *et al.*, 2002
nifHR	TTGTTSGCSGCRTACATSGCCATCAT	*nifH*	Roesch *et al.*, 2002

Table 2.2.1
Oligonucleotides used in this work.

2.2.6 Electrophoresis

Horizontal gel electrophoresis was performed on 0.7-2% agarose gels (GellyPhor, EuroClone), based on the length of the DNA fragments that had to be separated. DNA samples were electrophoresed in 0.5X TBE buffer. Gels were stained with EuroSafe Nucleic Acid Staining Solution (EuroClone), which was added directly to the gel before

the pouring (0.5 µl EuroSafe solution in 100 µl gel). After the electrophoresis, which was usually run at 100 V, gels were visualized and photographed with GENi device (Syngene, Cambridge, England, UK).

5x TBE buffer: Tris HCl 54 g/l
Boric acid 27.5 g/l
NaEDTA 4.65 g/l

2.2.7 Quantitative Real Time PCR experiments

Quantitative Real Time PCR (qRT-PCR) technique was used to quantify the levels of functional genes of the nitrogen cycle in soil samples. The assay was carried out with Power SYBR® Green PCR Master Mix (Applied Biosystems) in a Step One Real Time PCR System (Applied Biosystems) using 48 well plates or in the Quantstudio 12K Flex Real Time PCR System using 384 wells plates (Applied Biosystems). Gene-specific primers (listed in table 2.1.1) were used to amplify the desired specific genes. The quantification of the gene copy number was obtained with the standard curve method. With this method, a standard curve is created using known amounts of plasmids containing the gene of interest (such as in this case) or of genomic DNA, and subsequently the Ct values obtained for the unknown samples are interpolated in the standard curve and a gene copy number value is extrapolated.

2.2.7.1 Preparation of plasmids containing N-cycle functional genes for quantification in Real Time experiments

2.2.7.1.1 Cloning of N-cycle functional genes PCR products in a pGemT vector

As already stated, in order to permit the absolute quantification of the gene copy number in Real Time PCR experiments, a standard curve obtained with known amounts of DNA containing the desired gene is needed. To achieve this, PCR products obtained using the same primers used in Real Time experiments, were cloned in the vector pGEM-T (pGEM®-T Easy Vector Systems, Promega). The pGEM-T vector is convenient for the cloning of low quantity PCR products. Moreover, this vector confers resistance to ampicillin and permits the blue-white screening because it contains the multiple cloning site inserted in the *lacZ* gene.
PCR amplification was performed as described in paragraph 2.2.4, with the primers and the conditions reported in the table 2.2.3, and the obtained products were purified using the QIAquick PCR Purification kit (Qiagen). 3 µl of the purified fragments were then

ligated in the pGEM-T vector; the ligation of the diverse DNA fragments into the vector was performed in a total reaction volume of 10 µl using 1 µl (3 U) T4 DNA ligase provided in the kit, according to the manufacturer's recommendations.

2.2.7.1.2 Preparation of competent *Escherichia coli* cells

2.5 ml of overnight culture of *E. coli* S17-1 (Simon *et al*., 1983) were used to inoculate 2 flasks with 250 ml of Luria-Bertani broth (LB medium: peptone 10 g/l, NaCl 10 g/l, yeast extract 5 g/l; DIFCO, Sparks, MD, USA). Bacteria were grown at 37 °C with shaking for 2-3 hours until an OD_{600} of 0.6 was reached. After an incubation of 15 minutes on ice, the cells were centrifuged for 15 min at a speed of 4000 rpm, maintaining a temperature of 4° C. Each pellet was resuspended in 200 ml cold sterile water. After a centrifugation of 15 min at 4000 rpm at 4° C, the pellets were resuspended in 100 ml of cold sterile water. Cells were centrifuged again at 4000 rpm at 4 °C for 15 minutes. Each pellet was resuspended in 5 ml of a cold solution of glycerol 10% and centrifuged 15 min at 4000 rpm at 4 °C. 750 µl of the cold 10% glycerol solution were used to resuspend the final pellet, and finally cells were transferred to cold Eppendorf tubes in 80 µl aliquots, and stored at -80 °C.

2.2.7.1.3 Transformation of *E. coli*

The transformation was performed by adding 1.8 µl of ligation mixture to 80 µl competent *E. coli* cells. After an incubation of 5 min on ice, bacteria were subject to electroporation using the devices Pulse Controller and Gene Pulser (BIO-RAD) with the following settings: 2.5 kV, 200 Ohm, 25 µF. After the impulse, 800 µl LB medium were immediately added to the transformed cells. The sample was incubated at 37° C in 15 ml Falcon tubes for 1 hour. Cells were subsequently plated on selective LB-agar plates and incubated overnight at 37° C.

2.2.7.1.4 Isolation of plasmid DNA

Preparation of plasmid DNA was performed using the Qiagen Plasmid Midi Kit. Single colonies of transformed *E. coli* were picked from plates and inoculated in liquid LB medium provided with the selective antibiotic (ampicillin 100 µg/ml). Bacteria from 4 ml of the overnight culture were harvested by centrifugation and the supernatant was completely removed. Plasmid DNA purification was then performed according to the manufacturer's recommendations.

2.2.7.2 Quantitative Real Time PCR experiments performed with StepOne Real Time PCR System

When performing Real Time PCR experiments with StepOne Real Time PCR System (Applied Biosystems), the 20 µl reaction mixture consisted of 10 µl SybrGreen mix, 0.5 µM of the forward and the reverse primers, 0.2 mg/ml BSA, 1 µl template DNA, and sterile MilliQ water to reach the final volume. DNA extracted from soil was diluted 10 times for these reactions, both to dilute eventual humic acids that could have been present, and to obtain a lower final DNA concentration, a condition which is often suggested in this kind of experiments.

A typical cycling program is shown in the following table.

	Temperature	**Time**	**Cycle No.**	**Ramp Rate**
	52 °C	2 min	1x	100%
Enzyme activation	95 °C	15 min	1x	100%
Denaturation	95 °C	15 sec	40x	100%
Annealing	53-60 °C	30 sec		100%
Extension	72 °C	30-50 sec		100%
Fluorescence detection	77-80 °C	18 sec		100%
Denaturation	95 °C	15 sec	1x	100%
Starting temp.	60 °C	1 min	1x	100%
Melting step	60 °C	30 sec	1x	1%

Table 2.2.2
Cycling program used in Real Time PCR experiments performed in the StepOne Real Time PCR system.

The last three steps of the program are needed to obtain a melting curve to confirm the specificity of the reaction.
The step called “fluorescence detection” reported in the table was added to avoid the detection by the Real Time PCR machine of double stranded DNA not corresponding to the desired specific amplified fragment. This stratagem can be used when considering soil samples as unspecific amplification can more easily occur (Limpiyakorn *et al.*, 2011; Henry *et al.*, 2004; Szukics *et al.*, 2010; Keil *et al.*, 2011; Dandie *et al.*, 2011; Glaser *et al.*, 2010; He *et al.*, 2007; Santoro *et al.*, 2010; Liu *et al.*, 2010).
The primer pairs used in the Real Time experiments performed in the StepOne System, their concentration in the reaction mixes, and the corresponding annealing temperature and extension time are reported in the following table.

Primer pair	Gene	Amplicon's length	Primers' concentration	Annealing Temperature	Extension time
Arch_amoAF/ Arch_amoAR	Archaeal *amoA*	635 bp	0.5 µM	57°C	45 s
amoA1F/ amoA2R	Bacterial *amoA*	~ 500 bp	0.5 µM	57°C	40 s
nirS1F/ nirS-q-R	*nirS*	~ 300	0.5 µM	60°C	30 s
nosZF/nosZR	*nosZ*	706 bp	0.5 µM	56°C	45 s
nirK 1040/ nirK 876F	*nirK*	~ 160 bp	0.5 µM	58°C	30 s
nifHF/nifHR	*nifH*	432 bp	0.5 µM	53°C	45 s

Table 2.2.3
Primer pairs used in the Real Time PCR experiments performed in the StepOne System. In the table, the primer concentration used in the reaction mix, the annealing temperature and extension time are reported.

Primers' sequences and references are reported in table 2.2.1.

2.2.7.3 Quantitative Real Time PCR experiments performed with Quantstudio 12K Flex Real Time PCR System

Real Time PCR experiments were also performed with Quantstudio 12K Flex Real Time PCR System (Applied Biosystems). As stated in the paragraph 2.3.2 of the results, the optimization of Real Time amplification with lower reaction volumes was required.
First, different reaction volumes (5 µl, 10 µl, 15 µl, 20 µl) were tested and their amplification efficiencies were compared. The results were compared with the results obtained with a 25 µl reaction in the StepOne machine. For this optimization tests, the primer pair amoA1F/amoA2R (Table 2.2.1) was used in the reaction mixes reported in table 2.2.4.
For these Real Time PCR experiments, some parameters, such as the concentration of the primers or of BSA, or cycling conditions, were changed following the suggestions of the group of Environmental Genomics of the Helmholtz Zentrum in Munich (Germany), where part of the experiments for this thesis work were performed.

Mix 25 µl	Volume	Final
3% BSA	0.5 µl	0.6 mg/ml
Primer amoA1F 10 µM	0.75 µl	0.3 µM
Primer amoA2R 10 µM	0.75 µl	0.3 µM
Power SybrGreen	12.5 µl	1x
H_2O	9.5 µl	
DNA	1 µl	
Total Volume	25 µl	

Mix 20 µl	Volume	Final
3% BSA	0.4 µl	0.6 mg/ml
Primer amoA1F 10 µM	0.6 µl	0.3 µM
Primer amoA2R 10 µM	0.6 µl	0.3 µM
Power SybrGreen	10 µl	1x
H_2O	7.4 µl	
DNA	1 µl	
Total Volume	20 µl	

Mix 15 µl	Volume	Final
3% BSA	0.3 µl	0.6 mg/ml
Primer amoA1F 10 µM	0.45 µl	0.3 µM
Primer amoA2R 10 µM	0.45 µl	0.3 µM
Power SybrGreen	7.5 µl	1x
H_2O	5.3 µl	
DNA	1 µl	
Total Volume	15 µl	

Mix 10 µl	Volume	Final
3% BSA	0.2 µl	0.6 mg/ml
Primer amoA1F 10 µM	0.3 µl	0.3 µM
Primer amoA2R 10 µM	0.3 µl	0.3 µM
Power SybrGreen	5 µl	1x
H_2O	3.2 µl	
DNA	1 µl	
Total Volume	10 µl	

Mix 5 µl	Volume	Final
3% BSA	0.1 µl	0.6 mg/ml
Primer amoA1F 10 µM	0.15 µl	0.3 µM
Primer amoA2R 10 µM	0.15 µl	0.3 µM
Power SybrGreen	2.5 µl	1x
H_2O	1.1 µl	
DNA	1 µl	
Total Volume	5 µl	

Table 2.2.4
Reaction mixes tested to optimize the reaction volume in Real Time PCR experiments performed with Quantstudio 12K Flex Real Time PCR System (Applied Biosystems).

As reported also in the results (paragraph 2.3.2), considering the results from this first test done with different reaction volumes, it was decided to test serial dilution of DNA in two fixed reaction volumes of 10 µl and 20 µl. Two soil DNAs, of which the DNA concentration was known, were selected for this test: the first soil DNA had a initial concentration of 92.93 ng/µl, while the second of 124.06 ng/µl. Starting from a

concentration of 40 ng/µl DNA, serial 2-fold dilutions were done to a final quantity of 0.3125 ng DNA per reaction.

After these tests, Real Time PCR reaction in a volume of 10 µl was optimized for the functional genes of the nitrogen cycle, permitting the analysis of as many as 384 samples at a time in the QuantStudio 12K Flex System.

The 10 µl reaction mixture consisted of 5 µl Sybr Green mix, 0.2-0.5 µM of the forward and the reverse primers, 0.6 mg/ml BSA, 1 µl template DNA, sterile MilliQ water to reach the final volume. In some cases, DMSO was added at a final concentration of 2.5%, to improve amplification. DNA extracted from soil was diluted 50 times in this case, as the low reaction volume required a very low final DNA quantity (diluted template DNA had a concentration between 2.5 ng/µl and 0.625 ng/µl), as obtained with the optimization tests. Real Time PCR reaction mixes and conditions are reported in the following tables.

Gene	**Reagent**	**Volume**	**Final Concentration**
Bacterial *amoA*	3% BSA	0.2 µl	0.6 mg/ml
	amoA1F 10 µM	0.3 µl	0.3 µM
	amoA2R 10 µM	0.3 µl	0.3 µM
	Power SybrGreen	5 µl	1x
	H_2O	3.2 µl	
	DNA	1 µl	

Cycling conditions

	Temperature	**Time**	**Cycle No.**
	52 °C	2 min	1x
Enzyme activation	95 °C	10 min	1x
Denaturation	95°C	15 sec	40x
Annealing	60°C	20 sec	
Extension	72°C	40 sec	
Fluorescence detection	78°C	18 sec	

Table 2.2.5

Real Time PCR reaction mix and cycling conditions optimized for the bacterial *amoA* gene in the Quantstudio 12K Flex Real Time PCR System (Applied Biosystems).

Gene	Reagent	Volume	Final Concentration
Archaeal *amoA*	3% BSA	0.2 µl	0.6 mg/ml
	19F 10 µM	0.2 µl	0.2 µM
	CrenamoA616r 10 µM	0.2 µl	0.2 µM
	Power SybrGreen	5 µl	1x
	H_2O	3.4 µl	
	DNA	1 µl	

Cycling conditions

	Temperature	Time	Cycle No.
	52 °C	2 min	1x
Enzyme activation	95 °C	10 min	1x
Denaturation	95°C	15 sec	40x
Annealing	55°C	15 sec	
Extension	72°C	40 sec	

Table 2.2.6

Real Time PCR reaction mix and cycling conditions optimized for the archaeal *amoA* gene in the Quantstudio 12K Flex Real Time PCR System (Applied Biosystems).

Gene	Reagent	Volume	Final Concentration
nirK	3% BSA	0.2 µl	0.6 mg/ml
	DMSO 100%	0.25 µl	2.5%
	nirK876F 10 µM	0.2 µl	0.2 µM
	nirK 5R 10 µM	0.2 µl	0.2 µM
	Power SybrGreen	5 µl	1x
	H_2O	3.15 µl	
	DNA	1 µl	

Cycling conditions

	Temperature	Time	Cycle No.
	52 °C	2 min	1x
Enzyme activation	95 °C	10 min	1x
Denaturation	95°C	15 sec	5x
Annealing	63°C	20 sec	
Extension	72°C	20 sec	
Denaturation	95°C	15 sec	40x
Annealing	58°C	20 sec	
Extension	72°C	30 sec	
Fluorescence detection	78°C	18 sec	

Table 2.2.7

Real Time PCR reaction mix and cycling conditions optimized for the *nirK* gene in the Quantstudio 12K Flex Real Time PCR System (Applied Biosystems).

Gene *nirS*	Reagent	Volume	Final Concentration
	3% BSA	0.2 µl	0.6 mg/ml
	DMSO 100%	0.25 µl	2.5%
	nirS cd3af 10 µM	0.2 µl	0.2 µM
	nirSR3cd 10 µM	0.2 µl	0.2 µM
	Power SybrGreen	5 µl	1x
	H_2O	3.15 µl	
	DNA	1 µl	

Cycling conditions

	Temperature	Time	Cycle No.
	52 °C	2 min	1x
Enzyme activation	95 °C	10 min	1x
Denaturation	94°C	15 sec	39x
Annealing	57°C	20 sec	
Extension	72°C	45 sec	

Table 2.2.8

Real Time PCR reaction mix and cycling conditions optimized for the *nirS* gene in the Quantstudio 12K Flex Real Time PCR System (Applied Biosystems).

Gene *nosZ*	Reagent	Volume	Final Concentration
	3% BSA	0.07 µl	0.2 mg/ml
	nosZF 10 µM	0.5 µl	0.5 µM
	nosZR 10 µM	0.5 µl	0.5 µM
	Power SybrGreen	5 µl	1x
	H_2O	2.93 µl	
	DNA	1 µl	

Cycling conditions

	Temperature	Time	Cycle No.
	52 °C	2 min	1x
Enzyme activation	95 °C	10 min	1x
Denaturation	95°C	15 sec	40x
Annealing	56°C	50 sec	
Extension	72°C	45 sec	
Fluorescence detection	78°C	18 sec	

Table 2.2.9

Real Time PCR reaction mix and cycling conditions optimized for the *nosZ* gene in the Quantstudio 12K Flex Real Time PCR System (Applied Biosystems).

Each amplification reaction was concluded with the melt-curve stage (not reported in the tables), to confirm the specificity of the reaction.

Also in this case, with some primer couples, the "fluorescence detection" step was added, to ensure that the detected fluorescence corresponded to the specific PCR product.
Primers' sequences and references are reported in table 2.2.1.

2.2.7.4 Real Time PCR experiments run by external services

DNA extraction and Real Time PCR experiments regarding the analysis of N-cycle genes in soils subject to different water levels equipped with lysimeters (paragraph 2.3.4) were performed by an external private laboratory (Parco Tecnologico Padano, Lodi, Italy). The primers used were the same as described in paragraph 2.2.7.2. In this case, the quantification through the use of a standard curve was not performed, and the comparisons among different soil samples were done by taking into consideration an arbitrary number referred to as 'Quantity', calculated by the Real Time PCR software.

2.2.7.5 Statistical analyses on Real Time PCR results

Statistical analyses on Real Time PCR results were performed using the statistical program IBM® SPSS® Statistics Version 20. In specific, the means, the standard deviation and the standard error of the means were calculated and, through the Anova test, significant differences among the mean values were detected and analysed. Moreover, significant correlations among the considered genes were investigated calculating the Pearson's correlation coefficient (two-tailed).

2.2.8 Greenhouse fertilization trial

A greenhouse trial was set up in Summer 2012 to compare the effects of different fertilizers on plant growth and on soil bacterial communities. The fertilization experiment included 4 replicates, each composed of 2 single pots, of 14 treatments in a randomized design. The names and the description of the fertilizers are reported in the table 2.2.10.
The pots had a diameter of about 20 cm, and contained about 5.5 kg soil-sand mixture (see paragraph 2.2.8.1 for the chemical analysis of the used soil). The fertilizers were added in the upper 5 cm of the substrate, and subsequently a sod with grown *Cynodon dactylon* was placed over it. Apart from being added to an agricultural low-nutrient soil-sand mixture, 6 of the considered fertilizers were also added to soils, which had already been used for a similar test and that had been fertilized with the same fertilizers the year before.

Fertilizers were added to have a final nitrogen input of 150 kg/ha. Moreover, P_2O_5 and K_2O_5 were added to have final content of 50 kg/ha and 100 kg/ha, respectively. The fertilization trial lasted 2 months (June and July 2012), during which plant growth measurements and soil sampling were done.

Abbreviation	Name	N (%)	Composition
THL	Thermal Hydrolysed Leather	12.8	100% THL
Y	Yeast	6.1	100% Yeast
N	Neem	3.5	100% Neem
UTY	Urea-THL-Y	21.5	30% Urea, 60% THL, 10% Yeast
UTN	Urea-THL-N	21.1	30% Urea, 50% THL, 20% Neem
CC	Castor cake	4.3	100% Castor Cake
OATr	Oxy-ammino-triazine	50	100% OATr

Table 2.2.10
Fertilizers used in the greenhouse trial. In the table, the abbreviations used in this thesis work, the nitrogen content and the composition of the fertilizers are shown.

2.2.8.1 Chemical and physical analysis on the low-nutrient soil used for the fertilization trial

Before the beginning of the fertilization experiments, physical and chemical properties of soil were characterized. The analysis was performed by the private laboratory MAC - Minoprio Analisi e Certificazioni S.r.l. Results are shown in the table 2.2.11.

Test	Results	Unit of measurement
Fraction>2 mm (skeleton)	20	g/kg s.s.
Fraction 2-0.05 mm (sand)	203	g/kg s.s.
Fraction 0.05-0.002 (silt)	640	g/kg s.s.
Fraction<0.002 (clay)	157	g/kg s.s.
H_2O pH	8.1	pH unit
$CaCl_2$ pH	7.6	pH unit
Total limestone ($CaCO_3$)	380	g/kg s.s.

Test	Results	Unit of measurement
Active limestone	56	g/kg s.s.
Organic substance (calculated)	33	g/kg s.s.
Organic carbon (Dumas)	19.2	g/kg s.s.
Total nitrogen	1.4	g/kg s.s.
Carbon/nitrogen ratio (calculated)	13.7	
Cation exchange capacity	21.1	meq/100g s.s.
Calcium available for exchange	19.52	meq/100g s.s.
Magnesium available for exchange	2.78	meq/100g s.s.
Potassium available for exchange	0.38	meq/100g s.s.
Nitrogen available for exchange	0.03	meq/100g s.s.
Base saturation (calculated)	100	%
Calcium/magnesium ratio (calculated)	7	
Magnesium/potassium ratio (calculated)	7.3	
ESP (calculated)	0.14	%
Assimilable phosphorus	8	mg/kg s.s.

Table 2.2.11
Chemical and physical analysis of low-nutrient soil used in the fertilization trial.

2.2.8.2 Soil sampling

About 2 g soil were sampled in the centre of each pot, just under the *C. dactylon* sod, 5 times during the 2 months. In specific, sampling was performed before the distribution in the pots and the addition of the fertilizers (day 0), and after 1, 9, 30, and 58 days from the beginning of the trial. After the sampling, soil was immediately frozen in liquid nitrogen, and then stored at -80 °C until the extraction of nucleic acids (performed with the phenol-chloroform protocol, paragraph 2.2.1.3).

2.2.8.3 Plant growth measurements

Plant height and plant biomass (fresh weight and dry weight) were measured three times during the two months of the greenhouse experiment. Weight was assessed upon cutting the grass and weighing it on a precision scale before and after drying.

2.2.9 Terminal-Restriction Fragment Length Polymorphism (T-RFLP)

PCR amplification of the bacterial 16S rRNA gene was performed on DNA-RNA coextracted samples (diluted ten times) and on cDNA samples in a Mastercycler ep gradient (Eppendorf, Hamburg, Germany) with the following cycling conditions: initial denaturation at 94°C for 10 min followed by 30 cycles of denaturation (1 min at 94°C), annealing (1 min at 57°C) and extension (1 min 30 sec at 72°C) and by a final extension (10 min at 72°C). Each 50 µl reaction contained 1x reaction buffer, 2.5 mM $MgCl_2$, 0.2 µM of the primer pair 27F/1404R, 200 µM dNTPs, 2.5% DMSO, 0.6 mg/ml BSA, 2.5 U Taq DNA Polymerase (Invitrogen) and 1 µl of template DNA. The forward primer was labelled with 6-carboxyfluorescein (FAM). After confirming the amplification on an agarose gel, the PCR products were purified with the PCR clean-up Gel Extraction kit (Macherey-Nagel, Düren, Germany) and digested with the restriction enzyme *Msp*I (Thermo Scientific) as follows: in a 20 µl volume reaction, 10 µl of purified PCR product were digested with 10 U of the enzyme for 1 h 45 min at 37°C, followed by inactivation of the enzyme for 20 min at 80°C.
After the restriction, a second purification was performed, and the resulting samples were quantified with the NanoDrop (Thermo Scientific).
Sequencing plates were prepared as follows: 1 µl of sample (3 ng/µl for cDNA and 4 ng/µl for DNA) was added to 13 µl of HiDi Formamide containing MapMarker-1000 Rox size standard (BioVentures, Murfreesboro, TN, USA) diluted 300 times, and denatured 5 min at 95°C in a thermal cycler. Fragment separation was performed by capillary electrophoresis.
Electropherograms were visualized, and size and relative abundances of terminal restriction fragments (TRFs) were quantified using GeneMapper Software (Applied Biosystems). T-RFLP data were then processed with the T-REX (T-RFLP analysis expedited) online software (http://trex.biohpc.org/): peaks were filtered to eliminate background noise, aligned and a data-matrix was produced, from which it was possible to perform subsequent statistical analysis.

2.2.9.1 Statistical analyses on T-RFLP results

Statistical analyses on T-RFLP results were performed using the statistical programs R (version 2.15.1), Past (Paleontological Statistics Software Package, version 2.12), and IBM® SPSS® Statistics (version 20). Descriptive statistical analyses and PCA plots were performed with the program R. The calculation of diversity indices and the cluster analyses were done with Past Software. SPSS Statistics was used to compare the means calculated on diversity indices, and to investigate significant differences among them

through the use of Anova tests (differences were considered significant with a p-value<0.05).

2.2.10 454 sequencing

Amplicon pyrosequencing was performed on a 454 GS FLX Titanium system (Roche, Penzberg, Germany) under the following operating conditions.

2.2.10.1 Amplicon library preparation

64 DNA samples extracted from soil were selected for amplicon pyrosequencing, representing 4 biological replicates of 16 conditions. Barcoded amplicons for multiplexing were prepared using the primers Ba27f and Ba519r (Table 2.2.1) extended with the respective A or B adapters, key sequence and multiplex identifiers (MID) as recommended by Roche. As advised by Berry *et al.* (2011), a two-step PCR approach was selected, with the addition of the MID-primers only in the second PCR amplification, to avoid biases and increase reproducibility. For each DNA sample, 3 PCR replicates were done. The first PCR was performed in a Mastercycler ep gradient (Eppendorf) with the following cycling conditions: initial denaturation (94 °C, 10 min), followed by 22 cycles of denaturation (94 °C, 30 s), annealing (52 °C, 30 s) and extension (72 °C, 60 s) and by a final extension step at 72 °C for 10 min. Each 25 ml PCR reaction contained 1x PCR buffer (containing $MgCl_2$), 0.2 mM dNTPs, 1.25 U Fast Start HiFi Polymerase (Roche), 0.24 mg/ml BSA, 0.25 mM of each primer and 1 ml of template DNA (at a concentration of 20 ng/µl). The second PCR was performed with the same cycling conditions as the first, but with only 6 cycles of denaturation, annealing, extension. Each 25 ml PCR reaction contained 1x PCR buffer (containing $MgCl_2$), 0.2 mM dNTPs, 1.25 U Fast Start HiFi Polymerase (Roche), 0.24 mg/ml BSA, 0.25 mM of each MID-primer (Biomers) and 5 µl of the previous PCR product. Amplicons were purified using Agencourt AMPure-XP beads (Beckman Coulter, Brea, CA), and quantified with the Quant-iT PicoGreen dsDNA quantification kit (Invitrogen). The complete purification was checked, for a selection of the samples, with Agilent Bioanalyzer 2100 (Agilent technologies, Santa Clara, CA, USA). Amplicons were diluted to 10^9 molecules/µl and mixed in an equal volume in 4 pools of 16 samples.

2.2.10.2 Emulsion PCR, emulsion breaking and sequencing

Emulsion PCR, emulsion breaking and sequencing were performed applying the GS FLX Titanium chemistry following the supplier's protocol.

2.2.10.3 Data analysis and statistics

Raw sequence analyses were conducted using the QIIME pipeline (Caporaso *et al.*, 2010). After the filtering steps, about 246000 good quality sequences were used for further analyses, with a mean of about 3800 sequences per sample. Phylotypes were selected at 97% sequence similarity level and the taxonomic identity was determined using the RDP scheme. The QIIME software was also used to calculate and draw rarefaction curves based on the number of observed species and on the Shannon index.
The values of the presence percentage of each taxon were used for further analysis, to compare different soil samples and to deduce individual effects caused by different fertilizers on soil bacterial communities. In specific, the free source software MeV (version 4.8.1) was used to perform this kind of comparisons. One-way Anova was performed to detect the taxa, at different levels of taxonomy, which resulted to change significantly ($p\text{-value}<0.05$) in the different conditions (considering the 4 replicates of each condition as a single group). With the same software, Wilcoxon Rank Sum tests were performed to investigate significant differences between couples of conditions (fertilized soils with the unfertilized soil, and same soils sampled at the two considered sampling times).
Diversity indices were calculated considering the taxonomical level Phylum, using the Past Software (Paleontological Statistics Software Package, version 2.12), and statistical differences were searched with Anova tests performed with the software IBM® SPSS® Statistics (version 20). With this last software, the correlations among different bacterial phyla were also investigated, through the calculation of Pearson's correlation coefficient (two-tailed), and where considered significant with a $p\text{-value}<0.05$.

2.3 Results

2.3.1 DNA extraction from soil

2.3.1.1 PCR amplification of DNA extracted from soil

The importance of having a good quality DNA preparation for molecular techniques is widely known. Soil is a very peculiar matrix, and more than 10^9 microorganisms are present in a gram of this material. Moreover, as commented in the introduction, the diversity of soil bacterial species is astounding: in 2001, it has been estimated that about $4x10^6$ different taxa are contained in a ton of soil (Curtis *et al.*, 2002), while more recent estimates of the number of species vary between 2000 and 8.3 million per gram soil (Schloss and Handelsman, 2006; Gans *et al.*, 2005). DNA extraction from soil is challenging for many reasons: the wide diversity and abundance of bacterial species, and the presence of many compounds, such as humic acids, which bind to the DNA and interfere with enzymatic modifications of this macromolecule, are the most critical difficulties to overcome when manipulating this kind of samples. In specific, DNA preparations that are not pure often contain humic acids that impede or strongly inhibit amplification in PCR reactions.

In this work, many tests were made to better comprehend this issue and to find convenient solutions to this problem.

Figure 2.3.1.1 shows how the dilution in sterile MilliQ water of DNA extracted from different soil samples, which are used as templates for PCR, influence amplification efficiency.

DNA was extracted from 5 different soil samples using PowerSoil DNA isolation kit (Mobio, see Material and Methods), and 10-fold serial dilutions of the resulting eluted DNA were made until reaching the dilution of 1:10000. These dilutions, together with a positive and a negative sample, were used as templates for the amplification of the bacterial 16S rRNA gene in PCR reactions. The first observation that could be done looking at figure 2.3.3.1 is that amplification results were not the same for all DNA samples. This result highlights how the differences in soil matrix and composition strongly influence the efficacy of DNA extraction and purification, even if the method used is the same. This could cause ambiguities when comparing soils sampled in very different environments.

In this study case, undiluted DNA gave an intense amplification band only in one case, a very light band in 2 cases, and no amplification in 2 other cases. The dilution 1:100 gave strong amplification in all the cases but one, which is the same DNA sample that instead

gave strong amplification with the undiluted. In general, the brightness of gel bands diminished with sequential dilutions, but not in the case of DNA 3, where DNA dilution 1:100 worked as a worse template for PCR reaction than the dilutions 1:1000 and 1:10000. This last results is difficult to explain, as the results for DNA2. In fact, in these two cases, amplification is impaired at a certain dilution, even if at previous and following dilutions it worked well.

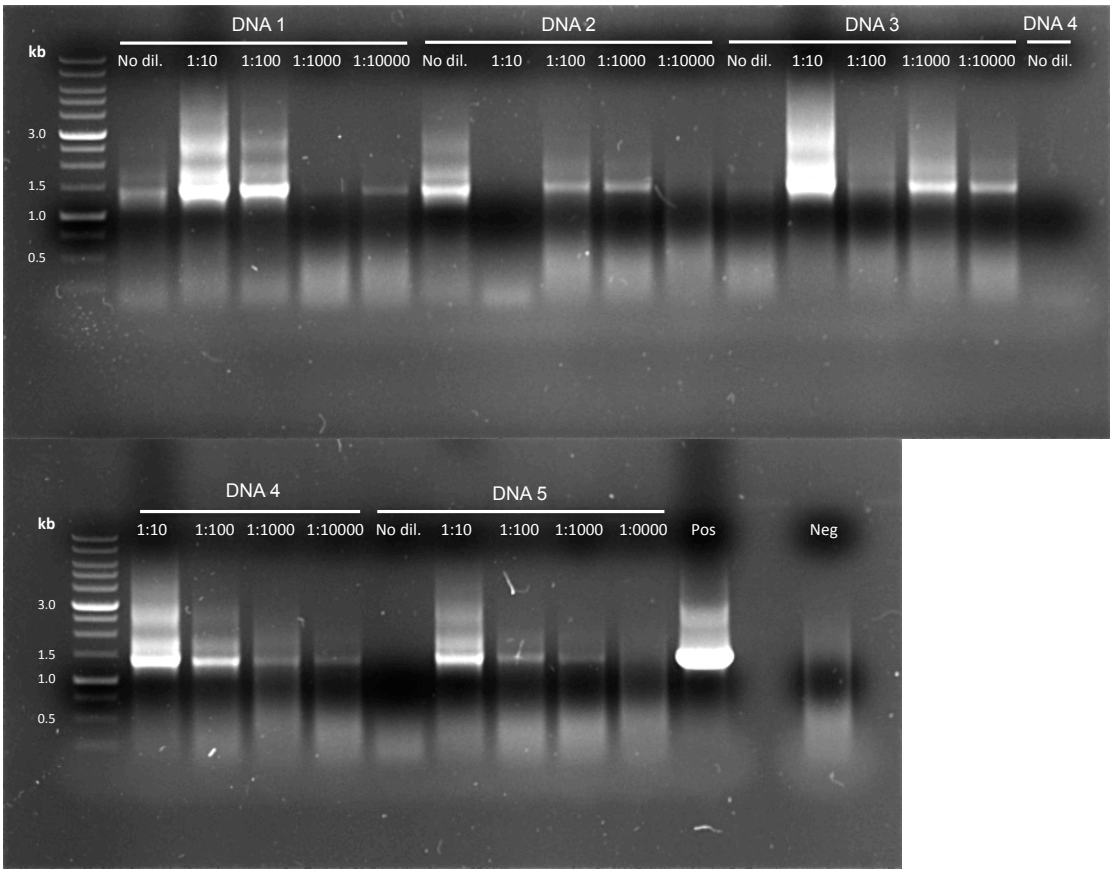

Figure 2.3.1.1

PCR amplification efficiency of 5 soil-DNA extracts: DNA templates were added to the reactions undiluted and at the dilutions of 1:10, 1:100, 1:1000, 1:10000 and the 16s rRNA gene was amplified.

This simple experiment confirms that, after DNA extraction with MoBio kit, it is necessary to perform PCR amplification with at least two dilutions (1:10 and 1:100) of the template soil DNA, as the efficiency of DNA extraction and the purification from humic acids depend on the soil type.

2.3.1.2 Evaluation of two different DNA extraction methods from soil: comparison between automatic DNA extraction and manual DNA extraction with a commercial kit

In this part of the work, two DNA extraction methods were taken into consideration, in order to evaluate their potentialities and their possible disadvantages. In particular, a new automatic DNA extraction method from soil was tested, and the required optimization was performed. The workstation BioSprint 96 and a modified protocol of the BioSprint 96 DNA Blood kit were used to perform automatic DNA extraction (see Material and Methods for the detailed protocol).

Six different soils were chosen to compare BioSprint DNA extraction Protocol and MoBio kit (these soils are the same analysed at paragraph 2.3.3). Three replicates were done for each soil sample with the two considered extraction methods.

The obtained DNA was quantified with Nanodrop and with Picogreen. These two nucleic acid quantification methods are different, as the first one performs a spectrophotometric measurement of the absorbance at 260 nm to detect DNA and at 280 nm and 230 nm to evaluate DNA purity, while with the second method a fluorescent dye (the Picogreen), which binds to double stranded DNA, is added to the DNA preparation and the emitted fluorescence is measured with a Spectrofluorimeter.

These two quantification methods gave different results also on the same DNA samples, with higher values obtained with nanodrop (Table 2.3.1.1, Figure 2.3.1.2). The incongruences between these two methods are caused by the fact that humic acids interfere with nucleic acid spectrophotometric quantification since they exhibit absorbance both at 260 nm and 230 nm (Jackson *et al*., 1997; Rajendhran and Gunasekaran, 2008). For this reason, quantification of DNA extracted from soil can be done with Nanodrop only when the DNA samples are very pure and contaminants such as humic acids have been eliminated.

Looking at figure 2.3.1.2 it could be noticed that samples extracted with MoBio gave similar quantification results with Nanodrop and with Picogreen, while the differences were really evident for soil samples extracted with BioSprint, with significantly higher values obtained with Nanodrop. This results, together with the values obtained for the ratios A_{260}/A_{280} and A_{260}/A_{230} for the BioSprint samples (Table 2.3.1.1), indicate that a relevant quantity of humic acids and contaminants remain in the final product of extraction, and therefore that more washing steps are needed.

	ng/µl Picogreen	ng tot in 250 mg soil Picogreen	ng/µl Nanodrop	ng tot in 250 mg soil Nanodrop	A_{260}/A_{280} Nanodrop	A_{260}/A_{230} Nanodrop
L1	6.4	1281.9	18.2	3640	1.56	1.66
L2	6.2	1240.9	22.2	4440	1.53	1.64
L3	6.3	1269.5	20	4000	1.54	1.34
L4	14.8	1482.8	17.5	1750	1.99	1.61
L5	12.1	1214.4	21	2100	1.96	2.1
L6	11.5	1146.8	16.9	1690	2.02	2.12
F1	2.4	480.7	13.5	2700	1.26	1.72
F2	4.3	863.1	14.9	2980	1.55	1.95
F3	4.0	806.5	15.6	3120	1.49	2.01
F4	7.6	759.9	10.8	1080	2.15	1.99
F5	6.9	692.5	12.5	1250	2.05	1.99
F6	5.7	566.4	11.3	1130	1.91	1.36
LN1	1.7	348.1	2.9	580	1.89	24.89
LN2	2.2	433.3	4.5	900	1.34	1.15
LN3	1.6	316.3	0.7	140	6.59	0.18
LN4	2.9	286.6	4.3	430	2.19	0.98
LN5	3.2	319.0	3.7	370	2.43	1.25
LN6	2.6	263.0	4.7	470	2.08	1.54
W1	8.8	1761.6	19.7	3940	1.55	1.05
W2	8.0	1602.6	21.1	4220	1.54	1.07
W3	8.3	1652.7	24.5	4900	1.53	1.25
W4	13.8	1383.0	19.2	1920	1.92	1.9
W5	24.5	2450.4	25.4	2540	1.96	2.21
W6	19.3	1932.6	21.9	2190	1.93	1.85
R1	6.6	1325.8	29.3	5860	1.46	0.94
R2	5.5	1099.4	33.6	6720	1.43	0.9
R3	5.6	1128.3	34.7	6940	1.45	0.93
R4	6.5	646.5	12.5	1250	1.85	1.22
R5	8.1	805.6	14.8	1480	1.72	1.06
R6	7.7	766.7	14.6	1460	1.76	1.14
D1	13.7	2742.9	34.1	6820	1.62	1.32
D2	9.0	1807.4	24.5	4900	1.53	1.26
D3	9.6	1923.7	24.5	4900	1.52	1.24
D4	10.4	1041.2	14.4	1440	1.99	1.89
D5	16.6	1658.4	22	2200	1.81	1.04
D6	13.3	1333.1	15.5	1550	2.1	2.77

Biosprint
MoBio

Table 2.3.1.1

Picogreen and Nanodrop quantification results of DNA extracted from soil with the two different extraction methods taken into account. In the table, A_{260}/A_{280} and A_{260}/A_{230} ratios automatically calculated with the Nanodrop software are also reported.

The average DNA extraction yield of MoBio kit and Biosprint are shown in figure 2.3.1.3. Also in this case the contrast between the results obtained with the two quantification methods is evident.

In fact, with Nanodrop, Biosprint resulted to have higher DNA yields, while with the fluorescent dye Picogreen the conclusion was the opposite, with higher DNA extraction yield with MoBio kit. In both cases, the difference between final DNA concentration obtained with the two methods was not significant, as revealed by the error bars. The quality of the extracted DNA was also evaluated loading the samples on a 0.7% agarose gel (Figure 2.3.1.4). From the gel, it is clear that the quality of the DNA extracted with MoBio is higher, while with Biosprint the fragmentation of the macromolecules is

strong. This last kind of situation can impair some molecular techniques, but does not give problems when fragmented DNA can be used.

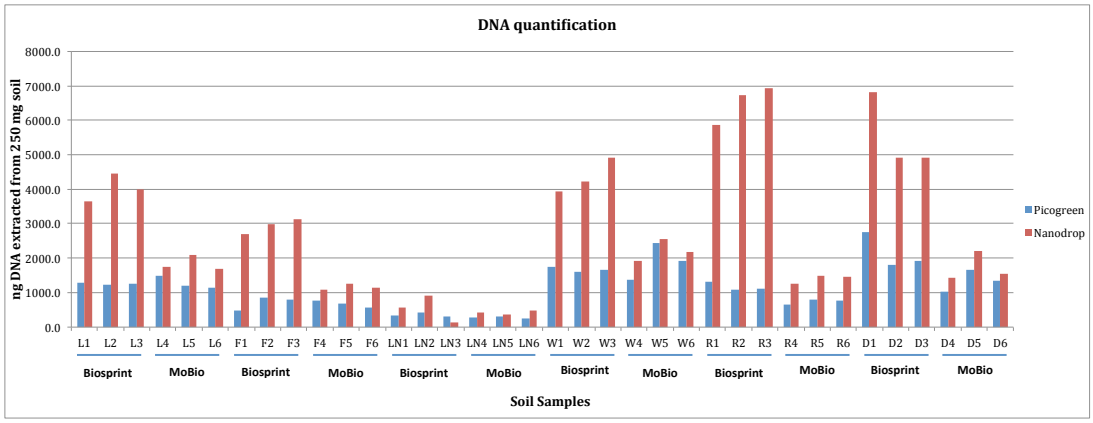

Figure 2.3.1.2

Quantification of DNA extracted from soil with the two different extraction methods. Quantification performed with Picogreen is compared to quantification performed with Nanodrop.

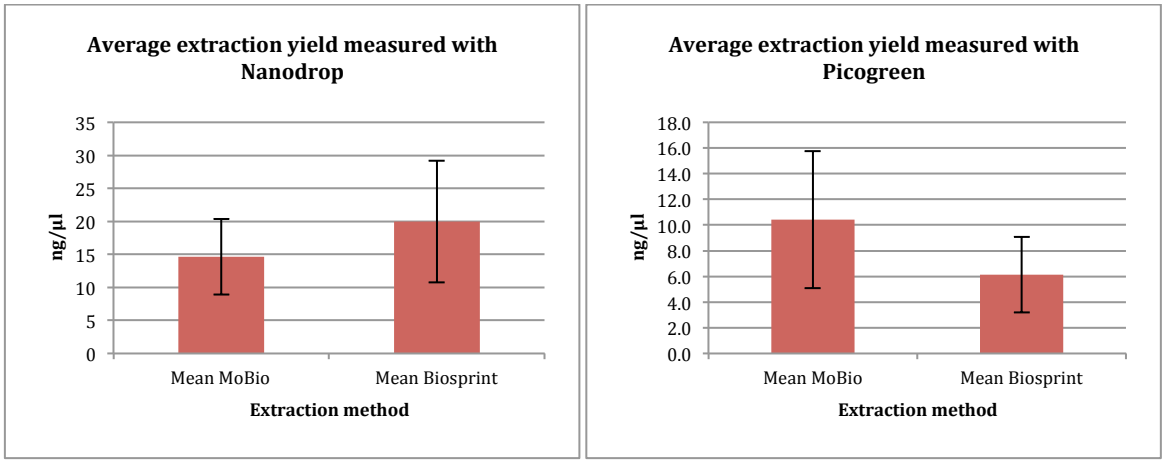

Figure 2.3.1.3

Average DNA extraction yields obtained with the two extraction methods, BioSprint 96 and MoBio, calculated on the values obtained with the two quantification methods Picogreen and Nanodrop.

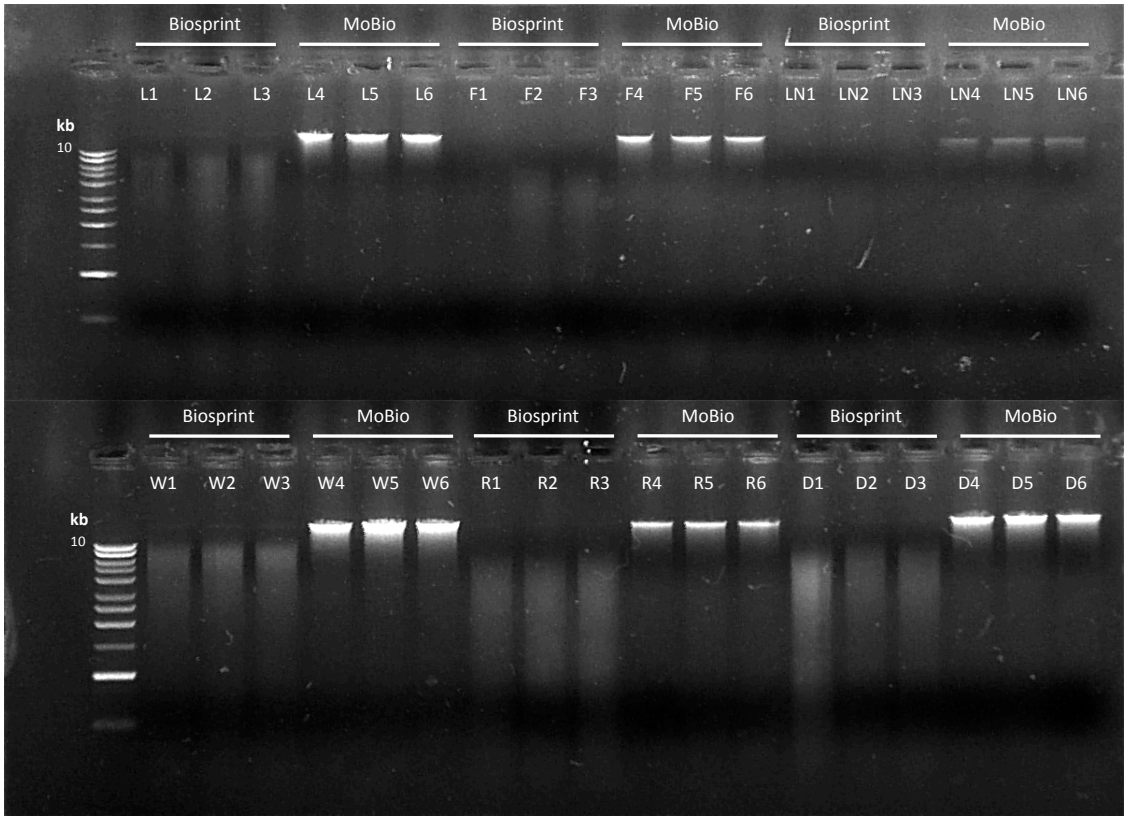

Figure 2.3.1.4

Visualization on 0.7% agarose gel of the DNA samples extracted from soil with the two different extraction methods.

To sum up the observations done in this paragraph, it can be said that the two extraction methods have different potentialities. Automatic DNA extraction with BioSprint could be a fundamental improvement for soil analysis, because it would permit the simultaneous extraction of DNA from 96 samples with limited manipulation and working time. This method could be used for molecular techniques where DNA fragmentation is not a problem, such as PCR amplification of short fragments, ARISA (Automated rRNA Intergenic Spacer Analysis), etc. However, when integer DNA is needed for further analysis, it is convenient to use other extraction methods, such as, for example, the MoBio extraction kit.

2.3.2 Optimization of Quantitative Real Time PCR in 10 µl reactions with Applied Biosystems QuantStudio machine

Part of the Real Time experiments was done with the QuantStudio 12K Flex Real Time PCR System using 384 wells plates (paragraph 2.3.6).
In order to optimize the use of this machine, and to diminish the costs of this kind of experiments, it was decided to lower the reaction volume to 5 µl or 10 µl.
Firstly, different reaction volumes (5 µl, 10 µl, 15 µl, 20 µl with QuantStudio and 25 µl with Applied Biosystems StepOne Real Time PCR System) were tested and their amplification efficiencies were compared. For this and the following tests, the gene bacterial *amoA* was used (see Material and Methods for detailes on the reaction mix).

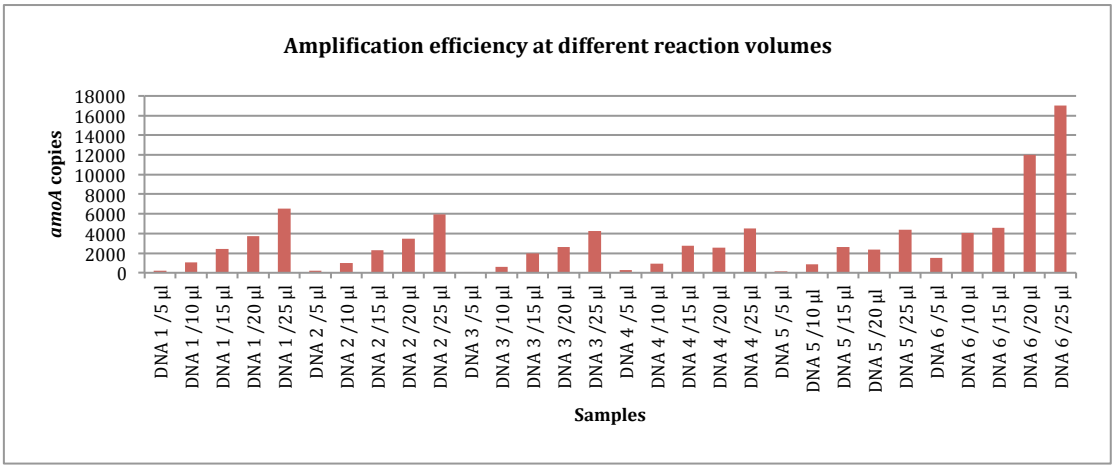

Figure 2.3.2.1
Real Time PCR amplification efficiency with diferent reaction volumes.

Results are shown in figure 2.3.2.1. It is evident that the efficiency of the amplification increased with the reaction volume, with a maximum detection of the gene copies with a volume of 25 µl. This fact was most probably due to a too high concentration of template DNA at lower volumes.
To overcome this problem, serial dilutions of DNA were tested in two fixed reaction volumes of 10 µl and 20 µl. Two soil DNAs, of which the DNA concentration was known, were selected for this test: the first soil DNA, called DNA 1 in figure 2.3.2.2, had a initial concentration of 92.93 ng/µl, while the second, called DNA 2, of 124.06 ng/µl. Starting from a concentration of 40 ng/µl DNA, serial 2-fold dilutions were done to a final quantity of 0.3125 ng DNA per reaction. The resulting copy number of each sample was ponderated to have the number of copies in 10 ng DNA, and the obtained values are reported in the graph in figure 2.3.2.2.

From these results, it was decided to use DNA concentrations between 2.5 ng/µl and 0.625 ng/µl with a volume of 10 µl to perform Real Time PCR experiments with the QuantStudio 12K Flex System.

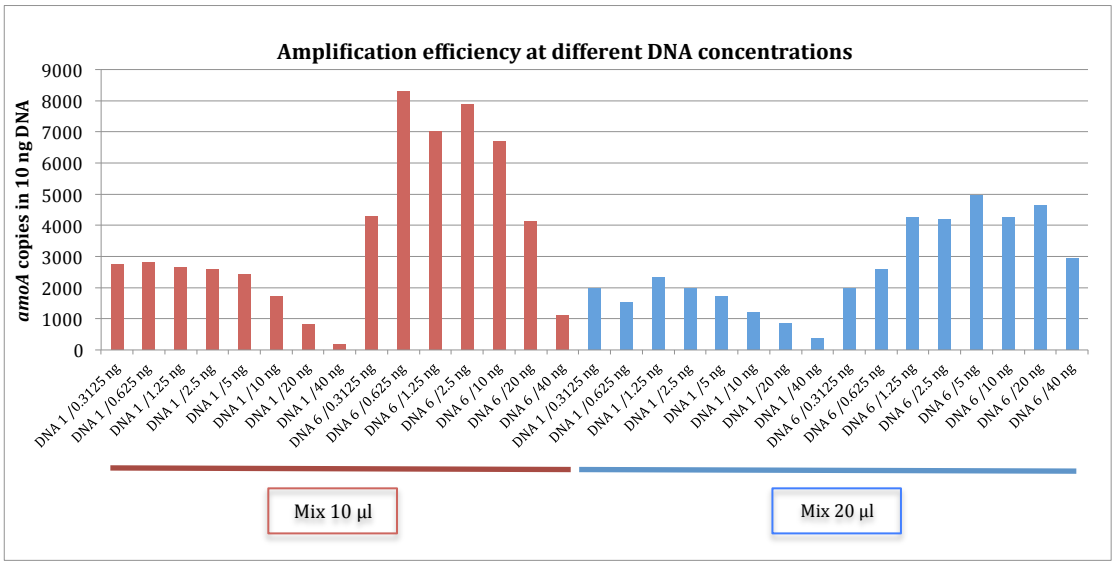

Figure 2.3.2.2

Real Time PCR amplification efficiency at different template DNA concentrations, tested with two different reaction mixes (of 10 and 20 µl). To compare the results in the different reactions, the gene copy number in 10 ng of DNA was calculated.

Thanks to this optimization it was possible to perform Real Time PCR experiments of 384 reactions, and in this way to significantly decrease time and costs for this kind of analysis.

2.3.3 Analysis of N-cycle genes in soils from different environments

Figure 2.3.3.1
Pictures of the 6 environments considered in this study. The lawn, the farm, the ditch, the rain garden and the woodlot were all in the same area, in Legnaro (Padova), Italy.

Nitrogen cycle genes were analysed in six soils sampled in neighbouring areas but subject to very different environmental conditions, in order to search for peculiarities of each environment and differences between mostly natural and artificially modified soils. Sampling was made in a lawn, in an agricultural field, in a ditch, in a small woodlot and in a rain-garden, which is a special flowerbed irrigated only with rainwater. As a control, a nutrient-limited soil (exhausted by unfertilized wheat cropping) was also taken into account. (Figure 2.3.3.1).
Six functional genes of the soil nitrogen cycle were tested: bacterial and archaeal *amoA* (ammonia monooxygenase A) for nitrification activity, *nirK*, *nirS* (two forms of nitrite reductase) and *nosZ* (nitrous oxide reductase) for denitrification activity, and *nifH* (nitrogenase) for nitrogen fixation. Both the nitrite reductase gene forms were considered for this study, as, even if they are functionally equivalent, they are present alternatively in different denitrifying organisms. The gene *nosZ* was also tested, as it represents the last step of denitrification, and therefore it can give a good indication of the completion of the process.
For this analysis, DNA was extracted from soils using the commercial kit MoBio. Using this extraction method, the average final DNA concentration was of about 10 ng/µl, and some DNA samples, such as the ones extracted from the low-nutrient soil, presented a concentration of about 3 ng/µl. Conceivably, the extraction efficiency will not be 100%, and therefore the gene copy numbers calculated per gram soil can present a certain

degree of underestimation. However as the chosen soils were of similar pedological nature, the technical efficiency loss should be assumed comparable and the relative proportions of the same gene across different soils should remain reliable.

2.3.3.1 Presence and abundance of nitrogen cycle functional genes in soils subject to different environmental conditions

All the genes analysed were detected in the 6 environments, with gene copy numbers ranging from 10^3 to $2x10^5$ per gram soil (Figure 2.3.3.3).
Relevant differences were found comparing natural soils and soils which were exposed to artificial or peculiar conditions.

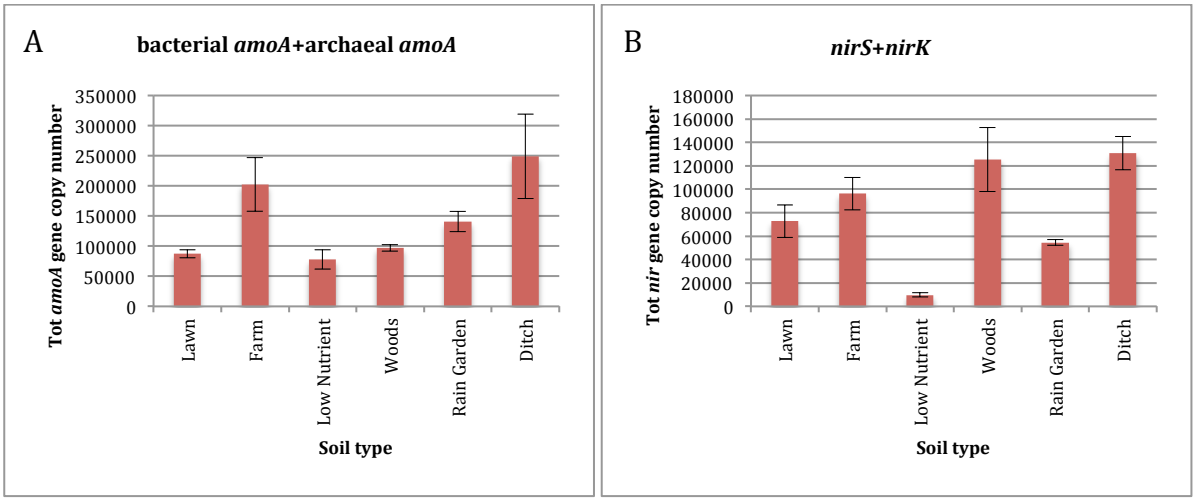

Figure 2.3.3.2
Presence of nitrification and denitrification functional genes in the considered soils. The genes corresponding to the same process were summed to obtain a more general view. The error bars represent the standard deviation.

Nitrifying microorganisms were present at higher levels in farm soil and in ditch soil (Figure 2.3.3.2 A). The significance of these differences was confirmed by the Anova test, reported in the table 2.3.3.1. In these two soils, archaea predominated over bacteria. Indeed, archaeal nitrifiers were about 3 times more abundant than bacterial nitrifiers in farm soil, and about 7 times in ditch soil (Figure 2.3.3.4 A, B).
Nitrification was present at progressively lower levels in the rain-garden, in the lawn, in the woods and in the low-nutrient soil. In this last sample, archaeal *amoA* gene was detected at a level about 6 times higher than the bacterial form. Nitrification was therefore mainly performed by archaea in farm soil, low-nutrient soil and ditch soil (Figure 2.3.3.4 B; Table 2.3.3.3 showing the significance of these differences).

			Sum of	df	Mean	F	Sig.
bacterial *amoA*+archaeal *amoA* * Soil type	Between Groups	(Combined)	7.282E+10	5	1.456E+10	7.762	.002
	Within Groups		2.251E+10	12	1.876E+09		
	Total		9.533E+10	17			
nirS+*nirK* * Soil type	Between Groups	(Combined)	3.159E+10	5	6.318E+09	18.778	.000
	Within Groups		4.037E+09	12	3.365E+08		
	Total		3.563E+10	17			

Table 2.3.3.1
The table shows the results obtained performing the test Anova on the total nitrification genes and on the total denitrification genes in respect to the analysed soil types.

Denitrification genes were found at high levels in all soil samples, with the exception of the low-nutrient soil, where they were present at a very low number (Figure 2.3.3.2 B and Figure 2.3.3.3 C, D, E). In this soil, the almost total absence of organic matter may explain the inability of these heterotrophic bacteria to grow.
The two forms of the gene encoding for nitrite reductase, *nirS* and *nirK*, were detected at similar levels in the lawn, in the woods and in the rain-garden; *nirS* predominated in the farm soil and in the low-nutrient soil (it was about 2 times more abundant than *nirK*), while the number of *nirK* copies was about 2.5 times higher than *nirS* in the ditch soil (Figure 2.3.3.4 C, significance of the differences shown in table 2.3.3.3).
The gene *nosZ*, representing the last step of denitrification, was present at similar degrees in all the soils, except the low-nutrient (Figure 2.3.3.3 E, Table 2.3.3.2).

			Sum of Squares	df	Mean Square	F	Sig.
amoA * Soil type	Between Groups	(Combined)	2.909E+10	5	5.818E+09	56.702	6.235E-08
	Within Groups		1.231E+09	12	1.026E+08		
	Total		3.032E+10	17			
archaeal *amoA* * Soil type	Between Groups	(Combined)	1.043E+11	5	2.086E+10	11.665	.000
	Within Groups		2.146E+10	12	1.788E+09		
	Total		1.258E+11	17			
nifH * Soil type	Between Groups	(Combined)	1.853E+10	5	3.707E+09	7.077	.003
	Within Groups		6.286E+09	12	5.238E+08		
	Total		2.482E+10	17			
nirS * Soil type	Between Groups	(Combined)	7.354E+09	5	1.471E+09	12.872	.000
	Within Groups		1.371E+09	12	1.143E+08		
	Total		8.725E+09	17			
nosZ * Soil type	Between Groups	(Combined)	1.843E+10	5	3.686E+09	14.028	.000
	Within Groups		3.153E+09	12	2.628E+08		
	Total		2.158E+10	17			
nirK * Soil type	Between Groups	(Combined)	1.489E+10	5	2.978E+09	20.653	.000
	Within Groups		1.730E+09	12	1.442E+08		
	Total		1.662E+10	17			

Table 2.3.3.2
The table shows the results obtained performing the test Anova on the considered nitrogen cycle genes in respect to the analysed soil types.

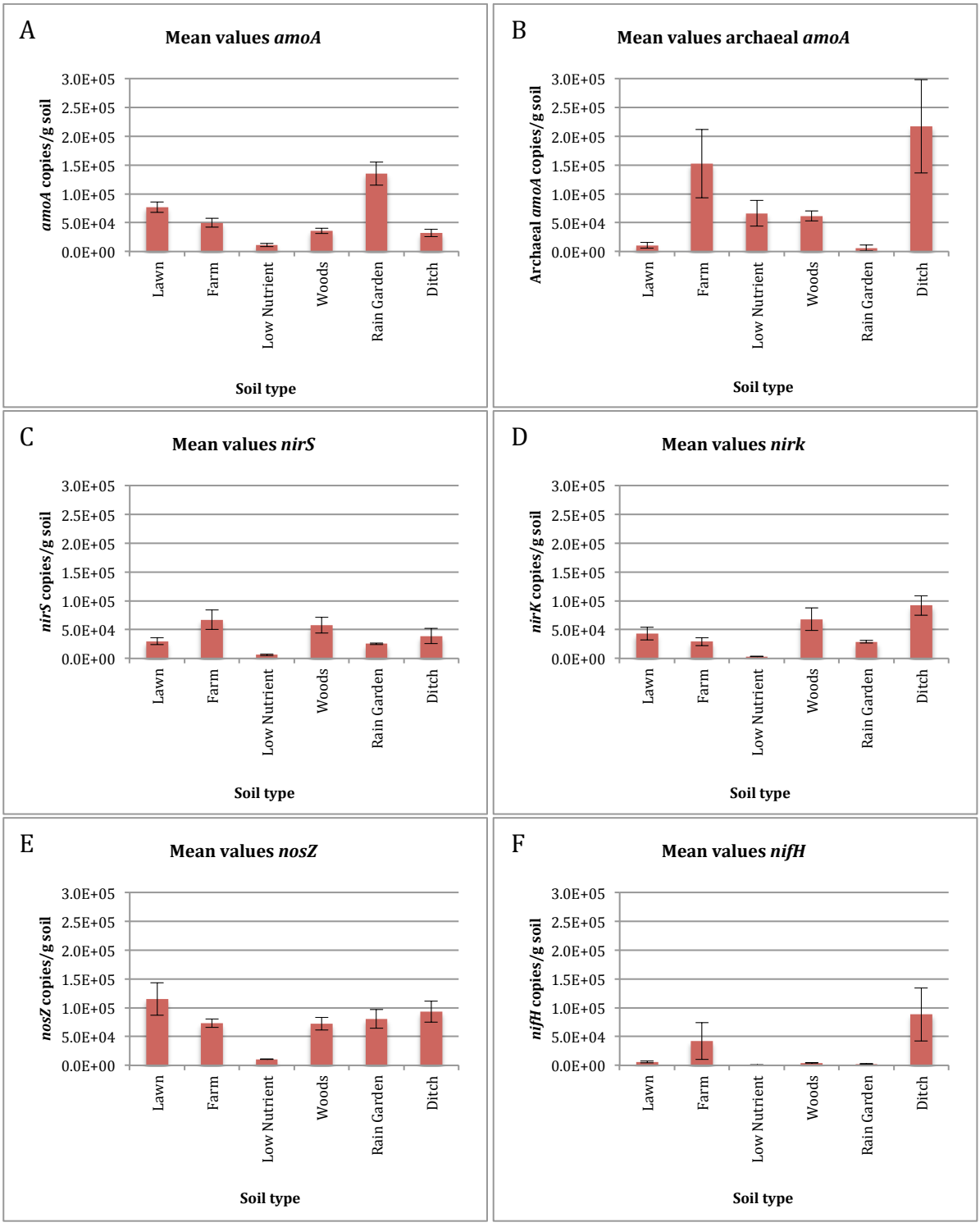

Figure 2.3.3.3

Presence of the six functional genes of the nitrogen cycle bacterial *amoA*, archaeal *amoA*, *nirS, nirK, nosZ* and *nifH*, in the considered soils. The error bars represent the standard deviation.

Comparing the copy number of the three considered denitrification genes (Figure 2.3.3.4 D, E, F), it was possible to infer that this process was near the equilibrium in the farm soil and in the low-nutrient soil, this last having had a very low total number of denitrifying organisms, as stated before. The reduction of nitrites to nitric oxide (NO) could have happened at slightly higher degrees in the woodlot and in the ditch, as the *nosZ*/*nirK*+*nirS* ratio was around 0.6 and 0.7 respectively.

The functional gene of nitrogen fixation, *nifH*, was detected in the farm soil and in the ditch soil at higher levels (around $5x10^4$ and $9\ x10^4$ copies per gram soil respectively)(Figure 2.3.3.3 F). This result was expected for the soil sampled in the farm, where leguminous plants are often cropped to enrich the soil. Rhizobia, bacteria able to establish symbiotic relationships with leguminous plants, are indeed able to fix nitrogen in root nodules. The even higher number of nitrogen fixing microorganisms observed in the ditch soil, on the other hand, could not be explained by this kind of agricultural management. However, *nifH* gene is present in a number of other bacterial species, such as *Rhodospirillum rubrum*, *Nostoc* sp., *Methanobacterium* sp., *Rhodobacter capsulatus*, which are microorganisms often isolated from soil. Free-living nitrogen fixers do not rely on the reducing conditions available in symbiotic plant nodules and must therefore take advantage of other temporarily anoxic interfaces. In this respect the fluctuating water level of the ditch could offer this low-redox potential possibility.

			Sum of Squares	df	Mean Square	F	Sig.
bacterial *amoA*/archaeal *amoA* * Soil type	Between Groups	(Combined)	3208.464	5	641.693	5.87	.006
	Within Groups		1311.432	12	109.286		
	Total		4519.896	17			
archaeal *amoA*/bacterial *amoA* * Soil type	Between Groups	(Combined)	137.369	5	27.474	7.34	.002
	Within Groups		44.906	12	3.742		
	Total		182.276	17			
nirK/*nirS* * Soil type	Between Groups	(Combined)	9.072	5	1.814	6.86	.003
	Within Groups		3.170	12	.264		
	Total		12.242	17			
nosZ/*nirK* * Soil type	Between Groups	(Combined)	12.489	5	2.498	14.6	.000
	Within Groups		2.041	12	.170		
	Total		14.530	17			
nosZ/*nirS* * Soil type	Between Groups	(Combined)	18.025	5	3.605	14.6	.000
	Within Groups		2.958	12	.247		
	Total		20.983	17			
nosZ/(*nirK*+*nirS*) * Soil type	Between Groups	(Combined)	2.653	5	.531	12.8	.000
	Within Groups		.497	12	.041		
	Total		3.151	17			

Table 2.3.3.3

The table shows the results obtained performing the test Anova on the ratios between different functional genes of nitrification and denitrification, in respect to the analysed soil types.

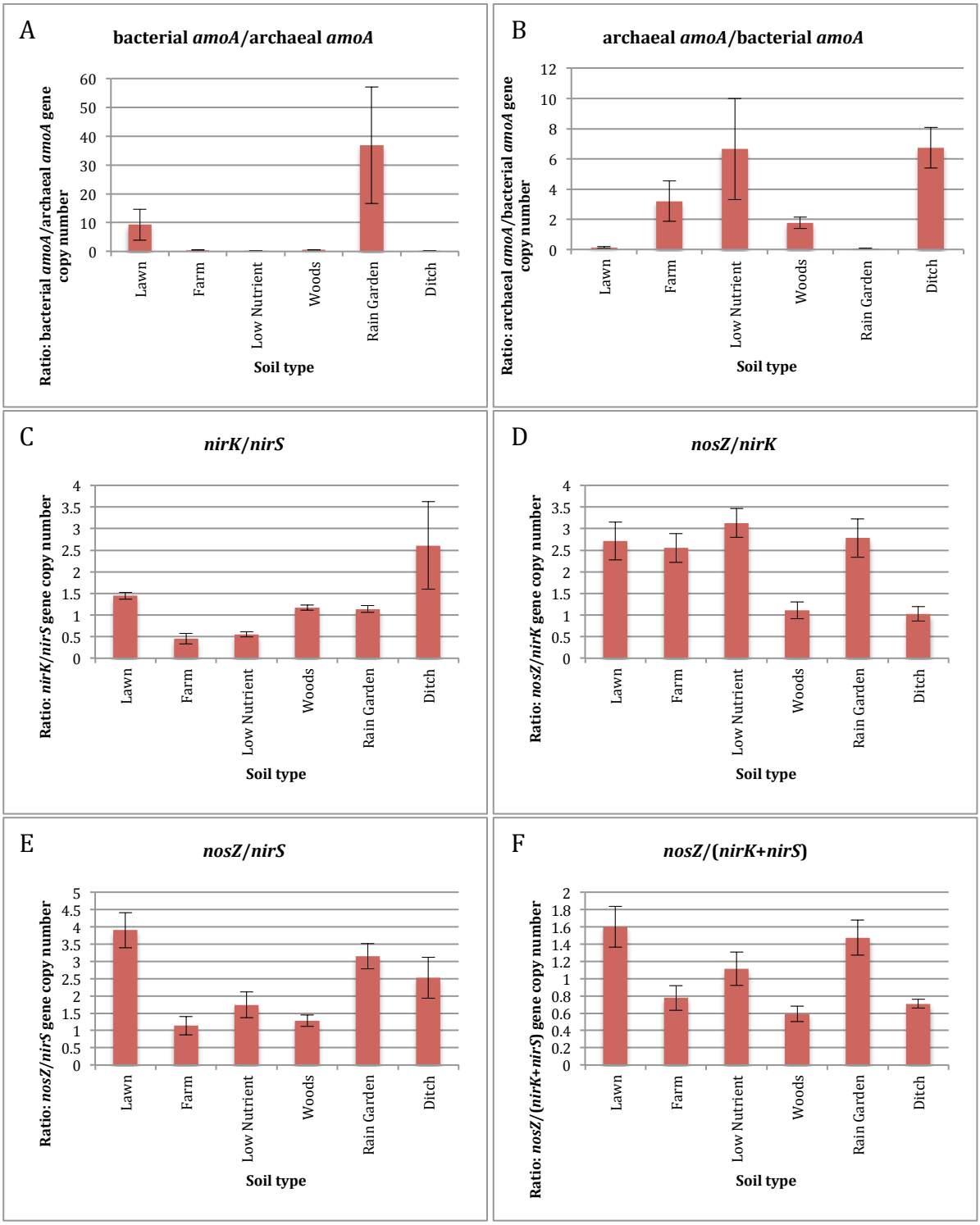

Figure 2.3.3.4

Ratios between functional genes involved in the same process of the nitrogen cycle. The ratio was made between genes performing the same step of the process, such as archaeal and bacterial *amoA* forms (A and B), and the two forms of nitrite reductase (C), and between genes performing different steps of the same process, such as *nirK* and *nirS* in respect to *nosZ* (D, E and F). The error bars represent the standard deviation.

2.3.3.2 Evaluation of the equilibrium of the nitrogen cycle in soils from different environments

Figure 2.3.3.5 gives an indication of the equilibrium of the nitrogen cycle, in general. In the lawn soil and in the woods soil, the ratio between nitrification and denitrification genes was near 1 (both considering the *nirK*/*nirS* and the *nosZ* steps), indicating that the two processes were near the equilibrium.

			Sum of Squares	df	Mean Square	F	Sig.
(bacterial *amoA*+archaeal *amoA*)/ (*nirK*+*nirS*) * Soil type	Between Groups	(Combined)	121.268	5	24.254	8.49	.001
	Within Groups		34.252	12	2.854		
	Total		155.521	17			
(bacterial *amoA*+archaeal *amoA*)/ *nosZ* * Soil type	Between Groups	(Combined)	84.545	5	16.909	21.4	.000
	Within Groups		9.441	12	.787		
	Total		93.986	17			

Table 2.3.3.4
The table shows the results obtained performing the test Anova on the ratios between the sum of functional genes involved in nitrification and those involved in denitrification, in respect to the analysed soil types.

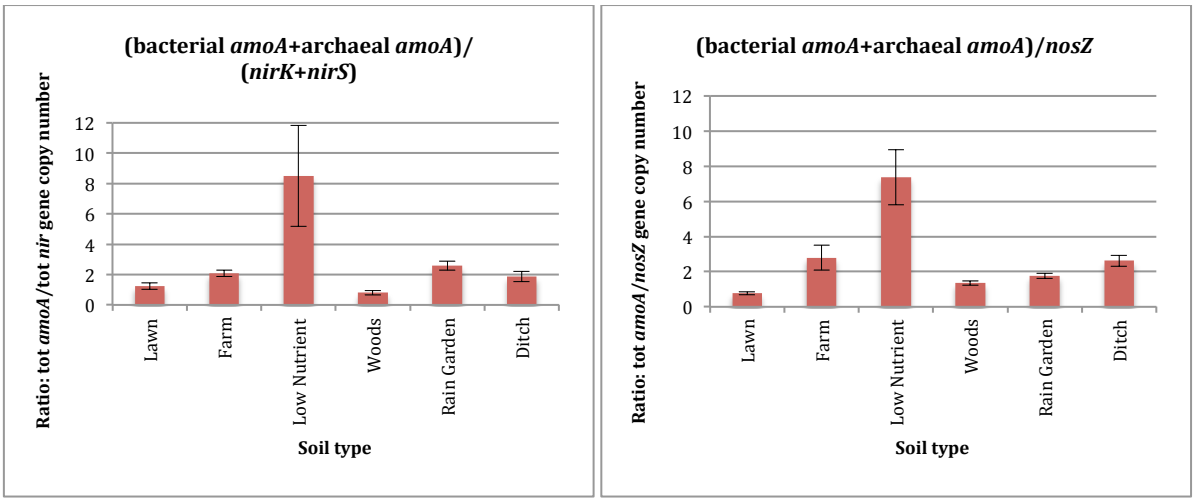

Figure 2.3.3.5
Ratios between functional genes involved in nitrification process and genes involved in denitrification process. These graphs give an indication of the equilibrium of the nitrogen cycle in the 6 soils. The error bars represent the standard deviation.

This is not the case for the other 4 soils, where nitrification genes exceeded denitrification genes, in particular in the low-nutrient soil, where denitrification was almost absent. Anova table 2.3.3.4 shows that the different equilibrium conditions in the 6 soils analysed were significantly different.

Summing up these observation, it can be said that the woodlot soil and the lawn soil, which were subject to the most natural conditions, among the six soils analysed, presented a more equilibrated situation when compared to the other soils. Here, in effect, nitrification-denitrification ratio is near the equilibrium.
Moreover, nitrification bacteria are present at a higher number in the lawn soil, in respect with nitrifying archaea.

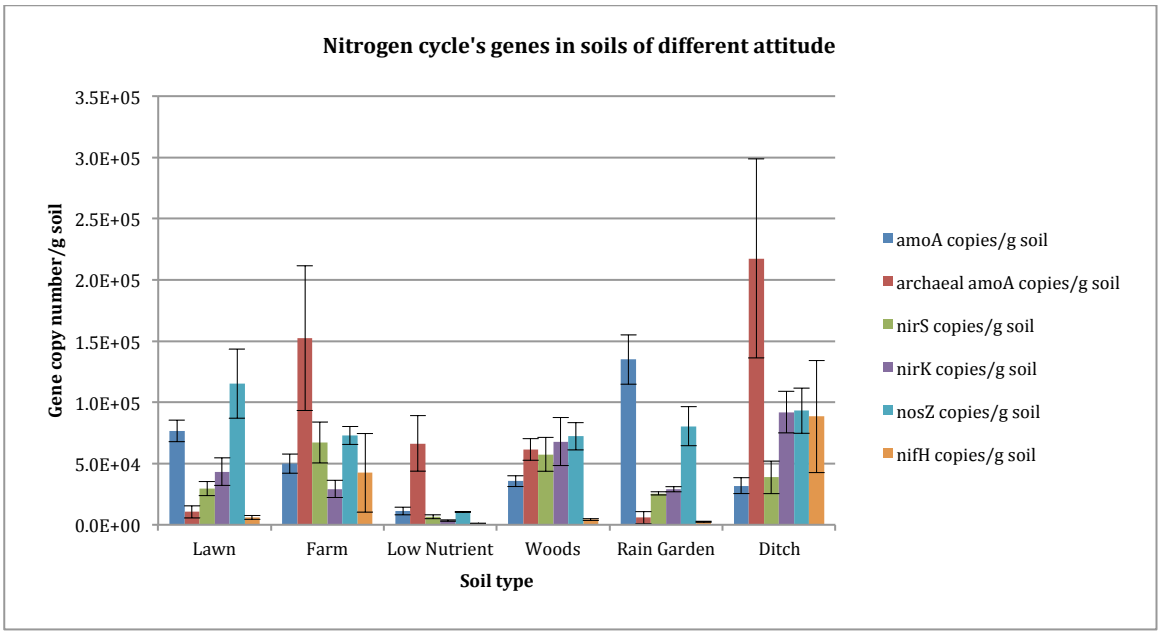

Figure 2.3.3.6
Presence of the six functional genes of the nitrogen cycle bacterial *amoA*, archaeal *amoA*, *nirS, nirK, nosZ* and *nifH*, in the considered soils. The error bars represent the standard deviation.

The low nutrient soil is an exhausted and impoverished soil. At these conditions, heterotrophic bacteria such as denitrifiers are not favoured. For this reason, nitrification functional genes far exceeded denitrification genes in number. This situation could be seen at a lower degree also for the farm soil, which has been cultivated for years and could therefore also present some nutrient depletion.
Moreover, in these two soils nitrification was performed mainly by archaea. This result can be explained by the better capacity of archaea to survive in adverse conditions.
The rain garden soil represents an intermediate case. Indeed, the soil used to build the rain garden was a mixture of the lawn soil and sand. This fact explains the similarities in the results obtained for this soil and for lawn soil (Figure 2.3.3.6). In both cases, bacterial *amoA* exceeded archaeal *amoA*, and nitrogen fixation gene *nifH* was present at a very low number.
The ditch soil was subject to peculiar and unstable conditions, such as fluctuating water table and agricultural nutrient runoff. In this environment, archaeal nitrifiers' number

was very high, and the disequilibrium between nitrification and denitrification was evident. It is noteworthy that nitrogen fixation organisms were also present in this soil as commented above.

2.3.3.3 Analysis of the correlation among nitrogen cycle genes in soils from different environments

Significant correlations among the 6 considered nitrogen cycle genes were searched, in order to determine whether in these conditions some microorganisms tended to grow in parallel, or if, on the other hand, the growth of some bacterial groups caused the decrease of some other. In the table 2.3.3.5, the results obtained calculating the Pearson's correlation coefficient for all these genes are shown.
Significant correlation (p-value<0.01), were found between the genes *nifH* and archaeal *amoA* and between the genes *nosZ* and *nirK*. The first strong correlation can be explained by the fact that nitrogen fixing bacteria were detected at high number in the farm soil and in the ditch soil, which are the same environments where also archaeal nitrifiers were present at a high number. It can be also recalled that the product of nitrogen fixation (ammonia) is also the substrate for nitrification.
The correlation between *nosZ* and *nirK* could be interpreted as a consequence of the fact that both genes belong to the same process.
Other positive correlations, significant at the 0.05 level, were found: *nifH* with *nirK*, bacterial *amoA* with *nosZ* and archaeal *amoA* with *nirK*. The correlation between genes involved in nitrification with genes involved in denitrification could be consistent with the fact that these two processes, being part of the same biogeochemical cycle and involving the same chemical species, are strictly connected. The end-product of nitrification (nitrate) is also the starting substrate of denitrification.
This kind of correlation analysis was performed previously in the work by Paranychianakis *et al*. (2013), which considered nitrifying and denitrifying microorganisms in relation to the presence of municipal solid waste compost. In those conditions, a strong positive correlation was detected between archaeal and bacterial nitrifiers, differently from the results obtained for the peculiar environments taken into account in this paragraph, where a negative correlation was detected. Moreover, Paranychianakis observed a positive correlation of denitrifying microorganisms with both groups of nitrifiers. This result is, instead, in line with our observations, and strengthens the hypothesis that the two major processes of the N-cycle, nitrification and denitrification, are linked and probably occur in the same environmental micro-niche.

Correlations

		nifH	*amoA*	Archaeal *amoA*	*nirS*	*nirK*	*nosZ*
nifH	Pearson Correlation	1	-0.274	0.856**	0.427	0.476*	0.255
			0.271	0	0.077	0.046	0.306
	N	18	18	18	18	18	18
amoA	Pearson Correlation	-0.274	1	-0.492*	-0.07	-0.114	0.496*
		0.271		0.038	0.783	0.653	0.037
	N	18	18	18	18	18	18
Archaeal *amoA*	Pearson Correlation	0.856**	-0.492*	1	0.453	0.475*	0.062
		0	0.038		0.059	0.046	0.808
	N	18	18	18	18	18	18
nirS	Pearson Correlation	0.427	-0.07	0.453	1	0.427	0.389
		0.077	0.783	0.059		0.077	0.111
	N	18	18	18	18	18	18
nirK	Pearson Correlation	0.476*	-0.114	0.475*	0.427	1	0.593**
		0.046	0.653	0.046	0.077		0.01
	N	18	18	18	18	18	18
nosZ	Pearson Correlation	0.255	0.496*	0.062	0.389	0.593**	1
		0.306	0.037	0.808	0.111	0.01	
	N	18	18	18	18	18	18

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 2.3.3.5

Pearson's correlation (2-tailed) among the considered genes.

2.3.4 Analysis of N-cycle genes in soils subject to different water levels equipped with lysimeters

Nitrogen cycle was studied through the detection of its functional genes also in soils coming from a lysimeter-trial. In this test, the crop plant *Zea mays* was grown in the presence of two fertilization conditions (low fertilization, with 170 kg/ha organic N + 80 kg/ha urea N, and high fertilization, with 250 kg/ha organic N + 118 kg/ha urea N) and with water table at three different depths (120 cm, 60 cm and no water table). Each condition was replicated twice, and soil sampling was done at three depths (20 cm, 60 cm and 120 cm) three times in a period of about one year (August 2011, May 2012 and September 2012).
The study of nitrogen cycle in the presence of water table is usually focused on denitrification occurring in groundwater. The analysis of nitrification, denitrification and nitrogen fixation genes in soils sampled at different depths, in the presence of a water table, is an innovative, unprecedented type of set up.
In this case, instead of calculating the copy number of each functional gene, the quantification was expressed using the built-in function of the Real Time analysis software which yields the parameter referred to as Quantity, which was used to compare results obtained for different soil samples for a certain gene. For this reason, the 5 genes considered, archaeal and bacterial *amoA*, *nirK*, *nosZ* and *nifH*, had to be considered separately for the analysis. It has to be stated that standard deviations obtained were in the majority of the cases rather high, as the replicates were only two for each condition and because soil bacterial communities often change considerably even in the same kind of soil and in the same conditions. In any case, the mean values reported in the graphs in this chapter give a good indication of the major changes that occurred in soils subject to different conditions and permit to notice some trends in the behaviour of the most important players in the nitrogen cycle.

2.3.4.1 Presence and abundance of archaeal and bacterial nitrifying microorganisms in soils subject to different water levels and to different fertilization managements, sampled at different depths during three seasons

Results obtained for the archaeal *amoA* gene are shown in the graph in figure 2.3.4.1. From a first observation of the results, it can be noticed that there was a periodicity in the growth of archaeal nitrifiers, as they grew better in May and in September, which are temperate months, than in August, where temperatures are often very high. Moreover, archaeal nitrifiers showed higher growth rates in the presence of stronger organic fertilization especially in the months of May and September. This result was not

expected, as nitrifiers are autotrophs and therefore they do not need organic matter to survive and multiply.
With high fertilization, the number of archaeal *amoA* gene diminished when the water table was at 60 cm, while this behaviour was not as pronounced in the case of lower fertilization. Another unexpected result was the high number of archaeal nitrifiers in the presence of a water table of 120 cm and with the sampling done at the same depth.

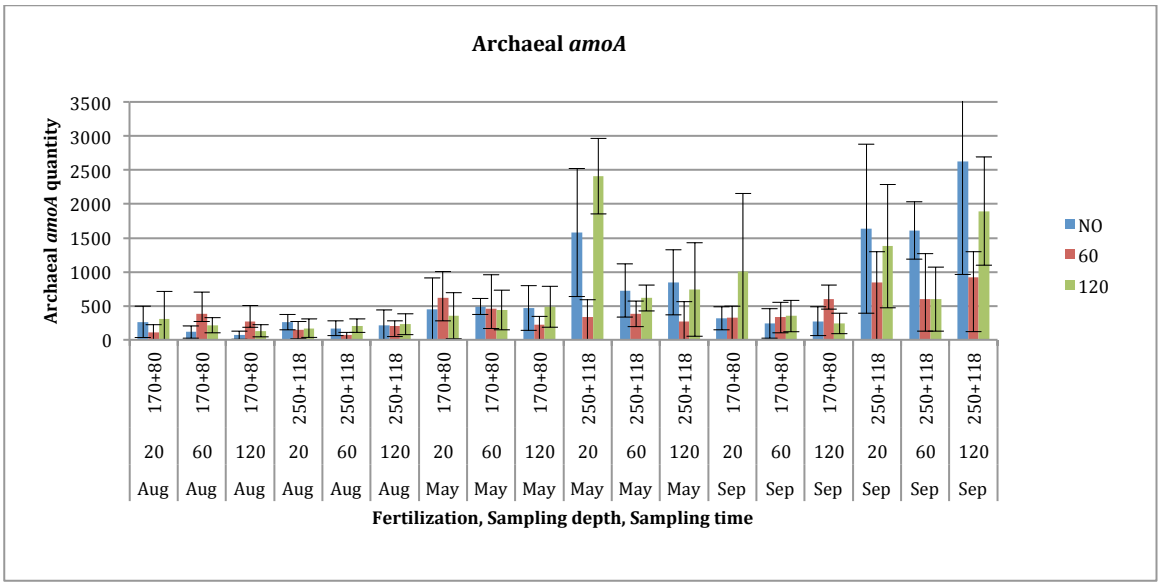

Figure 2.3.4.1
Presence of archaeal *amoA* gene in soils subject to different water levels. The blue columns represent the soils where no water table was present, the red columns the soils with a water table at 60 cm depth and the green columns the soils with water table at 120 cm depth. The error bars show the standard deviation between the two replicates of each condition.

Indeed, nitrification is known to happen in oxidizing conditions and not in the proximity of water. On the other hand, the high number of these organisms in soil exposed to the same conditions, but sampled at 20 cm depth, is not so surprising, as water normally never reached that level.

Results obtained for bacterial *amoA* gene showed some interesting differences when compared with those observed for archaeal gene (Figure 2.3.4.2). Here, the influence of the season is less evident, and bacterial nitrifiers were present on average at a higher number in August than in May. Nevertheless, highest values were detected in September, as with archaea.
The positive effect caused by the presence of high levels of fertilization on the growth of these microorganisms could be mainly observed in the months of May and September, while in August bacterial nitrifiers grew similarly in the two fertilization conditions. The impact of fertilization practices on bacterial nitrifiers' growth was also unexpected, as

they are autotrophic organisms and should therefore not be influenced by high quantities of organic matter (organic fertilization).

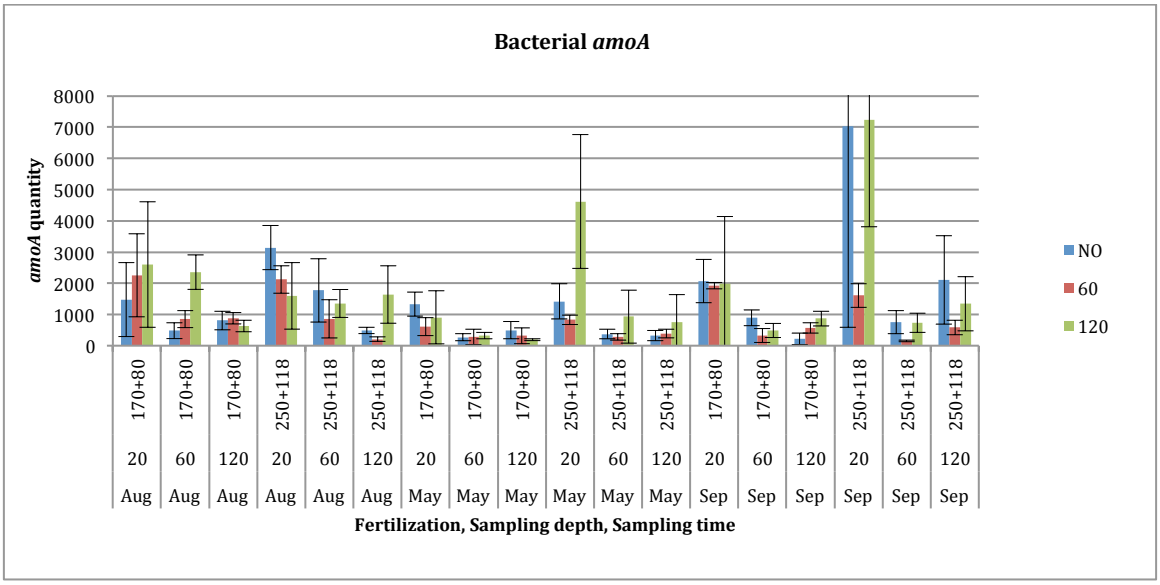

Figure 2.3.4.2

Presence of bacterial *amoA* gene in soils subject to different water levels. The blue columns represent the soils where no water table was present, the red columns the soils with a water table at 60 cm depth and the green columns the soils with a water table at 120 cm depth. The error bars show the standard deviation between the two replicates of each condition.

It could be hypothesized, on the other hand, that this effect was mainly due to the addition of urea, which is a fast source of ammonia that can be used by ammonia oxidizers for their metabolism and may cause their increase in number.

Also in this case, as with archaea, the presence of a water table at 60 cm impaired the increment of the number of *amoA* gene copies in most of the cases, while lysimeters where no water table was present and lysimeters with water tables at 120 cm exhibited high number of *amoA* gene copies.

The highest values obtained for *amoA* gene were at sampling depth of 20 cm, with a water table at 120 cm depth in September and in May, and with no water table in September.

2.3.4.2 Presence and abundance of denitrifying microorganisms in soils subject to different water levels and to different fertilization managements, sampled at different depths during three seasons

In figure 2.3.4.3, Real Time PCR results obtained for the gene *nirK*, which is coding for the enzyme nitrite reductase, involved in the second step of denitrification, are shown. The gene *nirS*, which encodes for a different form of nitrite reductase, was not considered in this study.

For *nirK* gene, the seasonality was not so pronounced, with the exception of the higher levels found in September. In August and in May the gene copy numbers detected were similar, with slightly higher values in August, as for bacterial nitrifiers.

Denitrifiers, which are heterotrophic microorganisms, survive and multiply better in the presence of organic matter, and this fact was confirmed by the results observed in this experiment, where *nirK* gene was present at higher level in the presence of high levels of organic matter in the form of manure.

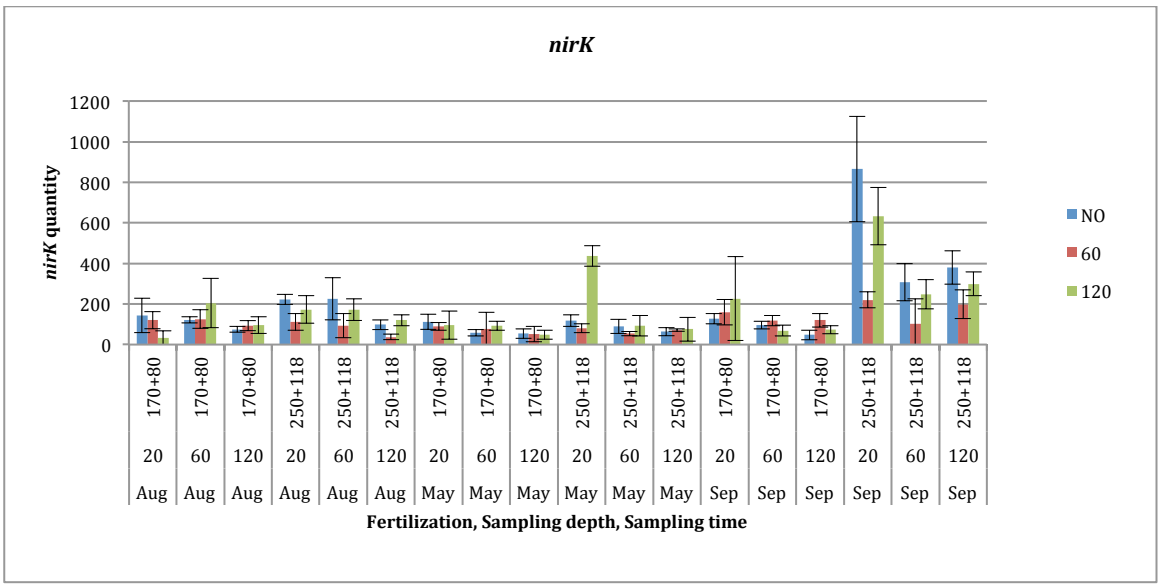

Figure 2.3.4.3

Presence of *nirK* gene in soils subject to different water levels. The blue columns represent the soils where no water table was present, the red columns the soils with a water table at 60 cm depth and the green columns the soils with a water table at 120 cm depth. The error bars show the standard deviation between the two replicates of each condition.

Denitrifiers are known to perform their metabolism in anoxic conditions, for example in deep substrata of soil or in areas where stagnant water is present. For this reason, it was expected to find higher *nirK* levels in the vicinity of water where lysimeters were present. On the contrary, higher values were found in lysimeters were the water table was absent, or in soils sampled at 20 cm where the water table was present. An

interpretation to this result could be found when comparing these results to those obtained for nitrifiers. In fact, it could be noticed that soil samples where nitrifying organisms were detected at high levels are the same where also denitrifying organisms were higher. The better survival of denitrifying organisms in the presence of nitrifiers could be due to the fact that the last supply the first with nitrites and nitrates, which are then used as electron acceptor for anaerobic respiration in their metabolism.

The gene *nosZ* represents the last step of denitrification, the reduction of nitrous oxide to elemental nitrogen gas, and results obtained for this gene (Figure 2.3.4.4) have therefore to be connected with those observed for the gene *nirK*. Indeed, the trends of the growth of denitrifying organisms representing the two different steps of the process were similar, with an increase of the growth in the presence of high organic fertilization also for *nosZ*, mostly evident in September, and a similar periodicity during the year.

Also this step of denitrification strangely seemed to be favoured in the absence of water table at 20 cm depth, and not in the proximity of water or at profound depths, and also in this case it could be supposed that the presence of microorganisms which provide substrates for their metabolism represented a favourable situation for the survival and increase of these denitrifying microorganisms.

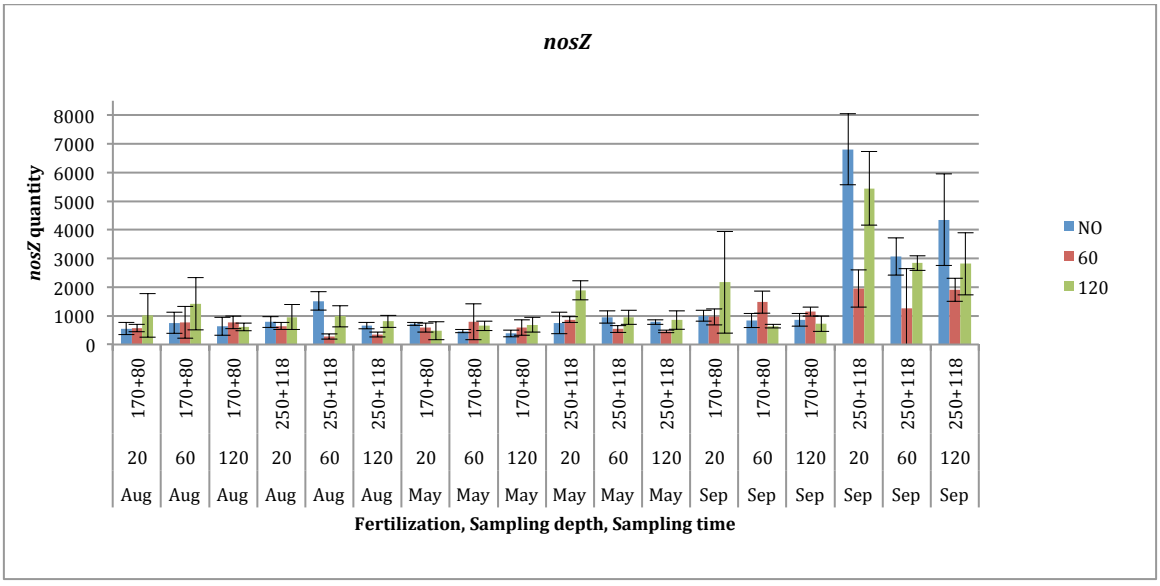

Figure 2.3.4.4

Presence of the *nosZ* gene in soils subject to different water levels. The blue columns represent the soils where no water table was present, the red columns the soils with a water table at 60 cm depth and the green columns the soils with a water table at 120 cm depth. The error bars show the standard deviation between the two replicates of each condition.

2.3.4.3 Presence and abundance of nitrogen-fixing microorganisms in soils subject to different water levels and to different fertilization managements, sampled at different depths during three seasons

In order to analyse nitrogen-fixing activity in soils subject to different water tables and fertilization conditions, *nifH* gene was also taken into account (Figure 2.3.4.5). Results obtained for this gene were quite different from those obtained for nitrification and denitrification genes, with some analogies in the periodicity, and interesting observations could be done.

Firstly, in this case the effect of fertilization, even though it is present and evident from the graph (Figure 2.3.4.5), was not so pronounced as before.

The level of *nifH* gene was found to be higher in the proximity of water, when the soil was sampled at the same depth of the water table, and under the water table, in most of the cases. This result is in line with the fact that nitrogen fixation has to be performed in strictly anaerobic conditions, and therefore bacteria performing this process grew better where water was higher. This situation was for the most very evident in September.

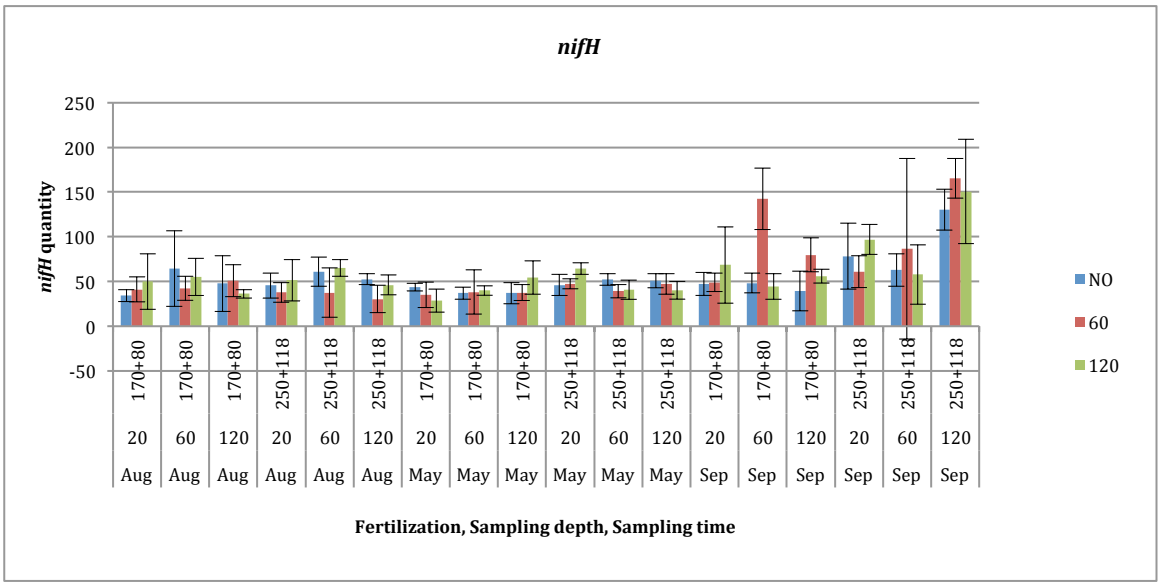

Figure 2.3.4.5

Presence of the *nifH* gene in soils subject to different water levels. The blue columns represent the soils where no water table was present, the red columns the soils with a water table at 60 cm depth and the green columns the soils with a water table at 120 cm depth. The error bars represent the standard deviation between the two replicates of each condition.

2.3.4.4 Influence of sampling season on N-cycle genes' abundance in soils subject to different water tables and fertilization conditions: statistical analysis

Statistical analysis was performed in order to evaluate the influence on the level of the nitrogen cycle's genes of the different conditions considered: the sampling date, the sampling depth, the fertilization type, and the water table depth.
The figure 2.3.4.6 shows separately the average quantity calculated for each gene at the three sampling dates, corresponding to three different seasons: Summer 2011, Spring 2012 and late Summer-early Autumn 2012. In the table 2.3.4.1, the results of the Anova test performed on the Real Time PCR amplification results considering the sampling date are shown. The considered nitrogen cycle genes exhibited significant differences amongst sampling seasons.

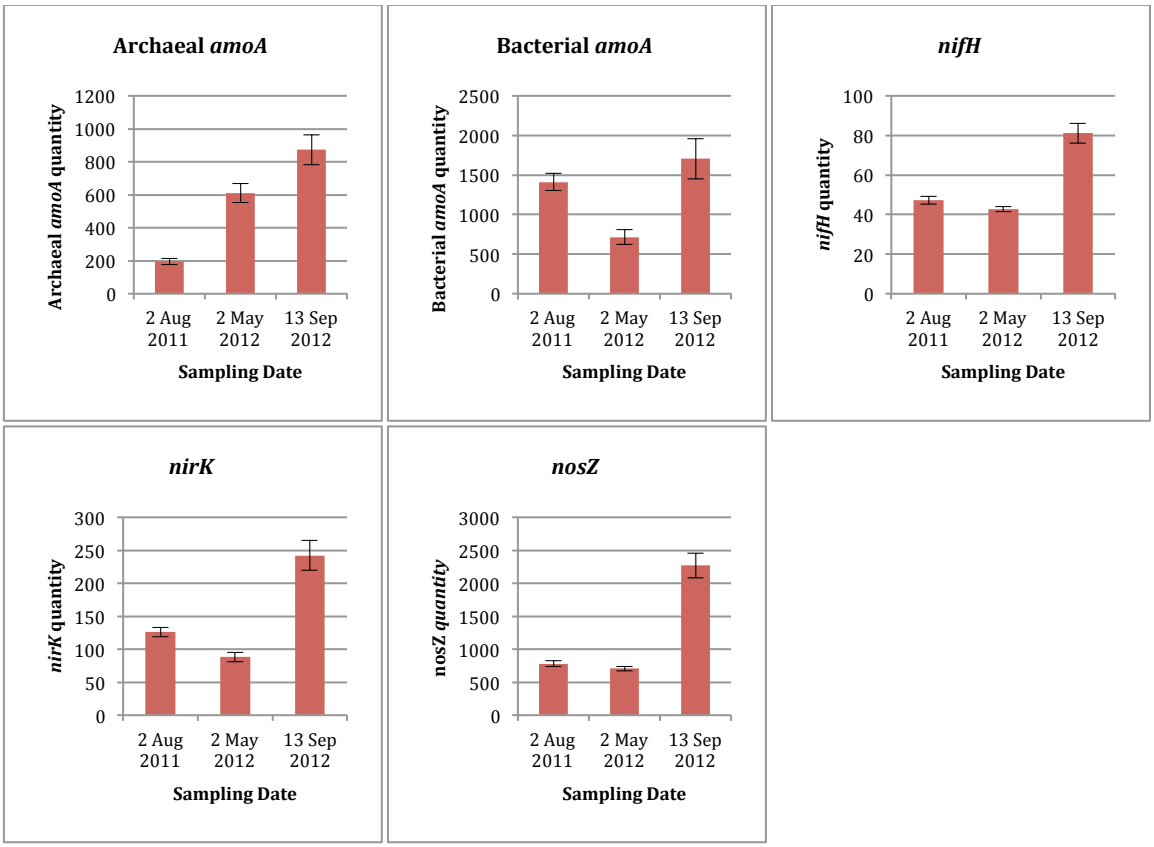

Figure 2.3.4.6
Presence of the five nitrogen-cycle genes at different sampling dates, representing three different seasons (Summer 2011, Spring 2012 and late Summer 2012). The columns represent the mean values calculated on the genes' quantities detected in all the soils, at all the sampling depths, at a certain day. The error bars represent the standard error of the mean.

These results confirmed some of the observations done before for each single gene. The archaeal nitrifiers were present at higher number in more temperate periods, such as May and September, while they seemed to be impaired in their growth in the presence

of high temperatures, in August. The bacterial nitrifiers, on the other hand, were disadvantaged in Spring, and were detected at higher levels in both August and September.
The genes *nifH*, *nirK* and *nosZ* showed a similar trend: they were detected at significantly higher level in September, while they were present at a similar degree in August and in May, with slightly lower levels in this last month.
In general, the microorganisms involved in the N-cycle were growing and surviving better in September, indicating that this month presented favouring conditions for them.

			Sum of Squares	df	Mean Square	F	Sig.
Archaeal *amoA* * Sampling date	Between Groups	(Combined)	2.42E+07	2	1.21E+07	29.324	.000
	Within Groups		1.26E+08	305	4.13E+05		
	Total		1.50E+08	307			
Bacterial *amoA* * Sampling date	Between Groups	(Combined)	5.15E+07	2	2.57E+07	8.861	.000
	Within Groups		8.83E+08	304	2.91E+06		
	Total		9.35E+08	306			
nifH * Sampling date	Between Groups	(Combined)	9.08E+04	2	4.54E+04	43.613	.000
	Within Groups		3.23E+05	310	1.04E+03		
	Total		4.14E+05	312			
nirK * Sampling date	Between Groups	(Combined)	1.33E+06	2	6.66E+05	31.603	.000
	Within Groups		6.51E+06	309	2.11E+04		
	Total		7.84E+06	311			
nosZ * Sampling date	Between Groups	(Combined)	1.61E+08	2	8.07E+07	61.421	.000
	Within Groups		4.07E+08	310	1.31E+06		
	Total		5.68E+08	312			

Table 2.3.4.1
The table shows the results obtained performing the test Anova on the considered nitrogen cycle genes in respect to the sampling date.

2.3.4.5 Influence of sampling depth on N-cycle genes' abundance in soils subject to different water tables and fertilization conditions: statistical analysis

Analysing the influence of sampling depth on the N cycle genes' level, it was evident that in the case of bacterial nitrifiers and of denitrifiers, the increase of soil depth resulted in a decrease of bacterial cells. Indeed, the abundance of bacterial *amoA*, *nirK* and, to a lesser extent, *nosZ*, was significantly higher when sampling was performed at 20 cm depth, whereas it decreased strongly already at 60 cm depth. This behaviour was particularly evident in the case of bacterial nitrifiers, which need oxygen for their metabolism, and are therefore favoured in oxidizing conditions. The significance of these differences was confirmed by the Anova test (see table 2.3.4.2).
In the case of archaeal nitrifiers, sampling depth had no significant influence on their abundance (see figure 2.3.4.7 and table 2.3.4.2). This could be due to an alternative metabolism of these microorganisms, and to their capacity to live in the presence of more harsh conditions.

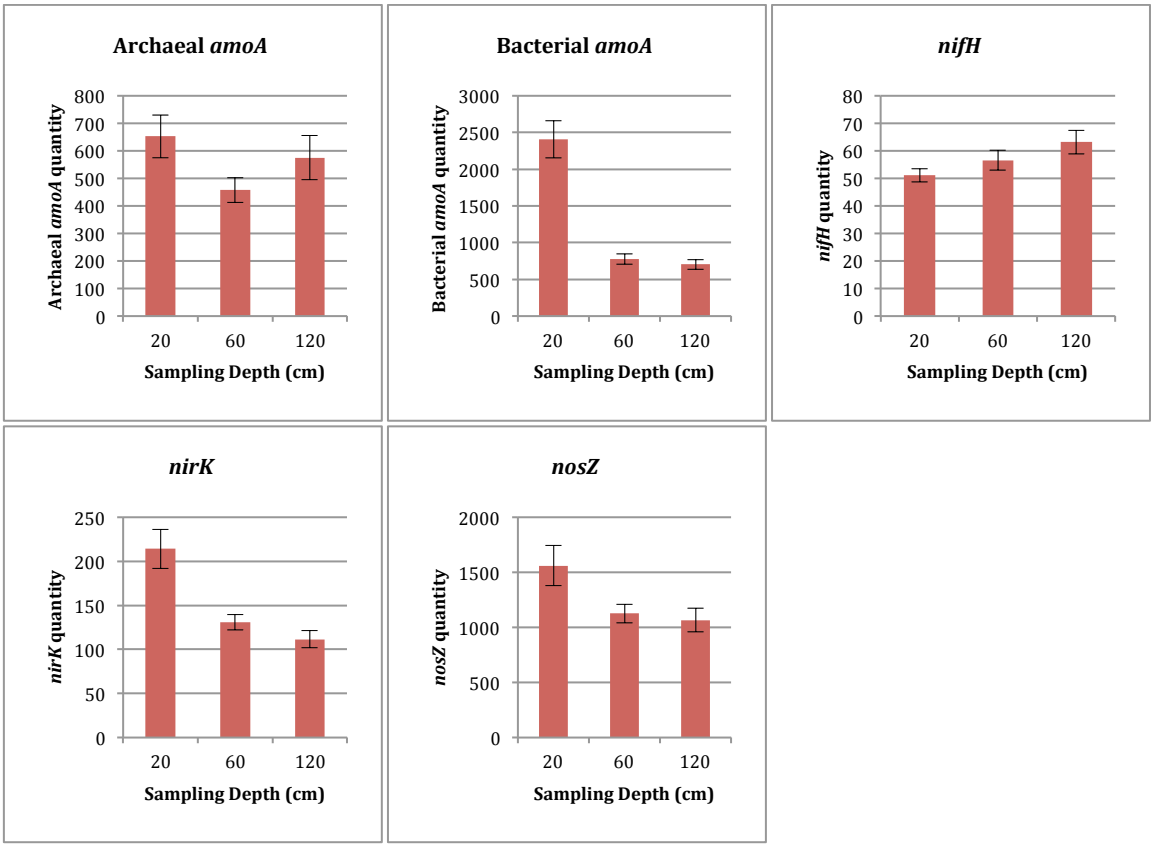

Figure 2.3.4.7

Presence of the five nitrogen-cycle genes at different sampling depths: 20 cm, 60 cm and 120 cm. The columns represent the mean values calculated on the genes' quantities detected in all the soil, at the three sampling days, at a specific sampling depth. The error bars represent the standard error of the mean.

			Sum of Squares	df	Mean Square	F	Sig.
Archaeal *amoA* * Sampling depth	Between Groups	(Combined)	2.02E+06	2	1.01E+06	2.075	.127
	Within Groups		1.48E+08	305	4.86E+05		
	Total		1.50E+08	307			
Bacterial *amoA* * Sampling depth	Between Groups	(Combined)	1.89E+08	2	9.44E+07	38.467	.000
	Within Groups		7.46E+08	304	2.45E+06		
	Total		9.35E+08	306			
nifH * Sampling depth	Between Groups	(Combined)	7.60E+03	2	3.80E+03	2.903	.056
	Within Groups		4.06E+05	310	1.31E+03		
	Total		4.14E+05	312			
nirK * Sampling depth	Between Groups	(Combined)	6.21E+05	2	3.11E+05	13.293	.000
	Within Groups		7.22E+06	309	2.34E+04		
	Total		7.84E+06	311			
nosZ * Sampling depth	Between Groups	(Combined)	1.52E+07	2	7.58E+06	4.249	.015
	Within Groups		5.53E+08	310	1.78E+06		
	Total		5.68E+08	312			

Table 2.3.4.2

The table shows the results obtained performing the test Anova on the considered nitrogen cycle genes in respect to the sampling depth.

Even if the differences in *nifH* levels at different sampling depth resulted to be non-significant (see table 2.3.4.2), a trend could be detected, showing the preference of

nitrogen-fixers to more anoxic conditions. Indeed, the abundance of these microorganisms raised with the increase of sampling depth.

2.3.4.6 Influence of fertilization conditions on N-cycle genes' abundance in soils subject to different water tables: statistical analysis

As already stated above, the influence of fertilization level was relevant for all the genes that were taken into account (see Fig 2.3.4.8 and Table 2.3.4.3).

			Sum of Squares	df	Mean Square	F	Sig.
Archaeal *amoA* * Fertilization	Between Groups	(Combined)	1.29E+07	1	1.29E+07	28.836	.000
	Within Groups		1.37E+08	306	4.49E+05		
	Total		1.50E+08	307			
Bacterial *amoA* * Fertilization	Between Groups	(Combined)	2.92E+07	1	2.92E+07	9.837	.002
	Within Groups		9.06E+08	305	2.97E+06		
	Total		9.35E+08	306			
nifH * Fertilization	Between Groups	(Combined)	1.58E+04	1	1.58E+04	12.353	.001
	Within Groups		3.98E+05	311	1.28E+03		
	Total		4.14E+05	312			
nirK * Fertilization	Between Groups	(Combined)	8.30E+05	1	8.30E+05	36.700	.000
	Within Groups		7.01E+06	310	2.26E+04		
	Total		7.84E+06	311			
nosZ * Fertilization	Between Groups	(Combined)	5.95E+07	1	5.95E+07	36.340	.000
	Within Groups		5.09E+08	311	1.64E+06		
	Total		5.68E+08	312			

Table 2.3.4.3
The table shows the results obtained performing the test Anova on the considered nitrogen cycle genes in respect to the fertilization conditions.

The presence of higher levels of fertilization, comprehending urea and manure, led to the increase in number of the microorganisms involved in nitrogen processing. As mentioned above, this result is not surprising for heterotrophic organisms such as denitrifiers, but was not expected for nitrifiers. However, the important growth in number of denitrifying organisms, which, being heterotrophs, are able to decompose organic matter and probably release also nitrogen forms such as ammonia, likely caused the concomitant increase of nitrifying organisms.

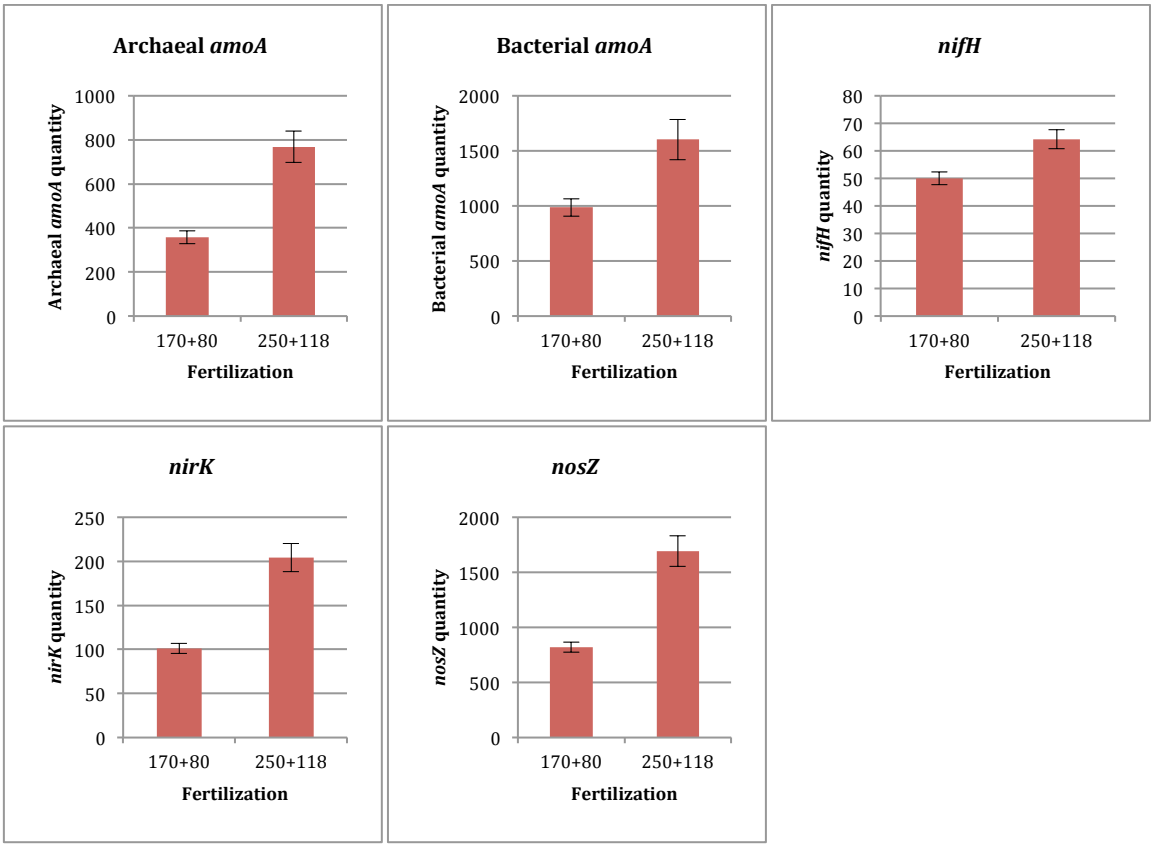

Figure 2.3.4.8

Presence of the five nitrogen-cycle genes in the presence of the two fertilization conditions: low fertilization, with 170 kg/ha organic N + 80 kg/ha urea N, and high fertilization, with 250 kg/ha organic N + 118 kg/ha urea N. The columns represent the mean values calculated on the genes' quantities detected in soils subject to the same fertilization treatments. The error bars represent the standard error of the mean.

2.3.4.7 Influence of water table level on N-cycle genes' abundance in soils subject to different fertilization conditions: statistical analysis

The presence of water table significantly affected the abundance of nitrifiers and denitrifiers, but did not have a clear effect on the growth of nitrogen-fixing microorganisms (Figure 2.3.4.9). For both nitrifying and denitrifying organisms, the mostly favourable conditions were in the absence of the water table, or when the water table was very deep (120 cm). In the presence of the water table at 60 cm depth, these microorganisms significantly decreased in number (Table 2.3.4.4).

As for the analysis of the effect of fertilization, these observations, which were expected for nitrifiers, result in some degree surprising for denitrifiers. In effect, anaerobic conditions, facilitated by the presence of the water table, should have encouraged the growth of denitrifying organisms.

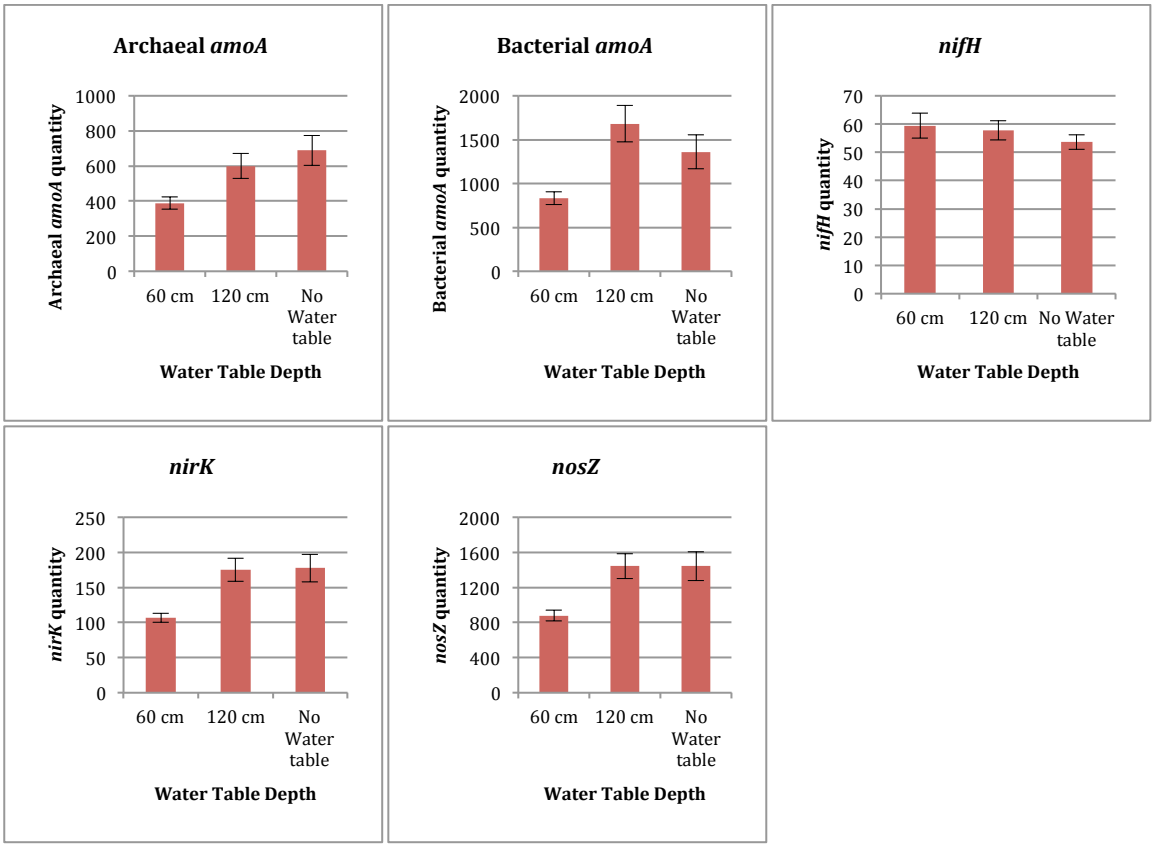

Figure 2.3.4.9

Abundance of the five nitrogen-cycle genes in the presence of water table at 120 cm, water table at 60 cm or in the absence of water table. The columns represent the mean values calculated on the genes' quantities detected in soils with the same kind of water table. The error bars represent the standard error of the mean.

			Sum of Squares	df	Mean Square	F	Sig.
Archaeal *amoA* * Water table depth	Between Groups	(Combined)	4.96E+06	2	2.48E+06	5.206	.006
	Within Groups		1.45E+08	305	4.77E+05		
	Total		1.50E+08	307			
Bacterial *amoA* * Water table depth	Between Groups	(Combined)	3.65E+07	2	1.83E+07	6.184	.002
	Within Groups		8.98E+08	304	2.95E+06		
	Total		9.35E+08	306			
nifH * Water table depth	Between Groups	(Combined)	1.85E+03	2	9.26E+02	.697	.499
	Within Groups		4.12E+05	310	1.33E+03		
	Total		4.14E+05	312			
nirK * Water table depth	Between Groups	(Combined)	3.41E+05	2	1.71E+05	7.030	.001
	Within Groups		7.50E+06	309	2.43E+04		
	Total		7.84E+06	311			
nosZ * Water table depth	Between Groups	(Combined)	2.24E+07	2	1.12E+07	6.355	.002
	Within Groups		5.46E+08	310	1.76E+06		
	Total		5.68E+08	312			

Table 2.3.4.4

The table shows the results obtained performing the test Anova on the considered nitrogen cycle genes in respect to the level of the water table.

2.3.4.8 Analysis of N-cycle genes' abundance in soils equipped with lysimeters, subject to different water tables and fertilization conditions: statistical analysis

In the figure 2.3.4.10, the mean values of the gene quantity, calculated for each single condition, are reported. In several cases, the variability between the two soils representing the two replicates of a particular condition is noticeable, because, as stated before, soils can vary significantly in their community structure even when they are subject to the same environmental conditions. In any case, some major trends could be highlighted.

First, as mentioned above, the abundance of the genes was in general higher with high fertilization. In particular, for the genes archaeal and bacterial *amoA*, *nirK*, and *nosZ*, this tendency was especially evident with the water table at 120 cm and without water table. Here, in effect, the levels of the genes were significantly higher than in all the other conditions.

With low fertilization, the growth of nitrogen-fixing bacteria seemed to be slightly favoured by the presence of the water table at 60 cm, even if the differences among the *nifH* levels in the soils subject to these fertilization conditions were not significant.

With high fertilization, instead, the condition represented by lysimeter 19, with the water table at 60 cm depth, and the soil represented by lysimeter 3, with the water table at 120 cm depth, presented significantly higher *nifH* levels, compared to the other soils subject to the same conditions.

			Sum of Squares	df	Mean Square	F	Sig.
Archaeal *amoA* * Lysimeter	Between Groups	(Combined)	2.76E+07	11	2.51E+06	6.040	.000
	Within Groups		1.23E+08	296	4.15E+05		
	Total		1.50E+08	307			
amoA * Lysimeter	Between Groups	(Combined)	1.20E+08	11	1.09E+07	3.961	.000
	Within Groups		8.14E+08	295	2.76E+06		
	Total		9.35E+08	306			
nifH * Lysimeter	Between Groups	(Combined)	4.33E+04	11	3.94E+03	3.204	.000
	Within Groups		3.70E+05	301	1.23E+03		
	Total		4.14E+05	312			
nirK * Lysimeter	Between Groups	(Combined)	1.68E+06	11	1.53E+05	7.454	.000
	Within Groups		6.16E+06	300	2.05E+04		
	Total		7.84E+06	311			
nosZ * Lysimeter	Between Groups	(Combined)	1.15E+08	11	1.04E+07	6.918	.000
	Within Groups		4.54E+08	301	1.51E+06		
	Total		5.68E+08	312			

Table 2.3.4.5

The table shows the results obtained performing the test Anova on the nitrogen cycle genes considered in respect to the different conditions.

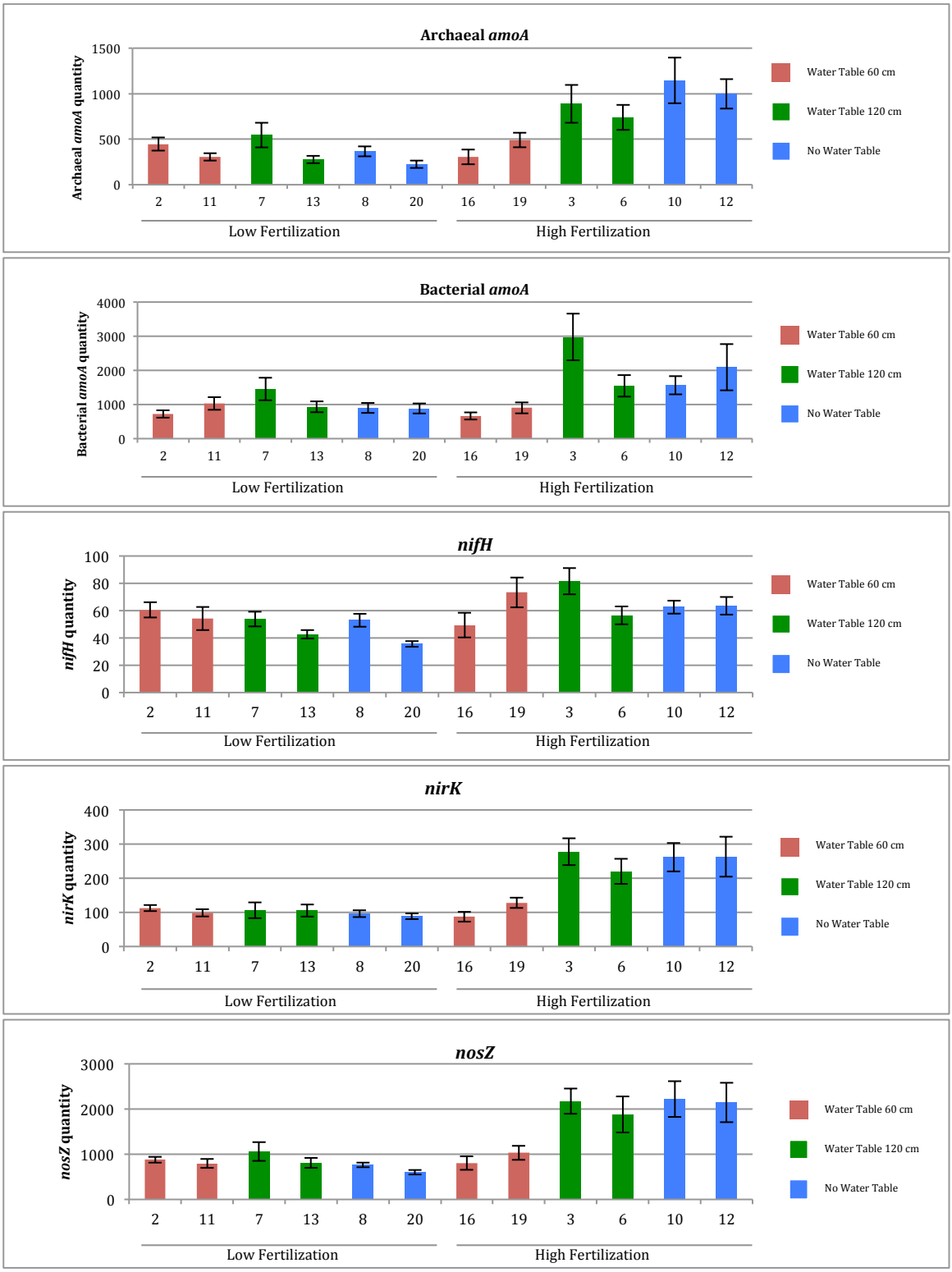

Figure 2.3.4.10

Presence of the five nitrogen-cycle genes in the 12 conditions considered in this study. The columns represent the mean values calculated on the genes' quantities detected in each soil at different sampling depths and at different sampling days. The error bars represent the standard error of the mean.

2.3.4.9 Analysis of the correlation among N-cycle genes in soils subject to different water levels and to different fertilization managements, sampled at different depths during three seasons

As stated before in this chapter, some functional genes had a similar behaviour and were changing similarly in the different soil samples. In the table 2.3.4.6, Pearson's correlation coefficients calculated within the considered genes are reported.
Firstly, a strong and significant correlation between the two denitrification genes *nirK* and *nosZ* confirms the observations done above.
Moreover, it could be observed that both the considered nitrification genes, archaeal and bacterial *amoA*, are significantly positively correlated with the two genes of denitrification, *nosZ* and *nirK*, and this result also supports the previous affirmation that the presence of nitrifying organisms and of their metabolites facilitates the growth of denitrifying organisms, which can in turn find a higher amount of the substrates they need for their metabolism. The presence of heterotrophic denitrifying microorganisms, on the other hand, probably also has a positive influence on the growth of nitrifying microorganisms.
Positive correlations could also be found for the gene *nifH* with each of the other nitrogen cycle genes considered, but these correlations, although being significant, are not very strong.
A positive correlation could also be observed between the bacterial and the archaeal *amoA* genes.

Correlations

		Archaeal *amoA*	**Bacterial *amoA***	***nifH***	***nirK***	***nosZ***
Archaeal *amoA*	Pearson Correlation	1	0.364**	0.452**	0.550**	0.601**
			0.000	0.000	0.000	0.000
	N	308	300	304	304	304
Bacterial *amoA*	Pearson Correlation	0.364**	1	0.224**	0.633**	0.567**
		0.000		0.000	0.000	0.000
	N	300	307	304	301	303
nifH	Pearson Correlation	0.452**	0.224**	1	0.398**	0.538**
		0.000	0.000		0.000	0.000
	N	304	304	313	307	309
nirK	Pearson Correlation	0.550**	0.633**	0.398**	1	0.870**
		0.000	0.000	0.000		0.000
	N	304	301	307	312	308
nosZ	Pearson Correlation	0.601**	0.567**	0.538**	0.870**	1
		0.000	0.000	0.000	0.000	
	N	304	303	309	308	313

** Correlation is significant at the 0.01 level (2-tailed).

Table 2.3.4.6
Pearson's correlation (2-tailed) among the 6 considered genes.

2.3.5 Effects of different fertilization practices on plant growth

A greenhouse trial was set up in Summer 2012 in order to evaluate the effects of different fertilizers on soil microbial communities, in the presence of the creeping perennial grass *Cynodon dactylon*. All the fertilizers tested (7) were added to an agricultural low-nutrient soil; moreover, 6 of the same fertilizers were also added to soils, which had already been used for a similar test and that had been treated with the same fertilizers the year before. These last samples were called "old" (abbreviated with "o" in the graphs), as the starting soil was not fertilized for the first time.
Soils sampled from these pots were analysed through Real Time PCR targeting the soil nitrogen cycle genes (chapter 2.3.6), T-RFLP experiments (chapter 2.3.7), and pyrosequencing (chapter 2.3.8). Moreover, the growth of the plants subject to the different treatments was measured (this chapter).
The growth of the plant *C. dactylon* was measured during the 2 months of the trial for the 14 different soil conditions.
Cynodon is a creeping perennial grass that was selected for this experiment as it is able to resist and grow in the presence of high temperatures and drought. In fact, the trial was performed between June and August 2012 in northern Italy. This plant has also a commercial value, as it is often used to cover sport and golf fields.
Height, fresh weight and dry weight were measured three times, and the results are shown in the column charts (Figure 2.3.5.1). The three measures gave the same trend of results.
The organic fertilizers Castor Cake (CC) and Yeast (Y) promoted plant growth at high degree, followed by Thermal Hydrolysed Leather (THL) and by the composite fertilizers UTY (Urea-THL-Yeast) and UTN (Urea-THL-Neem) (for more details on the composition of the fertilizers, see material and methods, paragraph 2.2.8). These last two, which are composite fertilizers, and are different only in their organic vegetable part (10% Yeast and 20% Neem respectively), promoted plant growth in a very similar way. Neem (N) and Oxy-amino-triazine (OATr), which are slow release fertilizers and are specifically used to decrease the loss of N in the environment through nitrification and denitrification, had lower effects on plant growth. In particular, the growth of the plant in the presence of OATr was very similar to the growth in the non-fertilized soil.
For almost all the treatments, a slight increase of plant growth could be noticed in the pots were fertilization had been performed with the same fertilizer the year before. This advantage was more evident in the cases of THL, Neem and Yeast, indicating an interesting pre-adaptation of the microbial community to specific fertilizers, which prompts their effects when used a year later. A different result was obtained for the organic fertilizer Castor Cake. In fact, in this case, plant growth in the "old" pots resulted to be very similar, if not even lower, when compared to the pots fertilized for the first

time. This fact could be explained by the establishment, in the presence of this treatment, of a less stable bacterial community.

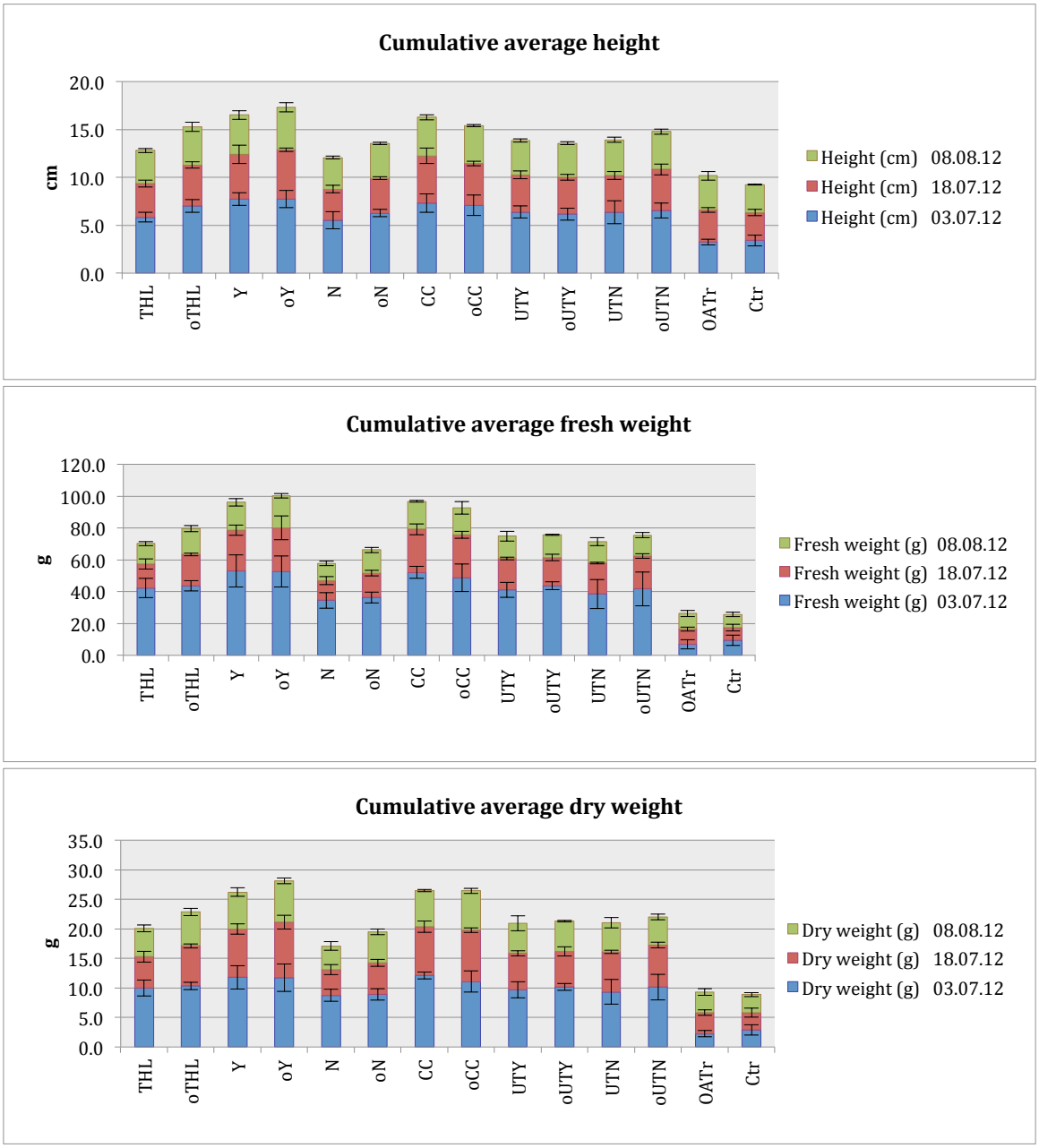

Figure 2.3.5.1

In the three graphs reported in this figure, the cumulative results obtained for the considered plant growth parameters are reported. Height, fresh weight and dry weight were measured three times during the two months of the fertilization trial. The columns represent the mean values obtained for the different treatments, obtained considering the plant growth in 8 treated replicate pots. The error bars report values of the standard deviation.

The significance of the differences between the growth results with the different treatments was confirmed by the test Anova (Table 2.3.5.1).

			Sum of Squares	df	Mean Square	F	Sig.
Cumulative average height * Treatment	Between Groups	(Combined)	278.0	13	21.4	14.127	.000
	Within Groups		63.6	42	1.5		
	Total		341.5	55			
Cumulative average fresh weight * Treatment	Between Groups	(Combined)	27981.0	13	2152.4	44.915	.000
	Within Groups		2012.7	42	47.9		
	Total		29993.7	55			
Cumulative average dry weight * Treatment	Between Groups	(Combined)	1773.0	13	136.4	45.916	.000
	Within Groups		124.8	42	3.0		
	Total		1897.7	55			

Table 2.3.5.1

In the table, the results obtained from the Anova test done considering the three plant growth parameters in respect to the different treatments are reported.

2.3.6 Analysis of N-cycle genes in an agricultural soil supplied with different kinds of fertilizers

It is well established that elevated N additions to ecosystems can have wide-ranging impacts on biogeochemical cycles, the emissions of greenhouse gases, and plant biodiversity (Tilman, 1987; Gough *et al*., 2000; Vitousek *et al*., 2002; Gilliam, 2006).
The effect of fertilization on the growth of nitrogen cycle microorganisms in agricultural soils was investigated in previous works (Peacock *et al*., 2001; Enwall *et al*., 2007; Hallin *et al*., 2009; Wessen *et al*., 2011; Fan *et al*., 2011; Gong *et al*., 2013; Habteselassie *et al.*, 2013), but none of these analyses focused on the impact of different types of organic, composite and inorganic commercialized fertilizers. Moreover, the evaluation of the effects of the addition of the treatments to previously fertilized soils is also unexplored. The changes in abundance of nitrogen cycle microorganisms, and in particular of ammonia-oxidizers, in response to N-additions was investigated also in other types of soils, such as forest soil (Wertz *et al*., 2012), rice paddy soils (Hussain *et al*., 2011; Chen *et al*., 2012; Wang *et al*., 2013), grasslands (Li *et al*., 2011).
Real Time PCR experiments were performed on soils sampled before the addition of the fertilizer (day 0), after 9 days (day 9), and after 58 days (day 58), at the end of the trial. Soils sampled at day 0, before the starting of the trial, were the 6 soils that had been fertilized with the 6 fertilizers in the previous experiment (before the addition of a new quantity of fertilizer) and the low-nutrient soil (called Ctr). As a consequence, the day 0 samples are only 7. The replicates of each treatment sampled at day 9 and at day 58 used for Real Time PCR experiments, instead, were 3, for a total of 42 samples for each of the two sampling times.
In this study, the following genes of the N-cycle were considered: bacterial and archaeal *amoA* for nitrification, and *nirK*, *nirS* and *nosZ* for denitrification. The nitrogen-fixation gene *nifH* was not considered, as this activity is not fundamental to evaluate the potentialities of a fertilizer.
For this analysis, DNA was extracted using a different method from the one used for the analysis reported at chapter 2.3.3. In fact, in this case, DNA and RNA were co-extracted using a phenol-chloroform based protocol (see material and methods, paragraph 2.2.1.3), as these samples had to be used also for T-RFLP experiments and for 454 sequencing. Differently from samples extracted with the MoBio commercial kit, with this method the mean DNA concentration obtained was around 70 ng/µl. The low-nutrient soil, which was extracted also with the MoBio kit giving DNA concentrations of about 3 ng/µl, resulted in a DNA sample having a concentration of 65 ng/µl using the phenol-chloroform based protocol. It is likely that this change in extraction method led to the obtainment of higher calculated gene copy number per gram soil. However, while this

could affect absolute estimations, the relative proportions among the values obtained for a given gene across different samples are regarded as reliably comparable.

2.3.6.1 Changes in the abundance of nitrogen cycle microorganisms present in fertilized and unfertilized soils over time

Statistical analyses were made to evaluate the effects of the different fertilizers on soil nitrifiers and denitrifiers during the two months of the trial.
First observations could be done when considering the mean values calculated for each sampling day (Figure 2.3.6.1, Table 2.3.6.1). Significant differences could be detected in the mean number of the copies of all the 5 genes considered. For the archaeal *amoA* gene, a slight decrease of the gene copy number could be detected over time. However, the number of archaeal nitrifiers remained very high, with a number of detected functional genes that was one to two orders of magnitude higher than for the other considered genes.
In general, the supplement of the treatments caused a significant decrease in the number of bacterial nitrifiers, which then tended to start a slight recovery after two months. This result could be due to the arrival of a relevant number of new bacterial cells with the fertilizers that altered the equilibrium of pre-existing ammonia-oxidizers.

			Sum of Squares	df	Mean Square	F	Sig.
Archaeal *amoA* * Day	Between Groups	(Combined)	8.58E+18	2	4.29E+18	3.564	.032
	Within Groups		1.06E+20	88	1.20E+18		
	Total		1.14E+20	90			
Bacterial *amoA* * Day	Between Groups	(Combined)	2.17E+15	2	1.09E+15	3.940	.023
	Within Groups		2.43E+16	88	2.76E+14		
	Total		2.64E+16	90			
nosZ * Day	Between Groups	(Combined)	2.45E+14	2	1.22E+14	5.029	.009
	Within Groups		2.14E+15	88	2.43E+13		
	Total		2.38E+15	90			
nirK * Day	Between Groups	(Combined)	6.92E+16	2	3.46E+16	4.424	.015
	Within Groups		6.89E+17	88	7.83E+15		
	Total		7.58E+17	90			
nirS * Day	Between Groups	(Combined)	4.56E+16	2	2.28E+16	16.750	.000
	Within Groups		1.20E+17	88	1.36E+15		
	Total		1.65E+17	90			

Table 2.3.6.1
The table shows the results obtained performing the test Anova on the considered nitrogen cycle genes in respect to the sampling day.

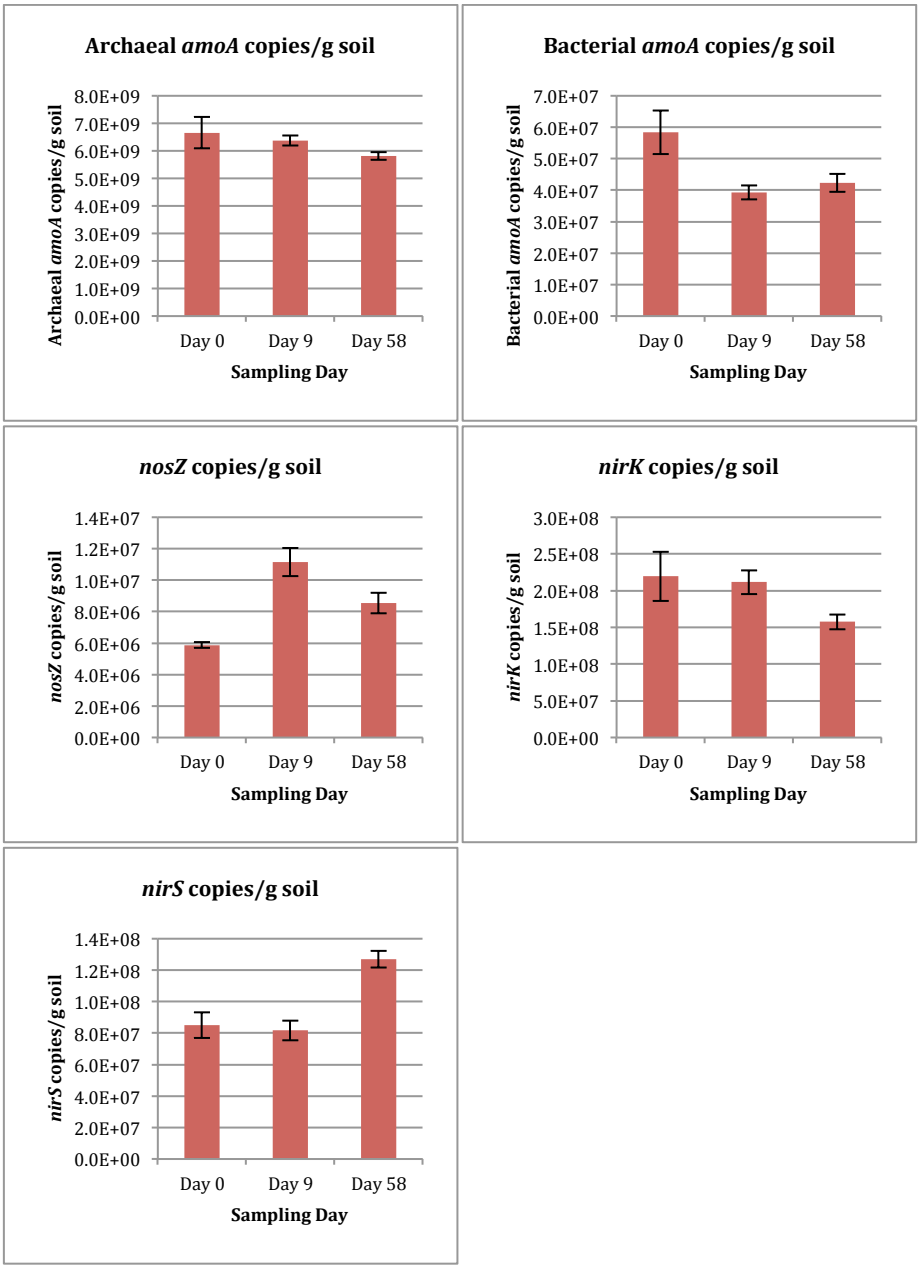

Figure 2.3.6.1

Presence of the five nitrogen-cycle genes at different sampling days: day 0, representing soils sampled before the distribution in the pots and the subsequent fertilization; day 9 and day 58, representing soils sampled 9 and 58 days after the addition of the fertilizers, respectively. The columns represent the mean values calculated on the genes' copy number detected in all the considered soil, sampled at a certain day. The error bars represent the standard error of the mean.

A different behaviour could be observed for the three genes of the denitrification process. The copies of the *nosZ* gene significantly increased in number 9 days after fertilization, and subsequently decreased over time. This gene was present at a considerably lower number at day zero, when compared with the four other genes, and

this fact could justify the ability of these microorganisms to grow in number more easily in the presence of favourable conditions.
The gene *nirK* decreased in copy number over time. More specifically, the number of *nirK* gene copies did not change significantly after 9 days, but was lower after 58 days. In any cases, the mean values remained in a range between $1.5x10^8$ and $2.5x10^8$. Differently from their counterpart, the *nirS* denitrifiers had an opposite trend in respect to the *nirK* denitrifiers, growing in time with a maximum after 58 days.

2.3.6.2 Presence of nitrogen cycle genes in untreated soil and in previously fertilized soils at day 0

In the figure 2.3.6.2, the values obtained for the gene copy number of each of the 5 considered genes at sampling day 0 are reported. The results were obtained from single soil samples, which represent the starting material that was used for the fertilization trial.
The archaeal *amoA* gene copy number was variable between around $4x10^9$ and about $9x10^9$, with higher values detected in the control soil, which had not been fertilized before (Ctr), followed by soil fertilized with the organic fertilizer Yeast the year before.
The soil sample, which, on the other hand, presented the lowest level of archaeal *amoA* gene, was the one treated with the organic fertilizer THL. These results could indicate a general negative influence over long time periods of the presence of a fertilizer, and in particular of the fertilizer THL, on the growth of archaeal ammonia-oxidizers. These results are in line with the recent findings that archaeal nitrifiers prefer low-nutrient environments, while bacterial nitrifiers positively respond to the presence of nutrients (Di *et al*., 2010; Schleper, 2010; Verhamme *at al*., 2011). Indeed, the trend was opposite in the case of bacterial *amoA* gene and of the denitrification *nirK* gene. In effect, in these cases the untreated soil presented the lowest number of gene copies, and higher levels in soils, which had been treated the year before. In particular, the soils previously treated with the organic fertilizers Castor Cake and THL resulted to have the highest levels of these genes. The number of copies of the gene *nosZ*, corresponding to the last step of denitrification, did not present significant differences in previously fertilized soils. In fact, the gene copy number in the untreated soil was very similar to that in the other old soils. However, later in this chapter, it will be observed that, after the addition of a new quantity of the fertilizers, the *nosZ* gene responded more strongly in previously fertilized soils.
In four of the formerly fertilized soils, *nirS* denitrifying bacteria were present at different levels, when compared to the unfertilized soil: in the soil previously fertilized with Castor Cake and in the soil previously fertilized with the organic, slow-release fertilizer

Neem, the number of *nirS* copies was higher, while in the soils fertilized with THL and with UTY the year before, *nirS* level was lower.

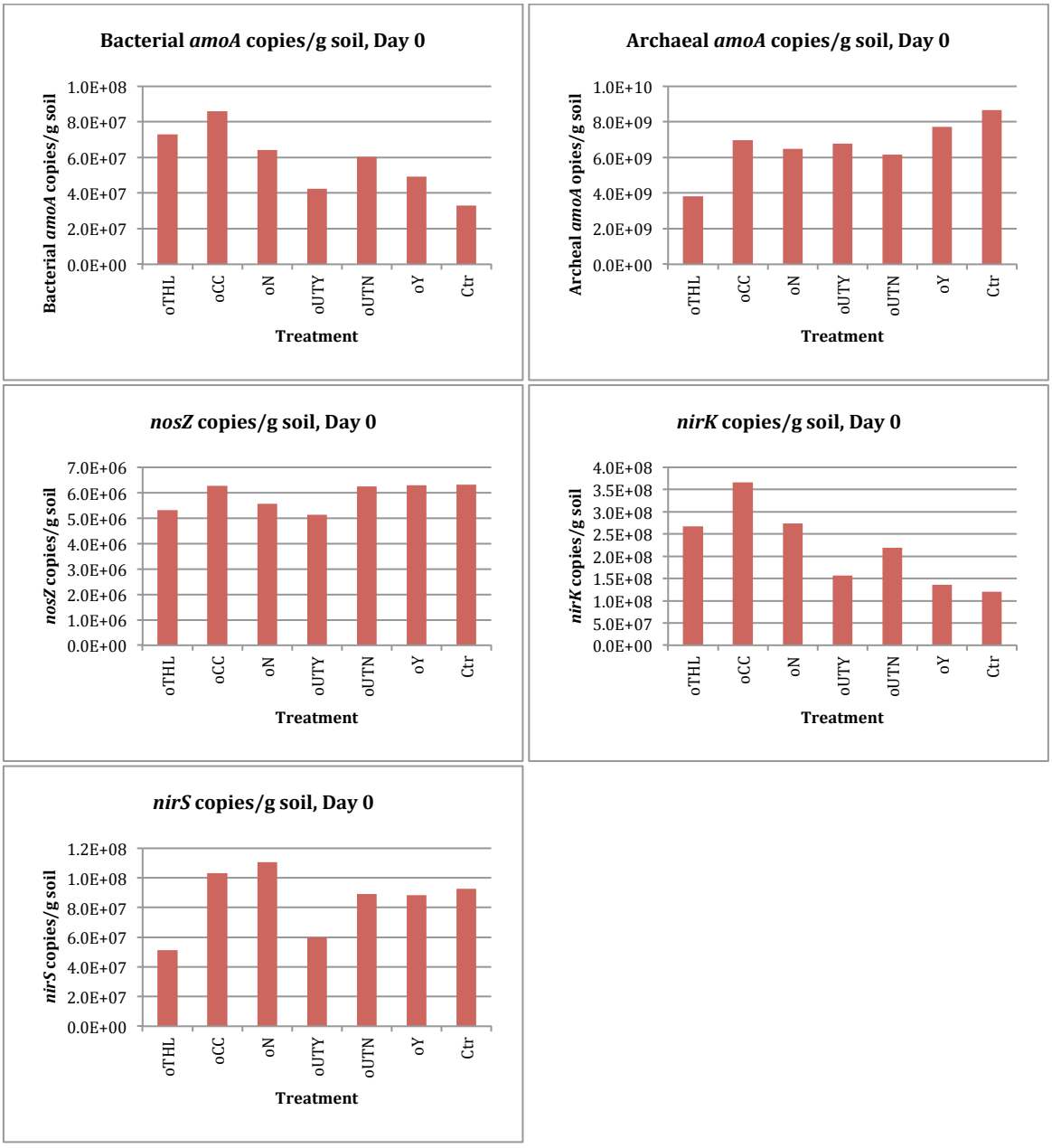

Figure 2.3.6.2

Abundance of nitrogen cycle genes in soils sampled before the starting of the fertilization trial. The reported values correspond to single soil samples, which represent the starting material that was then distributed in the pots, together with the fertilizers.

2.3.6.3 Changes in the abundance of nitrogen cycle microorganisms occurred after 9 days and after 58 days from the addition of the treatments

Figure 2.3.6.3 shows the mean values of the copy number of each gene in soils subject to the different treatments 9 days after the addition of the fertilizers. From table 2.3.6.2, it can be noticed that the differences between these mean values did not result to be significant. However, the observation of the trends at this sampling time could give interesting insights into the changes that occur to nitrogen cycle soil organisms a short period after fertilization.

As already observed previously, the archaeal ammonia-oxidizers were influenced only weakly by the presence of these kinds of fertilizers. From the graph in figure 2.3.6.3, it can be observed that the gene copy number of the archaeal *amoA* gene remained high in all the soil samples, with just a slight decrease in the soils that were fertilized the year before with the slow-release fertilizer Neem and with the two composite fertilizers UTY and UTN.

N-cycle bacteria were instead more influenced by the presence of fertilization, after 9 days. Considering bacterial ammonia-oxidizers, it could be noticed that, in the majority of the cases, the treatments caused an increase in number of these organisms. This increase in gene copy number was not detectable in the case of the slow-release fertilizer OATr and with the two composite fertilizers, containing urea. These last two fertilizers, however, caused an increase in the number of bacterial nitrifiers when they were added to previously fertilized soils (old). This effect, probably due to the presence of a previously established and adapted bacterial community, was visible also in the cases of the organic fertilizers Castor Cake and Yeast, after 9 days. The fertilizers THL and Neem, although inducing an increase in the *amoA* gene copy number, did not present this kind of result.

Denitrifying organisms, as expected, were also influenced by fertilization, as they are heterotrophic bacteria and are therefore depending on the presence of organic matter. At day 9, the effect was particularly evident for the gene *nosZ*, representing the last step of denitrification. Comparing the *nosZ* gene copy number in treated soils with the gene copy number in the control (Ctr) soil, a decrease could be detected only in the case of the fertilizer OATr, while with UTY and UTN the gene copy number was very similar to the control. Major increases, on the other hand, could be detected in the presence of the organic fertilizers and when the composite fertilizer UTY was added to the previously fertilized soil (oUTY). The effect of the use of the "old" soils was also visible in the case of the organic fertilizer Castor Cake, while with THL and Neem the effect was opposite.

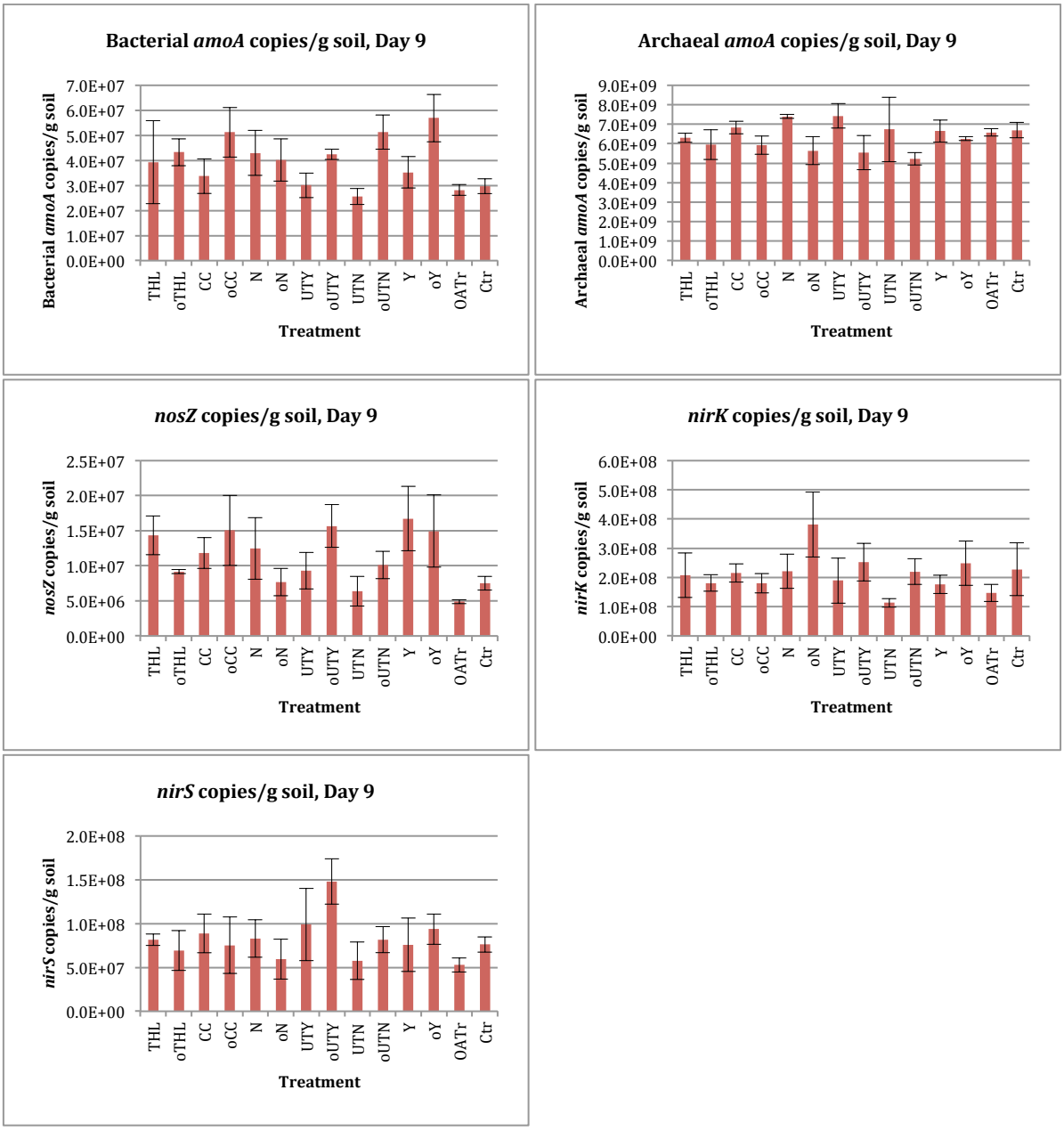

Figure 2.3.6.3

Presence of the 5 considered nitrogen cycle genes, archaeal and bacterial *amoA*, *nosZ*, *nirk*, and *nirS*, in soils sampled after 9 days from the addition of different fertilizers. The columns represent the mean values of the gene copy numbers detected in the three replicates, consisting of three pots with soil treated in the same way, at the considered sampling day. The error bars represent the standard error of the mean. In the graphs, the names of the treatments are substituted by the following abbreviations: THL= Thermal Hydrolysed Leather, oTHL = THL old, CC = Castor Cake, oCC = CC old, N = Neem, oN = N old, UTY = Urea-THL-Y, oUTY = UTY old, UTN = Urea-THL-N, oUTN = UTN old, Y = Yeast, oY = Y old, OATr= Oxy-amino-triazine, Ctr = control unfertilized soil. The "old" soils are soils, which had already been fertilized with the same treatment the year before.

			Sum of Squares	df	Mean Square	F	Sig.
Archaeal *amoA* * Treatment	Between Groups	(Combined)	1.70E+19	13	1.31E+18	1.006	.472
	Within Groups		3.65E+19	28	1.30E+18		
	Total		5.36E+19	41			
Bacterial *amoA* * Treatment	Between Groups	(Combined)	3.51E+15	13	2.70E+14	1.517	.172
	Within Groups		4.99E+15	28	1.78E+14		
	Total		8.50E+15	41			
nosZ * Treatment	Between Groups	(Combined)	5.68E+14	13	4.37E+13	1.518	.172
	Within Groups		8.06E+14	28	2.88E+13		
	Total		1.37E+15	41			
nirK * Treatment	Between Groups	(Combined)	1.49E+17	13	1.15E+16	1.016	.463
	Within Groups		3.16E+17	28	1.13E+16		
	Total		4.65E+17	41			
nirS * Treatment	Between Groups	(Combined)	2.12E+16	13	1.63E+15	1.015	.464
	Within Groups		4.49E+16	28	1.60E+15		
	Total		6.61E+16	41			

Table 2.3.6.2
The table shows the results obtained performing the test Anova on the nitrogen cycle genes' copy number detected 9 days after fertilization, in respect to the different treatments.

In the cases of *nirS* and *nirK* genes, the effects of the treatments were less extended after 9 days. In fact, for the *nirK* gene, only the treatment with Neem on previously fertilized soil caused a visible increase in the gene copy number, while for *nirS* this increase took place in soils fertilized for the second time with UTY. Both for *nirK* and *nirS*, some treatments, and in particular the composite fertilizer UTN and the oxy-amino-triazine OATr, seemed to cause a decrease in the number of denitrifying bacteria in the first 10 days of fertilization.
Considering the results obtained from soils sampled 58 days after the treatment (Figure 2.3.6.4), the situation observed for archaeal nitrifiers was very similar to the one observed at day 9. Indeed, also after 58 days, the number of archaeal *amoA* gene copies did not change significantly, when compared to the unfertilized soil (Ctr). Only in the presence of THL and Neem, added to the previously fertilized soils, and with OATr, a slight decrease in number could be observed.
Significant differences could be detected in the bacterial *amoA* gene copy number among different treatments (see table 2.3.6.3). Here, a relevant effect of the presence of formerly established microbial community in the soils, which had already been fertilized before, was observed. More in detail, the abundance of bacterial ammonia-oxidizers increased significantly in the soil samples "Neem old", "Castor Cake old", and "Yeast old". Similar effects were also detected, for the last two soil samples, at day 9, while for Neem the effect was delayed. This result could be explained by the fact that this last treatment is a slow-release fertilizer, and this fact could have caused a retard in the reactivation of the present nitrifying bacteria.

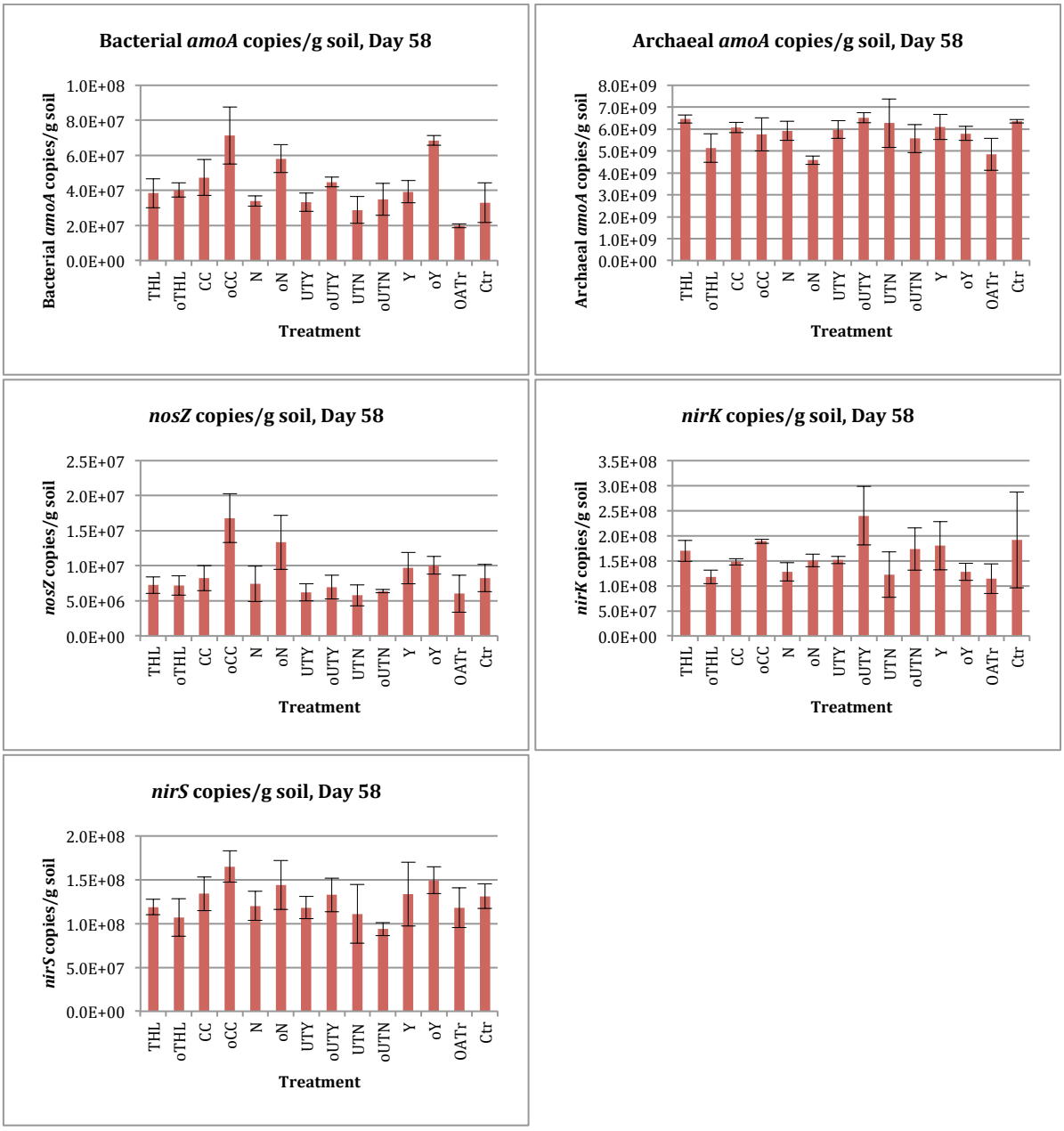

Figure 2.3.6.4

Presence of the 5 considered nitrogen cycle genes, archaeal and bacterial *amoA*, *nosZ*, *nirk*, and *nirS*, in soils sampled after 58 days from the addition of different fertilizers. The columns represent the mean values of the gene copy numbers detected in the three replicates, consisting in three pots with soil treated in the same way, at the considered sampling day. The error bars represent the standard error of the mean. In the graphs, the names of the treatments are substituted by the following abbreviations: THL= Thermal Hydrolysed Leather, oTHL = THL old, CC = Castor Cake, oCC = CC old, N = Neem, oN = N old, UTY = Urea-THL-Y, oUTY = UTY old, UTN = Urea-THL-N, oUTN = UTN old, Y = Yeast, oY = Y old, OATr= Oxy-amino-triazine, Ctr = control unfertilized soil. The "old" soils are soils, which had already been fertilized with the same treatment the year before.

The effect of the former fertilization was not so relevant, after 58 days, for the two composite fertilizers containing urea: UTY and UTN. With these two treatments, this kind of effect was detected, even if not at very high degree, after 9 days, indicating that the fast source of available N, urea, induced the growth of previously established nitrifiers rapidly, and that this effect decreased over time.

The only treatment that caused a decrease in number of bacterial ammonia-oxidizers after about two months was the slow-release fertilizer OATr. A reason for this results could be that, in this kind of soil, few bacteria able to decompose oxy-amino-triazine and thus to release NH_3 are present, and therefore the substrate for nitrification is present at low levels, and this fact does not induce bacterial nitrifiers to grow fast.

In general, the soils fertilized for the first time with the organic fertilizers tested here presented levels of nitrifying bacteria, which were similar to the non fertilized sample. This result likely indicates that this kind of fertilizers, of animal origin in the case of THL, of vegetable origin in the case of Castor Cake and Neem, and Yeast-based fertilizer, cause relevant effect on the nitrifying bacterial communities only after a long period, and after more than one application of the treatment.

The Anova test, reported in the table 2.3.6.3, indicated that another gene that was significantly influenced by the presence of different types of fertilization, was the denitrification gene *nosZ*. Also in this case, the two soils where major effects were detected where "Castor Cake old" and "Neem old". Therefore, a formerly set up denitrifying community was also present in these soils, and it increased in abundance especially after 2 months. In the other soil samples, *nosZ* gene was detected at similar or slightly lower level than in the control soil.

		Sum of Squares	df	Mean Square	F	Sig.
Archaeal *amoA* * Treatment	Between Groups (Combined)	1.38E+19	13	1.06E+18	1.195	.333
	Within Groups	2.49E+19	28	8.88E+17		
	Total	3.86E+19	41			
Bacterial *amoA* * Treatment	Between Groups (Combined)	8.43E+15	13	6.49E+14	3.418	.003
	Within Groups	5.31E+15	28	1.90E+14		
	Total	1.37E+16	41			
nosZ * Treatment	Between Groups (Combined)	3.80E+14	13	2.93E+13	2.139	.045
	Within Groups	3.83E+14	28	1.37E+13		
	Total	7.64E+14	41			
nirK * Treatment	Between Groups (Combined)	4.93E+16	13	3.80E+15	.837	.621
	Within Groups	1.27E+17	28	4.53E+15		
	Total	1.76E+17	41			
nirS * Treatment	Between Groups (Combined)	1.32E+16	13	1.02E+15	.759	.693
	Within Groups	3.75E+16	28	1.34E+15		
	Total	5.08E+16	41			

Table 2.3.6.3

The table shows the results obtained performing the test Anova on the nitrogen cycle gene copy number detected 58 days after fertilization, in respect to the different treatments.

In the cases of the denitrification genes *nirK* and *nirS*, the differences that can be observed in the Figure 2.3.6.4, were not statistically significant (see table 2.3.6.3). The number of *nirK* copies detected in the unfertilized soil was very variable in the three replicates, and therefore the influence of fertilization on this gene could not be clearly inferred. Also in the case of *nirS* gene, the comparison with the unfertilized soil was not easy, as the number of *nirS* copies was relatively high also in this soil sample.
The results reported in this paragraph are in agreement with observations published in recent scientific works. In detail, it was observed that ammonia-oxidizing bacteria and archaea react differently in the presence of fertilization. Archaeal nitrifiers, which were detected at high levels in these low-nutrient agricultural soils also before fertilization, did not respond with significant fluctuations to the addition of the fertilization treatments. These "indifference" of archaeal nitrifiers to external inputs was reported also in several other scientific reports (Habteselassie *et al*., 2013; Li *et al*., 2011; Chen *et al*., 2013; Gong *et al*., 2013; Hussain *et al*., 2011; Wang *et al*., 2013; Wertz *et al*., 2012; Fan *et al*., 2011). The same papers report, on the other hand, a significant response of bacterial nitrifiers to external treatments, similarly to the results reported here. From these observations, an hypothesis on the possible ecological strategy adopted from these two groups of microorganisms can be defined. Effectively, the presence of archaeal ammonia-oxidizers at high levels, especially in low-nutrient soils, as reported by Di *et al*., 2010, Schleper, 2010, and Verhamme *et al*., 2011, and their stability also in the presence of different treatments, could indicate that these microorganisms adopt a K-selection strategy. *K-strategists*, indeed, tend to be more abundant, to grow slowly in number and to have slower and minor responses to external inputs. Bacterial nitrifiers, on the other hand, seem to have adopted a R-selection strategy, which characterizes organisms living in the presence of unstable environments and able to reproduce at high rates, often exponentially, in the presence of favourable conditions. These kinds of organisms are usually present at lower number in their environment, in stable conditions. Similarly, also denitrifying microorganisms respond to the changing of environmental conditions (Chen *et al*., 2012; Hallin *et al*., 2009; Paranychianakis *et al*., 2013), and could be therefore considered *R-strategists*.

2.3.6.4 Analysis of the correlation among nitrogen cycle genes, in the presence of diverse fertilization treatments

Pearson's correlation coefficient was calculated among the five genes considered in this study, in order to determine if in these conditions some groups of bacteria tended to have similar or opposite growth trends. The results of this test are shown in table 2.3.6.4. The bacterial *amoA* gene presented significant positive correlations with the three genes of denitrification.

The correlation between the growth of bacterial nitrifiers and denitrifiers, which was detected before in this thesis work (paragraph 2.3.3.3 and 2.3.4.9), was confirmed also in this case. Apparently, nitrifying and denitrifying bacteria are strictly connected as, on the one hand, denitrifiers, when using complex organic matter, may release ammonia that could be then used by nitrifiers and, on the other hand, the products of nitrification, nitrates and nitrites, are the substrates used by denitrifying organisms.

Table 2.3.6.4 also shows the significant positive correlation of the *nosZ* gene, representing the last step of denitrification, with both *nirS* and *nirK*, the genes used upstream in the same process. This result was expected, as *nosZ* gene is known to be present in organisms presenting also the *nirS* or the *nirK* gene in their genome, while the opposite is not always true.

Correlations

		Archaeal *amoA*	**Bacterial *amoA***	***nirK***	***nirS***	***nosZ***
Archaeal *amoA*	Pearson Correlation	1.000	-0.038	0.050	0.117	0.077
			0.721	0.639	0.270	0.468
	N	91	91	91	91	91
Bacterial *amoA*	Pearson Correlation	-0.038	1.000	0.330**	0.295**	0.315**
		0.721		0.001	0.005	0.002
	N	91	91	91	91	91
nirK	Pearson Correlation	0.050	0.330**	1.000	0.106	0.240*
		0.639	0.001		0.318	0.022
	N	91	91	91	91	91
nirS	Pearson Correlation	0.117	0.295**	0.106	1.000	0.457**
		0.270	0.005	0.318		0.000
	N	91	91	91	91	91
nosZ	Pearson Correlation	0.077	0.315**	0.240*	0.457**	1.000
		0.468	0.002	0.022	0.000	
	N	91	91	91	91	91

**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

Table 2.3.6.4
Pearson's correlation (2-tailed) among the 5 genes considered.

2.3.6.5 Link between plant growth and abundance of microorganisms involved in the nitrogen cycle in the presence of fertilization

Pearson's correlation coefficient was also calculated considering together the Real Time PCR results on the genes of the nitrogen cycle, and the plant growth measurements obtained for the same pots (see paragraph 2.3.5 for plant growth results). These calculations were done in order to determine whether the abundance of given nitrogen cycle organisms could be connected with plant growth and health (table 2.3.6.5).
The three plant growth parameters taken into account, cumulative height, fresh weight and dry weight, resulted to be significantly positively correlated with the abundance of the bacterial *amoA* gene. Moreover, both cumulative fresh and dry weight were positively correlated with the denitrification gene *nosZ*. This last result was not expected, as the process of denitrification has the effect of subtracting nitrogen from soil, through its release in a gaseous form, and should therefore have a negative effect on soil fertility. A possible explanation for the positive correlation of denitrifying organisms with plant growth, is that these microorganisms, since they are heterotrophs and therefore contribute to the mineralization of the organic matter, likely release ammonia during this process, which can give a positive contribute to soil fertility.

Correlations

		Archaeal *amoA*	Bacterial *amoA*	*nosZ*	*nirS*	*nirK*
Cumulative height	Pearson Correlation	-0.075	0.316**	0.204	0.080	-0.002
		0.496	0.003	0.062	0.469	0.987
	N	84	84	84	84	84
Cumulative fresh weight	Pearson Correlation	-0.001	0.363**	0.329**	0.151	0.006
		0.993	0.001	0.002	0.170	0.959
	N	84	84	84	84	84
Cumulative dry weight	Pearson Correlation	-0.017	0.353**	0.330**	0.146	0.014
		0.878	0.001	0.002	0.186	0.901
	N	84	84	84	84	84

*. Correlation is significant at the 0.05 level (2-tailed).
**. Correlation is significant at the 0.01 level (2-tailed).

Table 2.3.6.5
Pearson's correlation (2-tailed) between the Real Time PCR results on the genes of the nitrogen cycle in the different soil samples, and the plant growth measurements obtained for the same pots.

To better comprehend the link between plant growth and nitrification and denitrification, the calculation of the Pearson's coefficient was done also considering separately the Real Time PCR results obtained for the soils sampled at the two different sampling points, day 9 and day 58. The results of these tests are shown in the tables 2.3.6.6 and 2.3.6.7.
Bacterial *amoA* gene resulted to be positively correlated with the three plant growth parameters only at day 9, with higher correlation values for fresh and dry weight, while

at day 58 only the plant height had a significant correlation with this gene, although not particularly strong. The denitrification gene *nosZ*, on the other hand, presented significant positive correlation, with relatively high values, for fresh and dry weight only after 58 days from fertilization.

Correlations Day 9

		Archaeal *amoA*	**Bacterial *amoA***	***nosZ***	***nirS***	***nirK***
Cumulative height	Pearson Correlation	-0.007	0.315*	0.052	0.172	0.161
		0.963	0.042	0.745	0.276	0.310
	N	42	42	42	42	42
Cumulative fresh weight	Pearson Correlation	0.026	0.438**	0.119	0.201	0.110
		0.871	0.004	0.451	0.202	0.488
	N	42	42	42	42	42
Cumulative dry weight	Pearson Correlation	0.042	0.427**	0.095	0.218	0.107
		0.793	0.005	0.549	0.165	0.501
	N	42	42	42	42	42

*. Correlation is significant at the 0.05 level (2-tailed).
**. Correlation is significant at the 0.01 level (2-tailed).

Table 2.3.6.6

Pearson's correlation (2-tailed) between the Real Time PCR results on the genes of the nitrogen cycle in the different soil samples obtained at sampling day 9, and the plant growth measurements obtained for the same pots.

Correlations Day 58

		Archaeal *amoA*	**Bacterial *amoA***	***nosZ***	***nirS***	***nirK***
Cumulative height	Pearson Correlation	0.001	0.325*	-0.189	0.244	0.035
		0.993	0.036	0.232	0.119	0.824
	N	42	42	42	42	42
Cumulative fresh weight	Pearson Correlation	-0.006	0.278	-0.103	0.449**	0.236
		0.970	0.074	0.515	0.003	0.132
	N	42	42	42	42	42
Cumulative dry weight	Pearson Correlation	-0.002	0.269	-0.114	0.439**	0.227
		0.991	0.085	0.474	0.004	0.148
	N	42	42	42	42	42

*. Correlation is significant at the 0.05 level (2-tailed).
**. Correlation is significant at the 0.01 level (2-tailed).

Table 2.3.6.7

Pearson's correlation (2-tailed) between the Real Time PCR results on the genes of the nitrogen cycle in the different soil samples obtained at sampling day 58, and the plant growth measurements obtained for the same pots.

Bacterial *amoA* gene, therefore, could be used as a premature indicator of soil quality, while *nosZ* gene could be a tardive indicator. At day 9, it is likely that, in soils, bacteria only went through one or two generations, so it is notable that bacterial nitrifiers present a significant correlation with plant growth already after such a short period. Day 58, on the other hand, represents a more settled situation. In this circumstance, the equilibrium that bacterial *nosZ*-denitrifiers reached over time is more in line with the plant growth results.

These results, together with the observations done before that the same two genes, bacterial *amoA* and *nosZ*, gave significant evidences in the influence of fertilization on soil nitrogen cycle, lead to the possible selection of these two genes as the best to evaluate the quality of soils from a nitrogen-cycle-equilibrium point of view. Moreover, bacterial *amoA* and *nosZ* genes could be useful proxies to predict crop productivity.

2.3.7 First insights into soil community changes in response to the presence of different kinds of fertilizers

2.3.7.1 T-RFLP results on DNA

The T-RFLP community profiles of 16S rRNA gene from soils subject to 14 conditions (different fertilization and different starting soils), sampled on day 0, day 1, day 9, day 30 and day 58, were singly analysed and subsequently compared among each other and with the unfertilized control soil (4 replicates each). Numerical data corresponding to the T-RFLP peaks obtained using GeneMapper software (Applied Biosystems) and the free source online software T-REX, were used for all the analyses.
Principal Component Analysis (PCA, results not shown) was performed in order to make a first evaluation of the distribution of the soil samples, and to define possible trends or groups. Analyses were made separately for each single treatment, and in all the cases it was possible to observe that soils sampled in the same day formed defined groups. The first two principal components used for these analyses could explain in all the cases between 30% and 40% of the variation. These low percentage values could be explained by the relevant differences among soil samples. Soil is indeed a very patchy matrix.
For the fertilizers THL, Castor Cake, Neem, and UTN, soils from day 9 separated neatly from all the others, soils from day 30 and those from day 58 grouped together while soils from day 1 formed a quite unite group which was in most of the cases in the vicinity of the day 30- and day 58-samples. These results could indicate that the major effect of these fertilizers on the bacterial community in soil occurred after about 10 days, and that after 1 month the community had already reached an equilibrium that did not change significantly during the following month.
UTY showed a slightly different situation, with the samples from day 1, day 30 and day 58 forming 3 separated but consecutive groups, and samples from day 9 rather apart. With the fertilizer OATr, the samples separating from the others were those sampled after 2 months. This difference could reflect the slow-release effect of this fertilizer. It was instead not possible to define groups or precise trends for soils treated with Yeast and for untreated soils (Ctr).
133 TRFs were obtained among all the 221 DNA samples considered.
The contribution of each TRF to the total diversity was measured summing the peaks' numerical data of a determined TRF in all the samples and calculating the percentage on the total. Only 4 TRFs (2.3%) were contributing for more than 6% to the total diversity, 26 (15%) for 1-5%, while the other 143 were all present in a percentage lower than 1%.
The sum of the TRFs numerical data was also calculated for each sample, as a measure of their bacterial richness.

Sampling time	Fertilizer	Contribution to microbial community (DNA)	Sampling time	Fertilizer	Contribution to microbial community (DNA)
1	THL	232.00	1	oUTY	9450.50
9	THL	4153.75	9	oUTY	-26.75
30	THL	1937.00	30	oUTY	-1957.75
58	THL	-1534.25	58	oUTY	-1998.00
1	OATr	2393.75	1	UTN	7995.00
9	OATr	611.75	9	UTN	-41.00
30	OATr	-1008.25	30	UTN	917.25
58	OATr	-1030.50	58	UTN	-1024.00
1	CC	1439.25	1	oUTN	12802.25
9	CC	876.50	9	oUTN	-274.50
30	CC	-564.00	30	oUTN	-906.25
58	CC	3253.25	58	oUTN	-960.25
1	oCC	16726.50	1	Y	7625.25
9	oCC	6482.75	9	Y	7242.25
30	oCC	1104.00	30	Y	422.50
58	oCC	1288.00	58	Y	-733.00
1	N	4963.25	1	oY	1048.50
9	N	-67.50	9	oY	7424.25
30	N	-1796.75	30	oY	937.25
59	N	-1705.00	58	oY	355.75
1	oN	19298.00	1	OATr	863.75
9	oN	1046.00	9	OATr	-1340.00
30	oN	330.50	30	OATr	-401.25
58	oN	83.25	58	OATr	-472.25
1	UTY	9823.00			
9	UTY	-1742.50			
30	UTY	264.50			
58	UTY	-1989.50			

Table 2.3.7.1

In the table, the sums of mediated contributions of every 16S TRF to each treated soil sample after subtraction of the same in the control soil sample are reported. The values reported give an indication of the increase or decrease of community richness and abundance in response to fertilization through the direct comparison with the unfertilized soil. In the table, positive values, which indicate a major quantity of 16S rRNA gene copies in the treated samples in respect to the untreated control sample, are coloured in black, while negative values are highlighted in red. In the figure, abbreviations are used to indicate the soil samples: the first letter "o" indicates that the fertilizer was added to "old soils", meaning soils fertilized in the same way the year before; the names of the fertilizers are indicated as follows: THL = Thermal Hydrolysed Leather, CC =Castor Cake, N = Neem, Y = Yeast, OATr = Oxy-amino-triazine, UTY = Urea-THL-Y, UTN = Urea-THL-N.

Peaks' numerical data of each TRF were mediated for the 4 replicates of each treatment, and these mean-data were used for further analyses.

In order to evaluate the effect of fertilization on the richness of bacterial communities, the sum of mediated contributions of every TRF to each treated soil sample was subtracted with the same in the control soil sample. In this way, it was possible to

determine when, among the 4 sampling times, soil bacterial communities were richer and most affected by the addition of the different fertilizers (Table 2.3.7.1).
In the table, positive values, which indicate a major quantity of 16S rRNA gene copies in the treated samples in respect to the untreated control sample, are coloured in black, while negative values are highlighted in red. At day 1, all the treated soils showed an increase in the number of microorganisms, which could be due to the presence of a considerable number of bacteria in the fertilizers. In most of the cases, this number decreased rapidly already after 10 days, and was negative at the last sampling point, indicating the readsorption of the bacteria coming from the fertilizers into the soil community.
For the soil samples Castor Cake old, Neem old, and Yeast old, the results were different: even if the ratio between TRFs-sum in the treated soils and in the untreated soil was decreasing over time, both at day 30 and at day 58 this value resulted to be positive, showing a better capacity of resisting to fluctuations of these soil communities, due to the presence of previously adapted microorganisms which had grown the year before after the addition of the same fertilizer.
Subsequently, DNA extraction and T-RFLP experiments were performed on pure fertilizers, in order to better explain these observations. It was possible to extract DNA from the fertilizers THL, Castor Cake, Neem, UTY and UTN, while extractions from Yeast and OATr resulted in no yeald of DNA.
Numerical data from these last T-RFLP experiments, together with mean values for each treatment, were used for further statistical analyses.
The neighbour joining tree obtained using Bray Curtis Distance (Figure 2.3.7.1) showed that the pure fertilizers formed a single cluster, which was located near another separated cluster composed by soils sampled at day 1. This result is in line with the previous observations and with the consideration that the fertilizers themselves would carry a noteworthy bacterial population.
From the neighbour joining tree the separation of the samples from day 9 in a cluster, and the mixture of samples from day 30 and day 58, were also confirming the PCA plots discussed above.

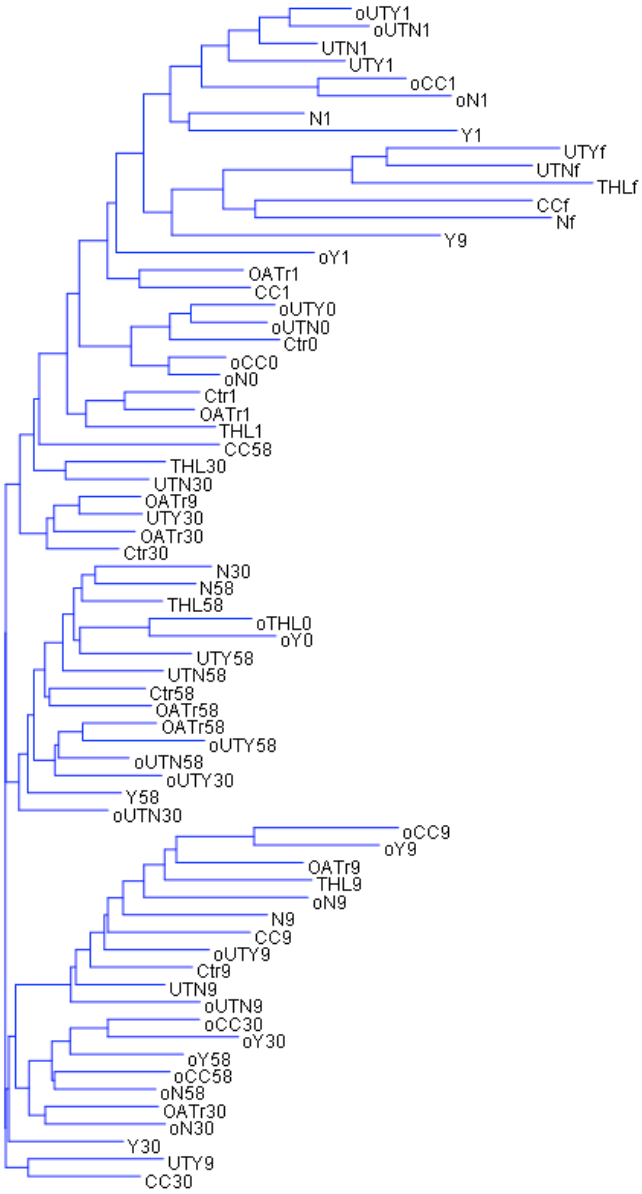

Figure 2.3.7.1

Neighbour joining tree obtained using the mean values for each condition (fertilizer type and sampling time). The Bray Curtis Distance was used to obtain the tree. In the figure, abbreviations are used to indicate the soil samples: the first letter "o" indicates that the fertilizer was added to "old soils", meaning soils fertilized in the same way the year before; the names of the fertilizers are indicated with THL = Thermal Hydrolysed Leather, CC =Castor Cake, N = Neem, Y = Yeast, OATr = Oxy-amino-triazine, UTY = Urea-THL-Y, UTN = Urea-THL-N, Ctr = unfertilized soil; the number indicates the sampling day (0, 9, 30 or 58).

2.3.7.2 Investigation of the changes at soil community level through analysis of diversity indices calculated from T-RFLP results

The main diversity indices were calculated for each single soil sample: taxa number S, Simpson's diversity index 1-D, Shannon's index H, and Evenness e^H/S. Statistical analyses were made on the values obtained, specifically to determine whether the differences among samples, or treatments, or sampling day were significant.
In figure 2.3.7.2, the mean values for each sample of the four considered indices are shown.
The mean of the number of taxa varied between 10 and 40 considering all the samples, and it could be noticed that in some cases a strong variation occurred also within replicates. Particularly evident are the high values of the standard deviations for the samples "Castor Cake old, day 1", "Neem old, day 1", "UTY old, day 1", "UTN old, day 1", "UTY, day 1", and "UTN, day 1". These strong variations could all be detected in soils sampled 1 day after the application of the treatments, and this fact could indicate a different immediate response of bacterial communities present in soils before the application, with the growth of a different number of bacterial species, an effect that could be influenced by the addition of a significant number of microorganisms present in these fertilizers, that in turn could be reflected in a different number of TRFs detected in T-RFLP experiments.
This situation occurred in four of the six soils that had already been treated with the same fertilizer the year before. Fluctuations in the number of taxa could be noticed in the treated soils during time, normally with higher values at day 1, indicating, once again, the contribution of the bacterial assemblages present in the fertilizers to soil community. Indeed, this trend is not clear in the control sample (indicated with Ctr in the graphs). However, the number of taxa did not increase at day 1 in the cases of Yeast and Yeast old. In these cases, the number of taxa was higher at day 9, and this fact could indicate that the addition of the fertilizer did not bring a vast number of new species to the soils. The increase in the number of taxa after about 10 days could indicate the need for a longer adaptation period for some species to grow in the presence of this peculiar fertilizer.
The test Anova, the results of which are reported in the table 2.3.7.2, indicated significant differences among samples' taxa number means.

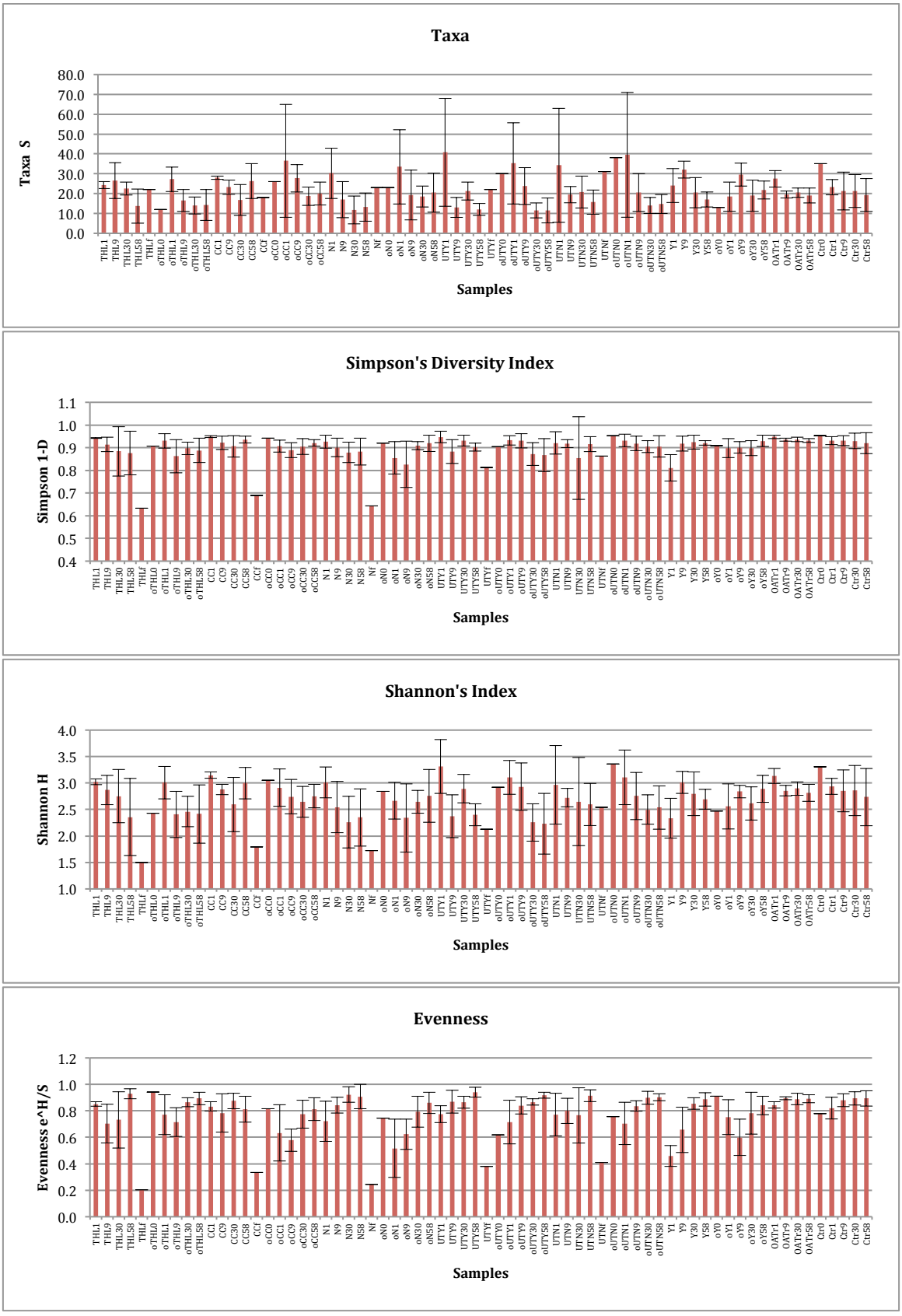

Figure 2.3.7.2

The four graphs report the results obtained for the four considered diversity indices (Taxa number S, Simpson's index 1-D, Shannon's index H and Evenness index e^H/S) in the soil

samples. Error bars represent the standard deviations. In the figure, abbreviations are used to indicate the soil samples: the first letter "o" indicates that the fertilizer was added to "old soils", meaning soils fertilized in the same way the year before; the names of the fertilizers are indicated with THL= Thermal Hydrolysed Leather, CC = Castor Cake, N = Neem, UTY = Urea-THL-Y, UTN = Urea-THL-N, Y = Yeast, OATr= Oxy-amino-triazine, Ctr = control unfertilized soil; the number indicates the sampling day (0, 9, 30 or 58).

Not many differences could be noticed when looking at the results obtained for the Simpson's diversity index. This index (1-D) represents the probability that two individuals randomly selected from the population will belong to different species. The only observation that could be done was that diversity is lower in the pure fertilizers, when compared to the soil samples. This result is not surprising, as the richness in species of soil is unlikely to be found in fertilizers, even if they are organic. However, the level of diversity of the fertilizer UTN, indicated with UTNf in the graph, is as high as some soil samples.

Simpson's index for all the soil samples considered resulted in a value between 0.8 and 0.95 (the maximum value is 1), indicating high diversity in all soils, treated or untreated. This is probably due to the high number of bacterial taxa detected.

The Shannon's index quantifies the uncertainty in predicting the species identity of an individual taken at random from the total population. It also indicates the diversity of the sample, but in a different way from Simpson's index.

In this case, we could observe more evident fluctuations of diversity among soil samples, although high standard deviations do not permit precise interpretations of the results. However, some trends could be observed; soils treated with the two composite fertilizers UTY and UTN showed similar fluctuations in diversity over time, with a gradual decrease from day 1 to day 58, both in the old samples and in soils treated for the first time.

Again, from these results, the lower level of diversity of the fertilizers could be detected.

Also in this case, the test anova, reported in the table 2.3.7.2, indicated that significant differences among these soil samples are present, for what is concerning the two diversity indices.

Another important ecological index considered in this analysis is Evenness. This index is a measure of the relative abundance of the different species making up the richness of a sample. The evenness of a population increases when the number of individuals is evenly distributed among the species present.

Firstly, we could report that also the evenness is significantly lower in the pure fertilizers, especially in the fertilizers THL and Neem. This fact could indicate the presence of a peculiar bacterial population in the fertilizers, most probably dominated by one or few single species. It could be hypothesized that THL and Neem represent

matrices that permit the growth of only few bacterial species, while Castor Cake, UTY and UTN could be slightly better growth media for bacteria.
In general, a first decrease in evenness after the application of the fertilizer, followed by its increase over time, could be observed. Nevertheless, also with this index, high standard deviations within the replicates was reported, most likely determined by the fact that soil is a patchy matrix and in different pots, even if treated in the same way, microbial population might evolve slightly differently.
The decrease in evenness after fertilization is mostly evident with the fertilizer Yeast, when comparing with the unfertilized soil (samples Ctr0 or Ctr1). This fact reflects the observation done above, that the treatment Yeast most probably changes significantly the population of soil.
The Anova test shows, also for this index, the significance of the differences among some samples (Table 2.3.7.2).

			Sum of Squares	df	Mean Square	F	Sig.
Taxa * Sample	Between Groups	(Combined)	12250.99	67	182.85	1.604	.008
	Within Groups		19151.50	168	114.00		
	Total		31402.49	235			
Simpson * Sample	Between Groups	(Combined)	.40	67	.01	2.474	.000
	Within Groups		.40	168	.00		
	Total		.80	235			
Shannon * Sample	Between Groups	(Combined)	19.78	67	.30	1.856	.001
	Within Groups		26.72	168	.16		
	Total		46.50	235			
Evenness * Sample	Between Groups	(Combined)	3.81	67	.06	5.167	.000
	Within Groups		1.85	168	.01		
	Total		5.66	235			

Table 2.3.7.2
In the table, the results obtained from the Anova test done considering the four diversity indices in respect to the different soil samples are reported.

The Anova test was performed also to detect possible significant differences in the mean values of the studied diversity indices considering the single treatments. As it is reported in the table 2.3.7.3, no significance was detected analysing the mean values of the treatments obtained for the indices Taxa, Simpson's index and Shannon's index. Differences were instead significant when considering evenness. Mean values and standard deviations are reported in the graph in figure 2.3.7.3. The highest values of evenness, with the lowest standard deviations, could be observed in the unfertilized sample, and in the soil treated with the slow-release fertilizer OATr. In the first case, the control soil, evenness was not influenced by the presence of any treatment, and therefore remained quite stable. For OATr, instead, this result could indicate a low effect on the equilibrium present in the soil before the addition of the treatment. Yeast, as already explained above in this paragraph, caused a decrease of evenness in soil, together with the soil samples "Castor Cake old" and "Neem old". The fact that these two

last samples presented lower values of evenness when compared to the respective non-old samples could be due to the persistence of a previously established bacterial community in the old soils that, especially in the first period after the treatment, responded more rapidly to the addition of it and grew faster than other less-adapted organisms, causing a decrease in evenness in the first days.

			Sum of Squares	df	Mean Square	F	Sig.
Taxa * Treatment	Between Groups	(Combined)	861.369	13	66.259	.482	.933
	Within Groups		30541.118	222	137.573		
	Total		31402.487	235			
Simpson * Treatment	Between Groups	(Combined)	.059	13	.005	1.370	.175
	Within Groups		.736	222	.003		
	Total		.796	235			
Shannon * Treatment	Between Groups	(Combined)	2.965	13	.228	1.163	.309
	Within Groups		43.539	222	.196		
	Total		46.504	235			
Evenness * Treatment	Between Groups	(Combined)	.696	13	.054	2.396	.005
	Within Groups		4.962	222	.022		
	Total		5.658	235			

Table 2.3.7.3

In the table, the results obtained from the Anova test done considering the four diversity indices in respect to the different soil treatments are reported.

Anova test performed to detect significant differences in the mean values of the studied diversity indices considering the sampling day (included the pure fertilizers) gave interesting results. In fact, the four diversity indices all gave significant results (Table 2.3.7.4). In figure 2.3.7.4, the mean values and the standard deviations obtained, are graphically shown.

The average number of taxa was higher in the soils sampled at day one, as already observed previously for some samples, with a high standard deviation, likely indicating, as stated before, different behaviours of soil communities in the different pots as a response to fertilization. The number of taxa then decreased gradually over time, on average. The average number of taxa in soils sampled at day 30 ad at day 58 is, surprisingly lower than in pure fertilizers.

As already observed before, the Simpson's index did not exhibit significant differences among soil samples, as all samples had values near 1. The only significant difference was found between soils and pure fertilizers, which presented a lower level of diversity. Similar results could be found observing the Shannon's index, even if here also a weak trend could be noticed, where diversity slightly decreased gradually over time, with very similar values after 30 and 58 days.

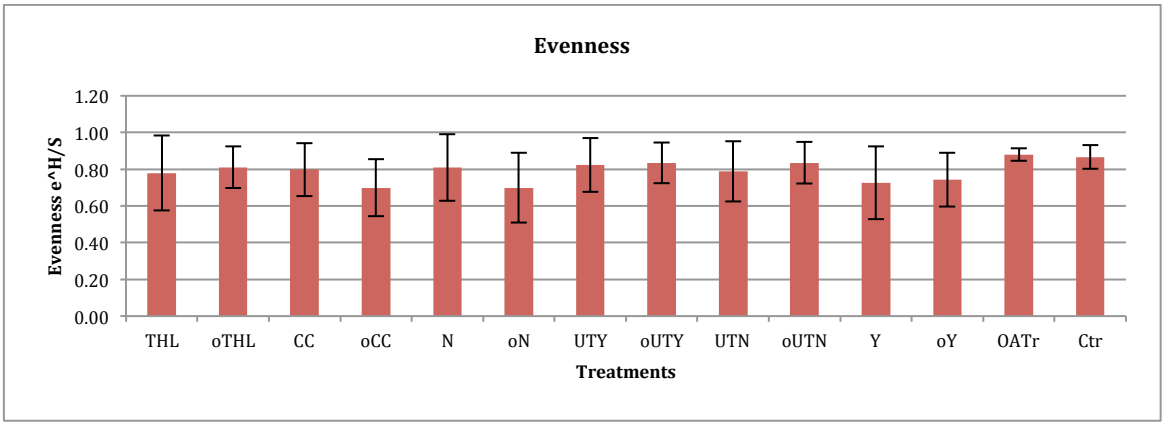

Figure 2.3.7.3

Mean values of the evenness index obtained for the different treatments. The error bars represent the standard deviations. In the figure, abbreviations are used to indicate the soil samples: the first letter "o" indicates that the fertilizer was added to "old soils", meaning soils fertilized in the same way the year before; the names of the fertilizers are indicated as follows: THL = Thermal Hydrolysed Leather, CC =Castor Cake, N = Neem, Y = Yeast, OATr = Oxy-amino-triazine, UTY = Urea-THL-Y, UTN = Urea-THL-N, Ctr = unfertilized soil.

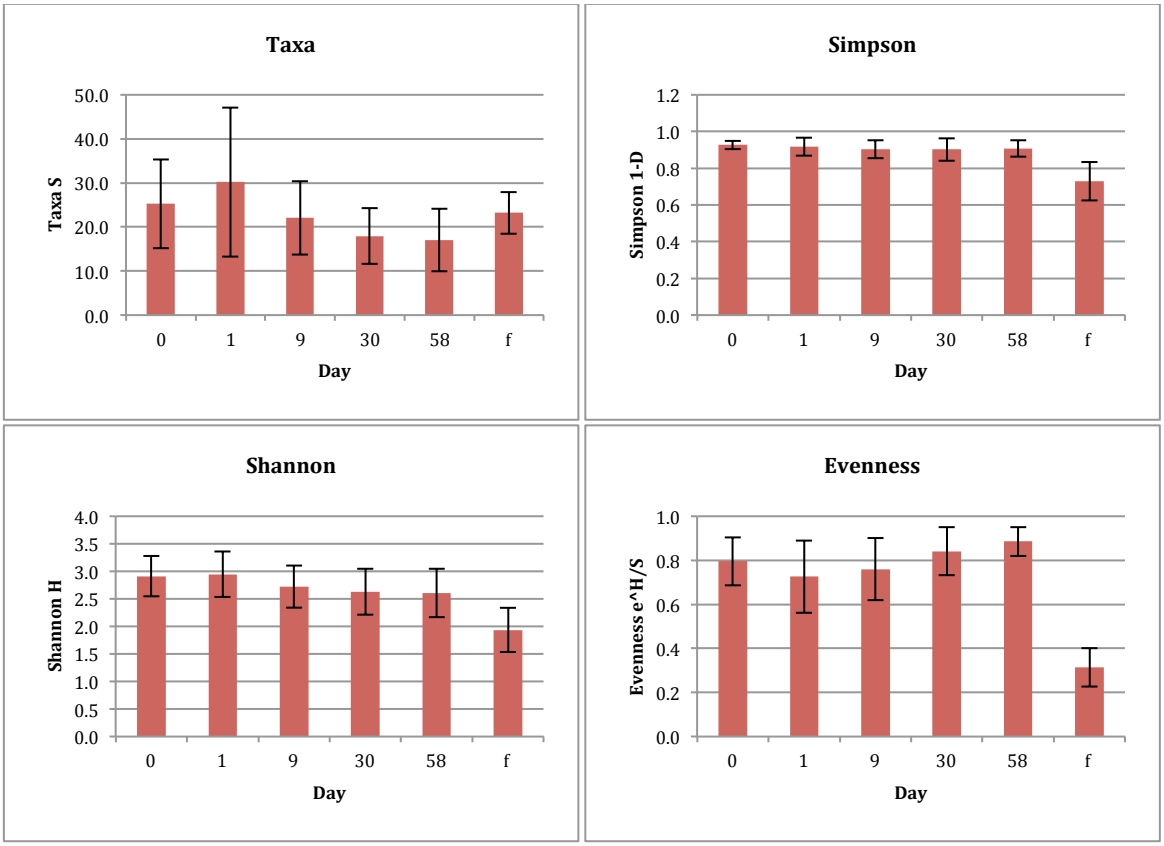

Figure 2.3.7.4

Mean values of the taxa number (S), Simpson's index (1-D), Shannon's index (H) and evenness index (e^H/S) obtained for the 5 sampling days and for the DNA samples extracted directly from the fertilizers. The error bars represent the standard deviations.

From the graph concerning evenness, the trend that seems to unfold is that it decreased just after the addition of the treatment, and therefore of the arrival of an important number of new microorganisms and of new substances, and subsequently increased over time to reach a more equilibrated situation after two months. Also from this graph, the low evenness of the pure treatments could be identified.

			Sum of Squares	df	Mean Square	F	Sig.
Taxa * Day	Between Groups	(Combined)	6133.437	5	1226.687	11.165	.000
	Within Groups		25269.050	230	109.865		
	Total		31402.487	235			
Simpson * Day	Between Groups	(Combined)	.167	5	.033	12.264	.000
	Within Groups		.628	230	.003		
	Total		.796	235			
Shannon * Day	Between Groups	(Combined)	7.297	5	1.459	8.562	.000
	Within Groups		39.206	230	.170		
	Total		46.504	235			
Evenness * Day	Between Groups	(Combined)	2.077	5	.415	26.669	.000
	Within Groups		3.582	230	.016		
	Total		5.658	235			

Table 2.3.7.4
In the table, the results obtained from the Anova test done considering the four diversity indices in respect to the sampling times are reported.

2.3.7.3 T-RFLP results on cDNA

16S rRNA gene amplification and T-RFLP experiments were also performed on cDNA preparation from the same soil samples.
The same kind of data processing was performed as for DNA samples.
132 TRFs were detected among the 187 samples from which amplification and subsequent processing was possible.
From the PCA plots, no precise groups could be defined, even if a trend could be seen, in which the soils sampled in the same day were positioned in the same area of the plot.
The grouping based on the sampling time was more evident from the neighbour joining tree obtained using Bray Curtis Distance (Figure 2.3.7.5); in fact, the 3 clusters corresponding to day 0, day 1 and day 9 were clearly separated from all the other soils sampled at day 30 and day 58.
As for the DNA, also for cDNA the contribution of each TRF to the total active community was calculated summing the numerical data corresponding to a determined TRF in all the samples, and subsequently calculating the percentage on the total. Three TRFs were found to have high presence percentage in the samples (14.6%, 12.5% and 9.78%), and therefore were corresponding to bacteria with high activity in those soils. Percentages between 6.5% and 1% were calculated for other 19 TRFs, while all the rest (150 TRFs) had a percentage of less than 1%.

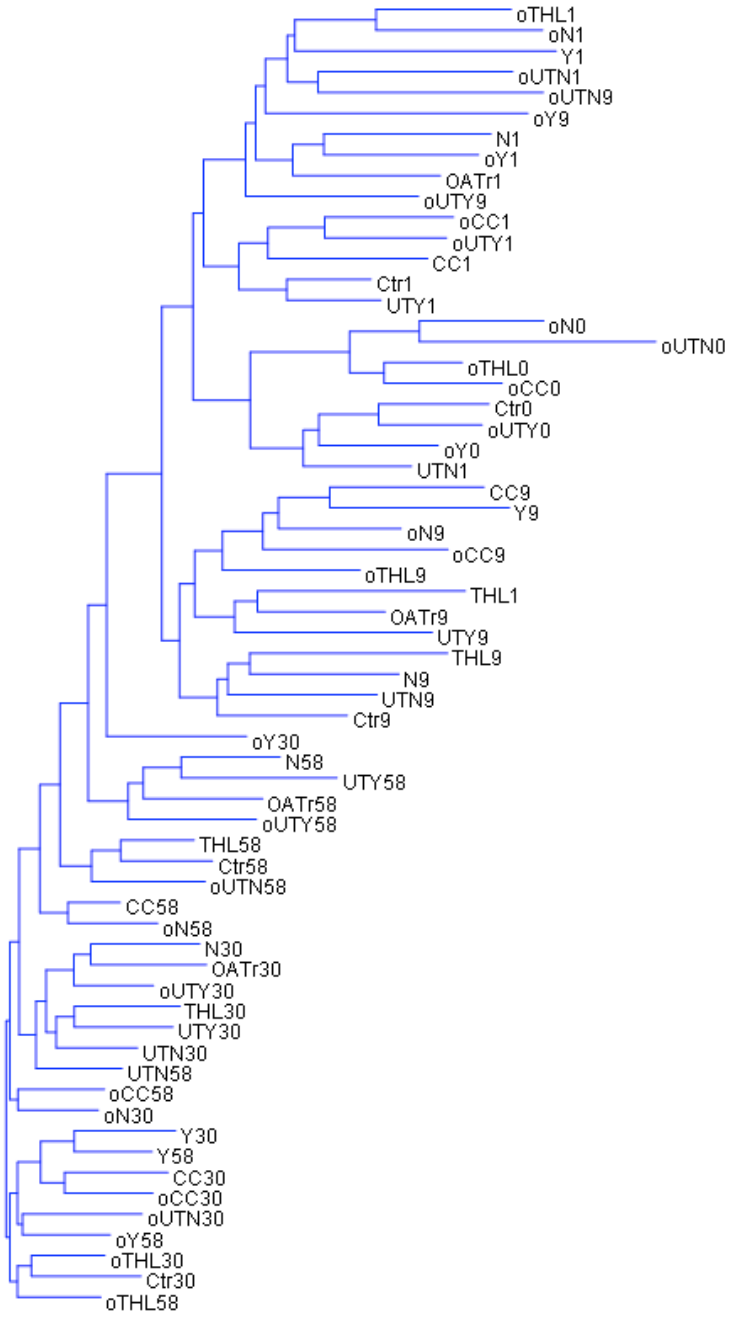

Figure 2.3.7.5

Neighbour joining tree obtained using the mean values for each condition (fertilizer type and sampling time). The Bray Curtis Distance was used to obtain the tree from the T-RFLP results obtained from cDNA samples. In the figure, abbreviations are used to indicate the soil samples: the first letter "o" indicates that the fertilizer was added to "old soils", meaning soils fertilized in the same way the year before; the names of the fertilizers are indicated as follows: THL = Thermal Hydrolysed Leather, CC =Castor Cake, N = Neem, Y = Yeast, OATr = Oxy-amino-triazine, UTY = Urea-THL-Y, UTN = Urea-THL-N, Ctr = unfertilized soil; the number indicates the sampling day (0, 9, 30 or 58).

2.3.8 Metagenomic analysis of soils subject to different fertilization treatments sampled few days or two months after the beginning of the fertilization trial

As already mentioned, N inputs to soil can have important impacts on the environment, and many studies have focused on their effects on plant growth, nitrogen and carbon cycles, and emissions of greenhouse gases in the atmosphere (Tilman, 1987; Gough *et al*., 2000; Vitousek *et al*., 2002; Gilliam, 2006). Less well understood are the impacts of fertilizers' inputs on soil bacterial communities, even though bacteria represent a major portion of living biomass in terrestrial ecosystems (Fierer *et al*., 2009) and their activities are intimately tied to belowground processes. Some scientific works have already reported analyses of bacterial community shifts in response to N amendments or particular fertilization practices (Roesch *et al*., 2007; Nemergut *et al*., 2008; Ramirez *et al*., 2010; Campbell *et al*., 2010; Fierer *et al*., 2012), but in none of these works the effect of different commercial fertilizers on soil bacterial community was evaluated.

In order to have a better insight into microbial communities of soils treated with the different fertilizers considered, it was decided to perform a sequencing experiment with the 454 sequencing machine GS FLX Titanium Series (Roche).

After the analysis of T-RFLP results, it was decided to concentrate on the structure of soil bacterial communities, and therefore on DNA sample, to avoid sequencing the "old" samples and to consider the soils sampled at time 9 and at time 58. Indeed, no significant differences were found between the "old" samples and those fertilized for the first time, and they were grouping together both in the PCA plots and in the neighbour joining tree. Day 9 and day 58 were selected, as the first one seemed to represent a moment at which the soil bacterial communities had changed in respect to the beginning of the experiment and the addition of the fertilizers, and the second one corresponded to the conclusive microbial community, at its final equilibrium.

Metagenomic analysis was performed on 64 soil samples, representing 4 replicates of 8 treatments at two sampling times (day 9 and day 58). About 450000 raw sequences were generated by the 454 sequencing run, and a mean number of about 3800 sequences per sample were subsequently analysed. Using the primer pair 27f and 519r (see material and methods), the first two hypervariable regions and part of the third were taken into account, permitting in most of the cases the identification of bacterial genera.

The rarefaction analysis (Figure 2.3.8.1 A) highlighted the presence of much complex soil bacterial communities, with extensive microbial diversity. Moreover, the shape of the rarefaction curves for the single samples indicated that a higher number of sequences would have been necessary to detect all the species present in these soils. The two curves that resulted to have a lower number of observed species represent two of the four replicates of the soil treated with Yeast fertilizer and sampled at day 9. This

result is in line with the observation that will be done later in this chapter (paragraph 2.3.8.1.2, paragraph 2.3.8.1.6), showing that the fertilizer Yeast caused a decrease in evenness and in soil diversity in the first days after its addition.

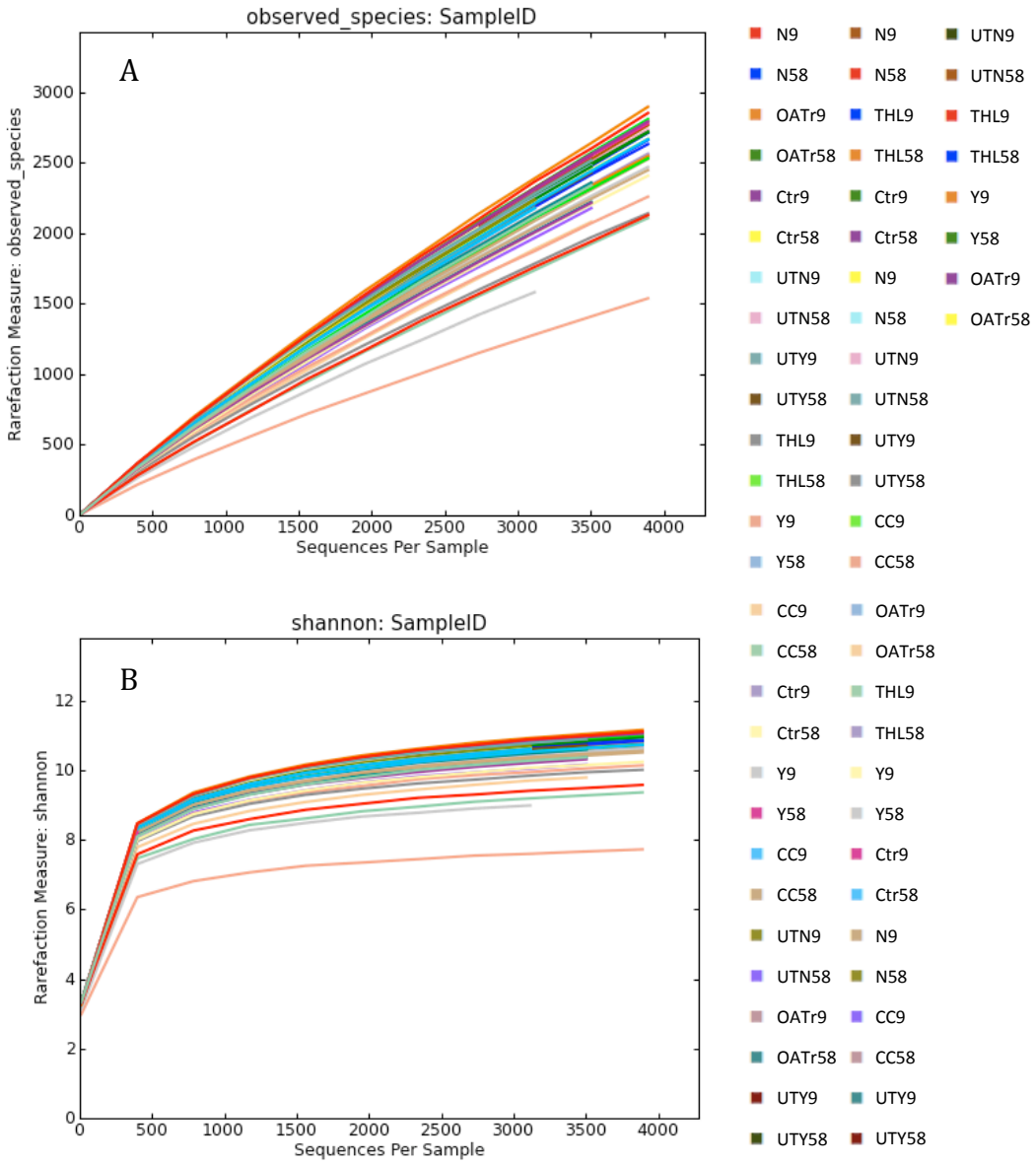

Figure 2.3.8.1

Diversity of the agricultural soil subject to different fertilization conditions. Rarefaction analysis (A) and Shannon indices (B) of 16S rRNA gene sequences from single soil samples. In the legend, the number represents the sampling time and the names of the fertilizers are indicated as follows: THL = Thermal Hydrolysed Leather, CC =Castor Cake, N = Neem, Y = Yeast, OATr = Oxy-amino-triazine, UTY = Urea-THL-Y, UTN = Urea-THL-N, Ctr = unfertilized soil.

In contrast, the Shannon index of community diversity stabilized within a relatively small number of sequences (Figure 2.3.8.1 B), suggesting that representative samples were obtained for estimating this diversity index.

This kind of situation, with rarefaction analysis showing that a much higher number of sequences would be required to approximate the number of OTUs in soil, was reported also in other scientific works (Campbell *et al*., 2010). Schloss and Handelsman (2006) suggested that as many as 18000 sequences per sample should be required to fully describe soil community diversity. However, this was not the main goal of this study, as here the focus is on the detection of the major changes in soil microbial communities induced by the presence of a certain fertilizer.

2.3.8.1 Analysis of soil bacterial communities at Phylum level

2.3.8.1.1 Analysis of microbial compositions of soils subject to different treatments

Considering the first level of taxonomy, the phyla, interesting observations could be done. Looking only at the effect of the treatment, and therefore the mean percentage over the four replicates and the two sampling points, some differences could be highlighted (Figure 2.3.8.2, Table 2.3.8.1).

While Proteobacteria were very abundant in all the treated soils and present at a percentage between 30% (in soil treated with Yeast) and 40% (in soil treated with UTY), more evident differences could be seen for Firmicutes, that were more abundant in the presence of the fertilizer Yeast (30%) and with the organic fertilizers THL (17%), Castor Cake (22%), and Neem (20%), while they were about 10% in the two composite urea-based fertilizers UTY, and UTN, and in the unfertilized soil, and only 6% in the oxy-amino-triazine fertilizer OATr. The relevant abundance of microorganisms belonging to the phylum Firmicutes in this kind of agricultural soil is rather interesting. In other types of soil, such as Tundra soil (Nemergut *et al*., 2008; Campbell *et al*., 2010), grassland sites and other agricultural soils (Ramirez *et al*., 2010; Fierer *et al.*, 2012) this bacterial group was not detected as one of the majors. In contrast, Roesch *et al*. (2007) listed Firmicutes in the prominent phyla present in soils.

The quantity of Acidobacteria and Actinobacteria also slightly changed in the presence of different fertilizers, and in particular they were less abundant in the sample Yeast. Bacteroidetes, on the other hand, were more abundant in this last sample (13%), while they were present at a percentage of 8 to 9 in THL, Castor Cake, Neem, OATr, unfertilized samples, and at a percentage of 6 to 7 in UTN and UTY. The growth of Chloroflexi, instead, seemed to be slightly impaired by organic fertilization, as they were more abundant (5%) in the unfertilized soil and in the soil fertilized with OATr, than in all the others (3% to 4%). The other phyla were present at percentages lower than 2% in all the samples.

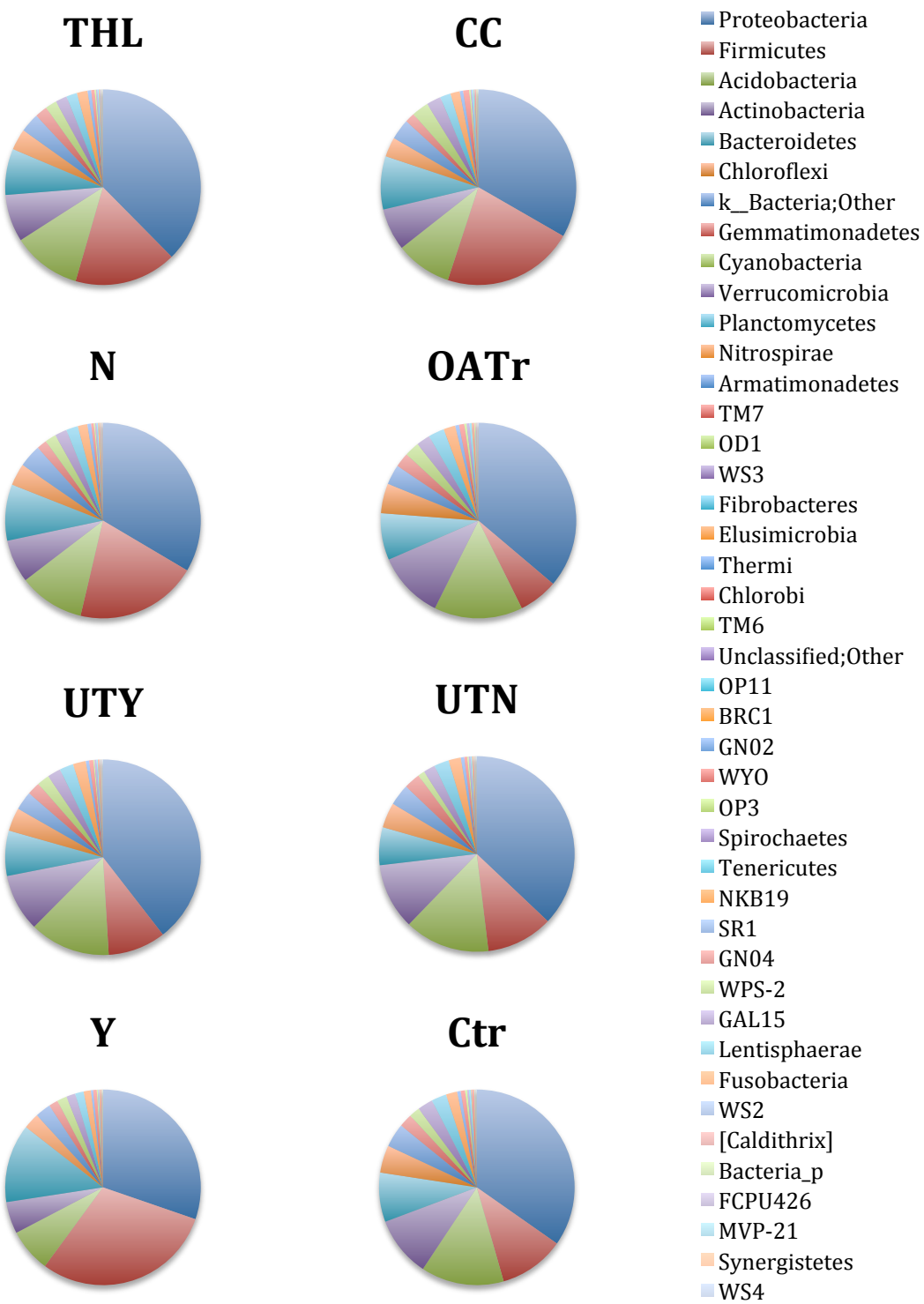

Figure 2.3.8.2

Pie charts showing the differences in bacterial communities of soils treated with different fertilizers or untreated. Graphs were constructed using the frequency of 16S rRNA sequences belonging to each bacterial group.

	Mean values of the % of the Phyla in soil samples							
Taxon	**THL**	**CC**	**N**	**OATr**	**UTY**	**UTN**	**Y**	**Ctr**
Acidobacteria	11.3884	9.2742	10.8795	14.6584	13.3610	14.1175	7.1891	13.7219
Actinobacteria	7.8231	6.9754	7.1161	11.1248	9.4916	10.9351	5.2591	9.9881
Armatimonadetes	0.6011	0.5466	0.6148	0.6304	0.5386	0.5642	0.3656	0.5850
Bacteroidetes	7.6492	8.8035	9.3080	7.7398	7.4780	6.2669	12.9895	8.1465
BRC1	0.0383	0.0577	0.0469	0.0647	0.0823	0.0489	0.0387	0.0542
[Caldithrix]	0.0029	0.0000	0.0000	0.0065	0.0000	0.0000	0.0000	0.0000
Chlorobi	0.0766	0.1609	0.1213	0.1940	0.1166	0.1204	0.1406	0.2853
Chloroflexi	3.5034	3.2463	3.6504	4.9368	3.8694	4.1642	2.6295	4.5463
Cyanobacteria	1.9212	2.8151	1.8610	2.4635	2.0582	1.1285	1.6312	1.7911
Elusimicrobia	0.1061	0.1184	0.1820	0.1907	0.1338	0.1542	0.1406	0.1553
FCPU426	0.0000	0.0000	0.0000	0.0000	0.0034	0.0000	0.0000	0.0000
Fibrobacteres	0.2239	0.2915	0.2233	0.3589	0.2230	0.2219	0.1301	0.3358
Firmicutes	16.9102	21.7613	20.1875	6.4983	9.5568	11.0292	29.8425	10.8331
Fusobacteria	0.0029	0.0000	0.0055	0.0000	0.0034	0.0038	0.0000	0.0000
GAL15	0.0059	0.0061	0.0110	0.0129	0.0000	0.0150	0.0000	0.0072
Gemmatimonadetes	2.0361	1.6155	1.6543	2.3827	2.1268	2.6896	1.5011	2.1774
GN02	0.0383	0.0425	0.0524	0.0679	0.0720	0.0489	0.0316	0.0506
GN04	0.0088	0.0091	0.0000	0.0129	0.0034	0.0038	0.0035	0.0072
Lentisphaerae	0.0059	0.0061	0.0028	0.0065	0.0069	0.0038	0.0070	0.0036
MVP-21	0.0000	0.0000	0.0000	0.0000	0.0103	0.0075	0.0000	0.0000
Nitrospirae	1.7679	1.5578	1.5853	1.9560	2.1302	2.0426	1.2093	1.8633
NKB19	0.0118	0.0061	0.0165	0.0226	0.0103	0.0150	0.0246	0.0108
OD1	0.2387	0.2520	0.2095	0.3815	0.1990	0.2332	0.1723	0.3647
OP11	0.0530	0.0121	0.0193	0.0679	0.0069	0.0188	0.0035	0.0253
OP3	0.0236	0.0456	0.0221	0.0517	0.0515	0.0301	0.0562	0.0289
Other	3.1882	3.4163	3.7552	3.3106	3.1010	3.5435	2.5874	3.9360
Planctomycetes	1.7856	1.7613	1.9658	2.6511	2.3052	2.4564	1.4167	2.5060
Proteobacteria	37.6098	33.3192	33.5070	36.1902	39.5102	37.0674	30.2819	34.7344
Spirochaetes	0.0236	0.0152	0.0441	0.0776	0.0755	0.0188	0.0070	0.0325
SR1	0.0118	0.0030	0.0028	0.0129	0.0137	0.0150	0.0105	0.0036
Synergistetes	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0036
Tenericutes	0.0236	0.0304	0.0221	0.0226	0.0343	0.0414	0.0141	0.0433
Thermi	0.0884	0.0820	0.0579	0.0388	0.0549	0.0564	0.0387	0.0614
TM6	0.0619	0.1002	0.0882	0.1293	0.0549	0.0865	0.0387	0.0939
TM7	0.5569	1.0052	0.4439	0.8729	0.6895	0.4815	0.5871	0.7186
Unclassified	0.0000	0.0030	0.0028	0.0032	0.0034	0.0038	0.0000	0.0072
Unclassified;Other	0.0530	0.0425	0.0634	0.0388	0.0515	0.0752	0.0387	0.0578
Verrucomicrobia	1.8828	2.3838	1.9686	2.2599	2.1885	1.9373	1.4905	2.5241
WPS-2	0.0088	0.0182	0.0000	0.0194	0.0000	0.0000	0.0070	0.0036
WS2	0.0029	0.0000	0.0055	0.0065	0.0103	0.0075	0.0035	0.0000
WS3	0.2387	0.2004	0.2592	0.4623	0.3396	0.3197	0.0984	0.2708
WS4	0.0000	0.0000	0.0000	0.0097	0.0034	0.0075	0.0000	0.0072
WYO	0.0265	0.0152	0.0441	0.0647	0.0309	0.0188	0.0141	0.0144

Table 2.3.8.1

Mean value, over the 4 replicates of each treatment and over time (day 9 and day 58), of the presence percentage of each Phylum in the analysed soils.

The major phyla detected in this soil were found to be predominating in general in soils (Roesch *et al*., 2007; Nemergut *et al*., 2008; Ramirez *et al*., 2010; Campbell *et al*., 2010; Fierer *et al*., 2012). These works, by comparing N fertilized soils with controls, also determined shifts in the abundance of determined bacterial groups, such as Bacteroidetes, some members of Proteobacteria and Acidobacteria (Ramirez *et al*., 2010; Fierer *et al.*, 2012), Gemmatimonadetes and Verrucomicrobia (Nemergut *et al*., 2008).

2.3.8.1.2 Analysis of the changes in bacterial soil composition over time at Phylum level

The figure 2.3.8.3 shows the changes in bacterial communities, at the level of Phylum, over time. Indeed, the abundance of some phyla was different at day 9 with respect to day 58 (Table 2.3.8.2, Table 2.3.8.3, Figure 2.3.8.3).
Acidobacteria raised in all the soil samples, except for OATr, where it remained stable. In general, Bacteroidetes and Firmicutes, which were very abundant at day 9, tended to decrease over time. This was particularly evident in soils treated with Yeast, where Firmicutes were present at significantly higher levels (45%) when compared to all other treatments (8% in OATr to 31% in Castor Cake). Fertilization with Yeast also caused a change in the number of Proteobacteria (22% to 38%), which, instead, remained stable with the other treatments. Less abundant phyla, on the other hand, increased from day 9 to day 58. These changes at the community level led to a gain in evenness over time.
From the table 2.3.8.4, another overview of the changes in the diversity of soil communities at a phylum level could be seen: the ratio between the percentage presence of each taxon at day 58 and at day 9 gives a good indication of the increase or decrease of single phyla over time, even if they were present at a low number. Phyla that were present only at day 58 are shown in white: here the reported number is the presence percentage of the phylum at day 58, not a ratio. The observations done before are evident also in this table, such as the decrease of Firmicutes and Bacteroidetes in the presence of all the treatments, and the increase over time of the number of Proteobacteria only in the presence of Yeast.
At a first sight of the table, and looking at the sums at its bottom, it is evident that the majority of the taxa increase over time (white, green and light green boxes). As stated before, indeed, less abundant phyla tended to increase in number. For example, the taxa OD1, which was present at day 1 at a percentage of less than 0.1 in almost all the samples (in OATr it was 0.2%), and OP11, which was present at day 9 at a percentage of less than 0.01 in all the samples except for Azortit, where it was around 0.02%, significantly increased in all the treated soils.
In the case of Tenericutes, this taxon decreased in abundance only in one sample, Neem. The phylum Nitrospirae, which comprehends nitrifying organisms, raised in number over time in all the samples, except for UTY, where it remained stable. At day 9, this taxon was present at a percentage of around 1.1-1.7% in all the treated and untreated soils, apart from the soil treated with Yeast, where it was present at around 0.6%, and the soil treated with UTY where it was slightly more abundant, with a percentage of 2%. After two months of treatment, the number of Nitrospirae was stable in all the samples at a % of around 2.

This kind of analysis, considering shifts in the microbial community composition after relatively short time from the addition of a specific fertilizer, had not been reported in literature, yet.

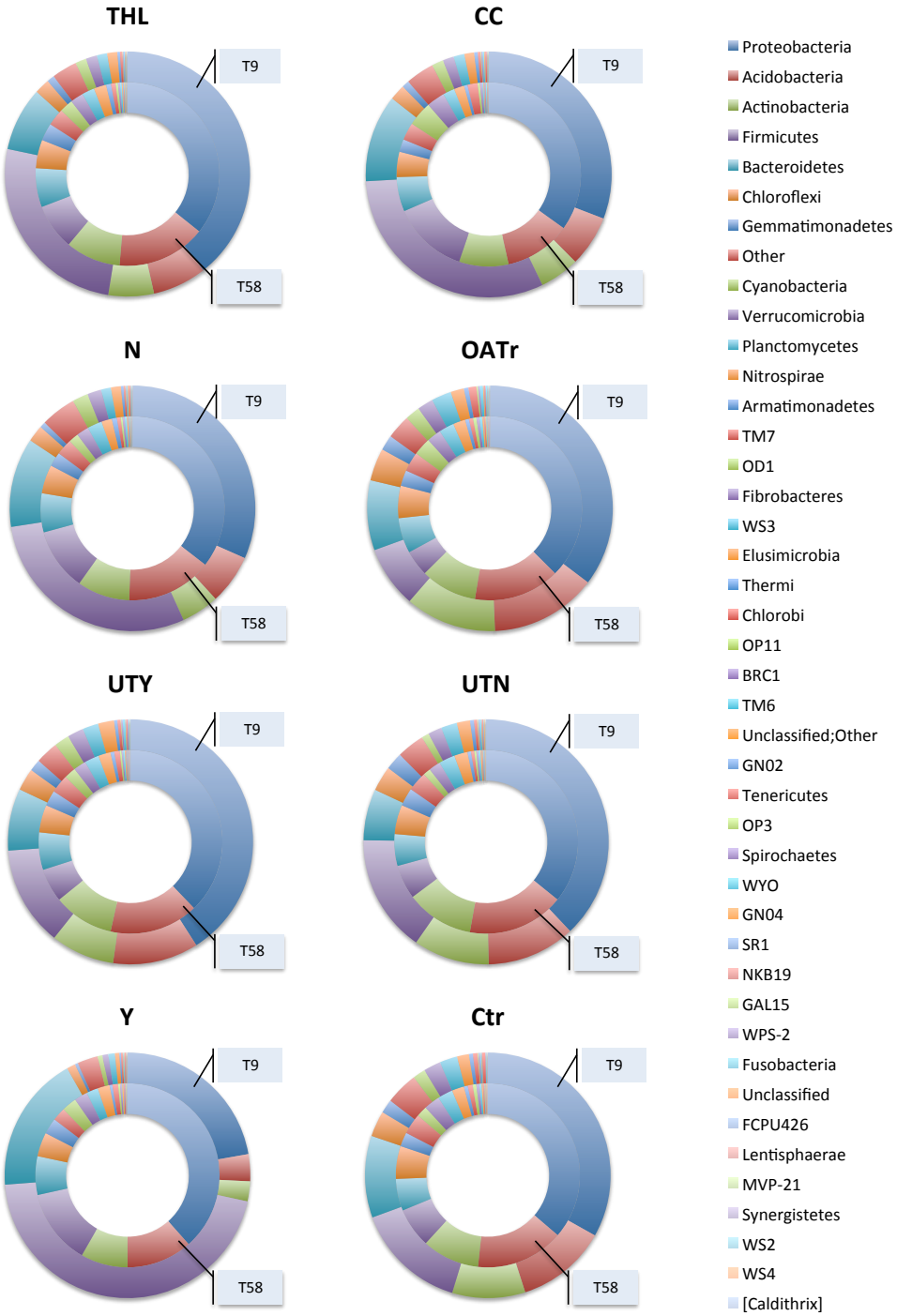

Figure 2.3.8.3
Double concentric pie charts showing the changes of soil bacterial communities at phylum level over time. The outer circle shows phyla present at day 9 in a soil subject to a certain fertilization practice, and the inner circle shows the bacterial community of the same soil after 58 days.

	Day 9, mean values of the % of the Phyla in soil samples							
Taxon	**THL**	**CC**	**N**	**OATr**	**UTY**	**UTN**	**Y**	**Ctr**
Acidobacteria	7.1546	6.7255	6.5480	14.0422	11.3017	11.6748	3.5576	11.9316
Actinobacteria	6.0020	5.2378	4.9860	12.0129	8.4077	10.0093	2.6296	9.6172
Armatimonadetes	0.3379	0.4591	0.3419	0.5961	0.4307	0.5064	0.2119	0.5146
Bacteroidetes	8.4350	11.4940	11.7614	9.2045	8.1803	6.7732	18.0550	10.7766
BRC1	0.0173	0.0557	0.0114	0.0742	0.0971	0.0348	0.0199	0.0615
[Caldithrix]	0.0060	0.0000	0.0000	0.0055	0.0000	0.0000	0.0000	0.0000
Chlorobi	0.0290	0.1946	0.1695	0.1394	0.1475	0.1207	0.1364	0.4257
Chloroflexi	2.0053	2.0712	2.1955	4.2849	2.9330	3.3107	1.1378	3.3902
Cyanobacteria	1.4503	1.5294	2.0795	1.9582	2.0130	0.9887	0.6381	1.6629
Elusimicrobia	0.0355	0.1142	0.1467	0.1201	0.0958	0.0894	0.0584	0.0806
FCPU426	0.0000	0.0000	0.0000	0.0000	0.0064	0.0000	0.0000	0.0000
Fibrobacteres	0.1071	0.1679	0.0938	0.2267	0.1866	0.1285	0.0416	0.1817
Firmicutes	25.7641	31.3048	29.3984	8.1064	13.1684	15.5369	45.3731	14.7424
Fusobacteria	0.0000	0.0000	0.0000	0.0000	0.0000	0.0071	0.0000	0.0000
GAL15	0.0000	0.0065	0.0104	0.0068	0.0000	0.0121	0.0000	0.0000
Gemmatimonadetes	0.9924	0.9584	0.7795	2.0231	1.5435	2.2935	0.4003	1.8133
GN02	0.0288	0.0290	0.0225	0.0723	0.0523	0.0071	0.0220	0.0417
GN04	0.0000	0.0129	0.0000	0.0174	0.0066	0.0000	0.0062	0.0134
Lentisphaerae	0.0115	0.0065	0.0056	0.0055	0.0000	0.0072	0.0055	0.0000
MVP-21	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Nitrospirae	1.3402	1.2889	1.3181	1.7545	2.1128	1.7509	0.5859	1.5764
NKB19	0.0061	0.0000	0.0221	0.0191	0.0066	0.0132	0.0136	0.0000
OD1	0.0534	0.0925	0.0765	0.2046	0.0803	0.1218	0.0590	0.1127
OP11	0.0058	0.0065	0.0052	0.0178	0.0000	0.0000	0.0000	0.0000
OP3	0.0058	0.0346	0.0160	0.0355	0.0576	0.0213	0.0289	0.0000
Other	3.3399	3.7678	4.6496	3.1687	2.9980	3.6571	2.6979	4.3061
Planctomycetes	1.3623	1.4303	1.3087	2.6186	2.0923	2.1161	0.8758	2.3212
Proteobacteria	39.3616	30.8481	31.6444	35.3115	40.9778	37.9865	22.2260	33.1405
Spirochaetes	0.0060	0.0000	0.0168	0.0183	0.0201	0.0218	0.0072	0.0155
SR1	0.0061	0.0049	0.0054	0.0000	0.0064	0.0216	0.0000	0.0000
Synergistetes	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0071
Tenericutes	0.0000	0.0099	0.0368	0.0000	0.0064	0.0213	0.0110	0.0000
Thermi	0.0239	0.0383	0.0275	0.0342	0.0448	0.0507	0.0216	0.0650
TM6	0.0657	0.0360	0.0594	0.1501	0.0590	0.0676	0.0383	0.1003
TM7	0.3314	0.3618	0.2371	1.0506	0.4200	0.4242	0.2317	0.6049
Unclassified	0.0000	0.0000	0.0000	0.0056	0.0000	0.0073	0.0000	0.0155
Unclassified;Other	0.0595	0.0363	0.0219	0.0414	0.0195	0.0952	0.0364	0.0289
Verrucomicrobia	1.4714	1.4504	1.8267	2.0926	2.1274	1.8122	0.7692	2.2657
WPS-2	0.0061	0.0049	0.0000	0.0291	0.0000	0.0000	0.0072	0.0071
WS2	0.0060	0.0000	0.0000	0.0120	0.0065	0.0073	0.0000	0.0000
WS3	0.1483	0.2051	0.1227	0.4213	0.3558	0.2616	0.0895	0.1590
WS4	0.0000	0.0000	0.0000	0.0055	0.0000	0.0072	0.0000	0.0000
WYO	0.0233	0.0166	0.0550	0.1125	0.0382	0.0346	0.0072	0.0208

Table 2.3.8.2

Mean value, over the 4 replicates of each treatment, of the presence percentage of each Phylum in the analysed soils, at sampling day 9.

	Day 58, mean values of the % of the Phyla in soil samples							
Taxon	**THL**	**CC**	**N**	**OATr**	**UTY**	**UTN**	**Y**	**Ctr**
Acidobacteria	15.6416	11.6242	15.0747	15.1322	15.6346	17.0073	11.5497	15.4331
Actinobacteria	9.6802	8.6784	9.2339	9.9198	10.6714	11.9194	8.2664	10.0792
Armatimonadetes	0.8677	0.6083	0.8931	0.6601	0.6555	0.6191	0.4892	0.6714
Bacteroidetes	6.8689	6.0603	6.8580	6.1983	6.5958	5.5928	6.7890	5.6076
BRC1	0.0609	0.0599	0.0849	0.0563	0.0629	0.0648	0.0775	0.0457
[Caldithrix]	0.0000	0.0000	0.0000	0.0063	0.0000	0.0000	0.0000	0.0000
Chlorobi	0.1213	0.1235	0.0752	0.2554	0.0844	0.1219	0.2086	0.1871
Chloroflexi	5.0072	4.4302	5.0472	5.6885	4.8535	5.1705	4.2221	5.7657
Cyanobacteria	2.3711	4.3428	1.7256	3.1161	2.0751	1.3288	2.6169	1.9037
Elusimicrobia	0.1764	0.1093	0.2113	0.2734	0.1689	0.2311	0.2382	0.2363
FCPU426	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Fibrobacteres	0.3402	0.4177	0.3638	0.5161	0.2552	0.3392	0.2785	0.5205
Firmicutes	8.0896	13.2683	10.9668	4.5329	5.9084	6.0142	13.3105	6.9927
Fusobacteria	0.0058	0.0000	0.0118	0.0000	0.0068	0.0000	0.0000	0.0000
GAL15	0.0120	0.0054	0.0103	0.0202	0.0000	0.0141	0.0000	0.0151
Gemmatimonadetes	3.0874	2.2316	2.5214	2.7666	2.7558	3.1834	2.8268	2.4903
GN02	0.0472	0.0571	0.0792	0.0639	0.0898	0.0964	0.0313	0.0628
GN04	0.0177	0.0070	0.0000	0.0069	0.0000	0.0084	0.0000	0.0000
Lentisphaerae	0.0000	0.0054	0.0000	0.0075	0.0144	0.0000	0.0058	0.0070
MVP-21	0.0000	0.0000	0.0000	0.0000	0.0218	0.0162	0.0000	0.0000
Nitrospirae	2.1920	1.8112	1.8396	2.1542	2.1641	2.3515	2.1102	2.1450
NKB19	0.0171	0.0113	0.0118	0.0274	0.0135	0.0154	0.0305	0.0233
OD1	0.4181	0.4045	0.3400	0.6032	0.3223	0.3694	0.3547	0.6381
OP11	0.1009	0.0207	0.0318	0.1249	0.0143	0.0449	0.0060	0.0547
OP3	0.0415	0.0524	0.0300	0.0703	0.0419	0.0385	0.0733	0.0624
Other	3.0540	3.0839	2.8539	3.4647	3.2352	3.4359	2.3556	3.6403
Planctomycetes	2.2164	2.0535	2.5826	2.6676	2.5408	2.8416	2.0670	2.6694
Proteobacteria	35.7481	34.9088	35.5059	37.4951	37.8486	35.8293	38.4401	36.2732
Spirochaetes	0.0411	0.0300	0.0690	0.1544	0.1333	0.0160	0.0058	0.0540
SR1	0.0173	0.0000	0.0000	0.0290	0.0206	0.0091	0.0740	0.0084
Synergistetes	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Tenericutes	0.0459	0.0529	0.0057	0.0521	0.0620	0.0686	0.0125	0.0877
Thermi	0.1532	0.1190	0.0950	0.0421	0.0651	0.0602	0.0756	0.0524
TM6	0.0586	0.1728	0.1136	0.1077	0.0492	0.1103	0.0305	0.0811
TM7	0.7873	1.7568	0.6545	0.7220	0.9619	0.5542	0.9813	0.8701
Unclassified	0.0000	0.0067	0.0061	0.0000	0.0069	0.0000	0.0000	0.0000
Unclassified;Other	0.0476	0.0495	0.1090	0.0356	0.0829	0.0503	0.0371	0.0916
Verrucomicrobia	2.2958	3.1978	2.1680	2.5080	2.2149	2.0659	2.3247	2.8102
WPS-2	0.0115	0.0322	0.0000	0.0075	0.0000	0.0000	0.0058	0.0000
WS2	0.0000	0.0000	0.0103	0.0000	0.0144	0.0084	0.0060	0.0000
WS3	0.3288	0.1945	0.3808	0.5010	0.3253	0.3939	0.0813	0.3953
WS4	0.0000	0.0000	0.0000	0.0126	0.0068	0.0089	0.0000	0.0163
WYO	0.0293	0.0121	0.0352	0.0000	0.0219	0.0000	0.0176	0.0084

Table 2.3.8.3

Mean value, over the 4 replicates of each treatment, of the presence percentage of each Phylum in the analysed soils, at sampling day 58.

Taxon	THL Day58/ Day9	CC Day58/ Day9	N Day58/ Day9	OATr Day58/ Day9	UTY Day58/ Day9	UTN Day58/ Day9	Y Day58/ Day9	Ctr Day58/ Day9
Other	0.91	0.82	0.61	1.09	1.08	0.94	0.87	0.85
Bacteria_p	0.0000	0.0067	0.0061	0.00	0.0069	0.00	0.0000	0.00
[Caldithrix]	0.00	0.0000	0.0000	1.14	0.0000	0.0000	0.0000	0.0000
Acidobacteria	2.19	1.73	2.30	1.08	1.38	1.46	3.25	1.29
Actinobacteria	1.61	1.66	1.85	0.83	1.27	1.19	3.14	1.05
Armatimonadetes	2.57	1.33	2.61	1.11	1.52	1.22	2.31	1.30
Bacteroidetes	0.81	0.53	0.58	0.67	0.81	0.83	0.38	0.52
BRC1	3.51	1.08	7.47	0.76	0.65	1.86	3.89	0.74
Chlorobi	4.18	0.63	0.44	1.83	0.57	1.01	1.53	0.44
Chloroflexi	2.50	2.14	2.30	1.33	1.65	1.56	3.71	1.70
Cyanobacteria	1.63	2.84	0.83	1.59	1.03	1.34	4.10	1.14
Elusimicrobia	4.97	0.96	1.44	2.28	1.76	2.59	4.08	2.93
FCPU426	0.0000	0.0000	0.0000	0.0000	0.00	0.0000	0.0000	0.0000
Fibrobacteres	3.18	2.49	3.88	2.28	1.37	2.64	6.70	2.87
Firmicutes	0.31	0.42	0.37	0.56	0.45	0.39	0.29	0.47
Fusobacteria	0.0058	0.0000	0.0118	0.0000	0.0068	0.00	0.0000	0.0000
GAL15	0.0120	0.83	0.99	2.98	0.0000	1.17	0.0000	0.0151
Gemmatimonadetes	3.11	2.33	3.23	1.37	1.79	1.39	7.06	1.37
GN02	1.64	1.97	3.51	0.88	1.72	13.55	1.42	1.51
GN04	0.0177	0.54	0.0000	0.40	0.00	0.0084	0.00	0.00
Lentisphaerae	0.00	0.83	0.00	1.36	0.0144	0.00	1.06	0.0070
MVP-21	0.0000	0.0000	0.0000	0.0000	0.0218	0.0162	0.0000	0.0000
Nitrospirae	1.64	1.41	1.40	1.23	1.02	1.34	3.60	1.36
NKB19	2.81	0.0113	0.53	1.43	2.06	1.17	2.24	0.0233
OD1	7.82	4.37	4.45	2.95	4.01	3.03	6.01	5.66
OP11	17.47	3.19	6.11	7.00	0.0143	0.0449	0.0060	0.0547
OP3	7.18	1.51	1.88	1.98	0.73	1.80	2.54	0.0624
Planctomycetes	1.63	1.44	1.97	1.02	1.21	1.34	2.36	1.15
Proteobacteria	0.91	1.13	1.12	1.06	0.92	0.94	1.73	1.09
Spirochaetes	6.80	0.0300	4.10	8.46	6.63	0.74	0.81	3.48
SR1	2.84	0.00	0.00	0.0290	3.23	0.42	0.0740	0.0084
Synergistetes	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.00
Tenericutes	0.0459	5.35	0.15	0.0521	9.73	3.22	1.14	0.0877
Thermi	6.41	3.11	3.45	1.23	1.45	1.19	3.49	0.81
TM6	0.89	4.80	1.91	0.72	0.83	1.63	0.79	0.81
TM7	2.38	4.86	2.76	0.69	2.29	1.31	4.24	1.44
Verrucomicrobia	1.56	2.20	1.19	1.20	1.04	1.14	3.02	1.24
WPS-2	1.89	6.52	0.0000	0.26	0.0000	0.0000	0.81	0.00
WS2	0.00	0.0000	0.0103	0.00	2.20	1.14	0.0060	0.0000
WS3	2.22	0.95	3.10	1.19	0.91	1.51	0.91	2.49
WS4	0.0000	0.0000	0.0000	2.28	0.0068	1.24	0.0000	0.0163
WYO	1.26	0.73	0.64	0.00	0.57	0.00	2.45	0.40
Unclassified;Other	0.80	1.36	4.97	0.86	4.26	0.53	1.02	3.16
L2: Day58/Day9	13	12	15	11	13	13	14	20
	2	7	5	6	6	3	3	5
	3	4	2	8	6	5	5	3
	8	8	7	11	10	17	3	9
	17	12	14	7	8	5	18	6
	0	0	0	0	0	0	0	0

Legend	x<0.5	0.5<=x<0.85	0.85=<x<1.15	1.15<=x<2	2<=x<20	x>020
	Taxon absent at day 9. In these boxes, the % of the taxon in the sample at day 58 is shown, not the ratio.					

Table 2.3.8.4

In the table, the ratios between the presence percentage of each phylum at day 58 and at day 9 are reported, and each box is coloured depending on the resulting value (from green, when the percentage of the phylum is higher at day 58 than at day 9 and therefore when it has raised over time, to red, when the percentage of the phylum is lower at day 58 and therefore when it has

decreased over time). At the bottom of the table, the number of phyla that changed to a certain extent (and coloured in a corresponding way) in each treated soil is reported.

2.3.8.1.3 Changes induced by fertilization treatments on soil bacterial community at Phylum level

In order to have a better insight into the changes at phylum level caused by the presence of a particular fertilization, the ratio between the presence percentage of each taxon in each fertilized soil sample (the average value of the two sampling times was used in this calculation) and the presence percentage of the same taxon in the unfertilized soil was done and reported in the table 2.3.8.5. Some differences and analogies in the effects of the different fertilizers could be noticed.

The abundance of Proteobacteria was similar in all the soil samples, and the ratio between the fertilized soils and the unfertilized control gave values around 1. The growth of Firmicutes was favoured by the presence of the organic fertilizers THL, Castor Cake, Neem and Yeast, while it was slightly impaired in the presence of the slow-release fertilizer OATr and did not change significantly in the presence of the two composite fertilizers (containing urea) UTY and UTN. A possible explanation of these results could be that the presence of nitrogen in complex macromolecules, such as in organic fertilizers, led to the prevailing of particular groups of organisms that had the ability to metabolize them (in this case Firmicutes), while the presence of Urea, which is an easy source of nitrogen both for plants and for microorganisms, did not favour a specific phylum.

Acidobacteria and Actinobacteria had a similar behaviour: in the presence of the 4 single-matrix organic fertilizers their number was lower when compared to the unfertilized soil, while with OATr, UTY and UTN their growth and survival did not seem to be influenced. This result is in contrast with the observations done by Ramirez *et al*. (2010), who found opposite behaviours of these two groups, and in particular an increase of Actinobacteria and a decrease of Acidobacteria with N inputs. A similar trend could be found also for two less abundant phyla: Chloroflexi and Planctomycetes. The microorganisms of the phylum Bacteroidetes grew better in the presence of the fertilizer Yeast as a source of nitrogen, phosphorus and potassium, while they decreased in number when the fertilizer UTN was used. This group of bacteria is considered copiotrophic (or R-selected), and its growth in abundance in the presence of N fertilization has already been reported (Nemergut *et al*., 2008; Ramirez *et al*., 2010; Campbell *et al*., 2010; Fierer *et al*., 2012).

Taxon	THL/ Ctr	CC/ Ctr	N/ Ctr	OATr/ Ctr	UTY/ Ctr	UTN/ Ctr	Y/ Ctr
Proteobacteria	1.08	0.96	0.96	1.04	1.14	1.07	0.87
Firmicutes	1.56	2.01	1.86	0.60	0.88	1.02	2.75
Acidobacteria	0.83	0.68	0.79	1.07	0.97	1.03	0.52
Actinobacteria	0.78	0.70	0.71	1.11	0.95	1.09	0.53
Bacteroidetes	0.94	1.08	1.14	0.95	0.92	0.77	1.59
Chloroflexi	0.77	0.71	0.80	1.09	0.85	0.92	0.58
Other	0.81	0.87	0.95	0.84	0.79	0.90	0.66
Gemmatimonadetes	0.94	0.74	0.76	1.09	0.98	1.24	0.69
Cyanobacteria	1.07	1.57	1.04	1.38	1.15	0.63	0.91
Verrucomicrobia	0.75	0.94	0.78	0.90	0.87	0.77	0.59
Planctomycetes	0.71	0.70	0.78	1.06	0.92	0.98	0.57
Nitrospirae	0.95	0.84	0.85	1.05	1.14	1.10	0.65
Armatimonadetes	1.03	0.93	1.05	1.08	0.92	0.96	0.62
TM7	0.77	1.40	0.62	1.21	0.96	0.67	0.82
OD1	0.65	0.69	0.57	1.05	0.55	0.64	0.47
WS3	0.88	0.74	0.96	1.71	1.25	1.18	0.36
Fibrobacteres	0.67	0.87	0.67	1.07	0.66	0.66	0.39
Elusimicrobia	0.68	0.76	1.17	1.23	0.86	0.99	0.91
Thermi	1.44	1.34	0.94	0.63	0.89	0.92	0.63
Chlorobi	0.27	0.56	0.43	0.68	0.41	0.42	0.49
TM6	0.66	1.07	0.94	1.38	0.58	0.92	0.41
Unclassified;Other	0.92	0.74	1.10	0.67	0.89	1.30	0.67
OP11	2.10	0.48	0.76	2.69	0.27	0.74	0.14
BRC1	0.71	1.07	0.87	1.19	1.52	0.90	0.71
GN02	0.76	0.84	1.04	1.34	1.42	0.97	0.63
WYO	1.84	1.05	3.05	4.48	2.14	1.30	0.97
OP3	0.82	1.58	0.76	1.79	1.78	1.04	1.95
Spirochaetes	0.73	0.47	1.36	2.39	2.32	0.58	0.22
Tenericutes	0.54	0.70	0.51	0.52	0.79	0.95	0.32
NKB19	1.09	0.56	1.53	2.09	0.95	1.39	2.27
SR1	3.26	0.84	0.76	3.58	3.80	4.17	2.92
GN04	1.22	1.26	0.00	1.79	0.47	0.52	0.49
WPS-2	2.45	5.05	0.00	5.37	0.00	0.00	1.95
GAL15	0.82	0.84	1.53	1.79	0.00	2.08	0.00
Lentisphaerae	1.63	1.68	0.76	1.79	1.90	1.04	1.95
Fusobacteria	0.0029	0.0000	0.0055	0.0000	0.0034	0.0038	0.0000
WS2	0.0029	0.0000	0.0055	0.0065	0.0103	0.0075	0.0035
[Caldithrix]	0.0029	0.0000	0.0000	0.0065	0.0000	0.0000	0.0000
Bacteria_p	0.00	0.42	0.38	0.45	0.47	0.52	0.00
FCPU426	0.0000	0.0000	0.0000	0.0000	0.0034	0.0000	0.0000
MVP-21	0.0000	0.0000	0.0000	0.0000	0.0103	0.0075	0.0000
Synergistetes	0.00	0.00	0.00	0.00	0.00	0.00	0.00
WS4	0.00	0.00	0.00	1.34	0.47	1.04	0.00

L2 Treatment/Ctr							
	4	5	6	2	8	3	13
	17	16	14	6	5	10	14
	9	9	12	12	17	18	4
	5	6	5	12	5	5	4
	3	2	1	6	3	2	3
	0	0	0	0	0	0	0

Legend	x<0.5	0.5<=x<0.85	0.85=<x<1.15	1.15<=x<2	2<=x<20	x>020
	Taxon absent in Ctr (not fertilized). In these boxes, the % of the taxon in the sample is shown, not the ratio.					

Table 2.3.8.5

In the table, the ratios between the average presence percentage of each phylum in each treated soil with the average presence percentage of the same phylum in the unfertilized soil are reported, and each box is coloured depending on the resulting value (from green, when the percentage of the phylum is higher in the treated soil and therefore when fertilization increases the growth of those organisms, to red, when the percentage of the phylum is lower in the treated

soil than in the control. At the bottom of the table, the number of phyla changed to a certain extent (and coloured in a corresponding way) in each treated soil is reported.

Armatimonadetes were negatively influenced only by the presence of Yeast, a behaviour which is similar to that obtained for Nitrospirae, that were present at a lower number in soils treated with Yeast and Castor Cake, when compared to the unfertilized soil. The decrease in abundance of this phylum in response to the presence of N fertilization was reported also in the work by Ramirez *et al*. (2010). The two urea-containing fertilizers (UTY, UTN) and OATr had a positive effect on the growth of the organisms of the phylum WS3, while this same candidate phylum was strongly impaired by the presence of Yeast.
The phylum Chlorobi was disadvantaged in the presence of fertilization in general, as it was present at a lower percentage in all the fertilized samples, when compared to the unfertilized.
The fertilizer OATr promoted the growth of many low-number phyla (18), and in particular OP11, WYO, WPS-2, Spirochaetes, SR1 and NKB19. Apart from Spirochaetes, the other 5 phyla cited are candidate divisions, as no organisms of these phyla have ever been cultivated yet.
The fertilizer Yeast, on the other hand, impaired the growth of 27 phyla, both abundant and low-number taxa, and promoted the growth of only 7 taxa. These results could reveal a loss of diversity caused by this fertilizer on this soil bacterial community, even if Anova tests on Simpson diversity index did not show significant differences among the 8 treatments (Table 2.3.8.10).

2.3.8.1.4 Detection and analysis of significant differences at Phylum level between fertilized and unfertilized soil samples

Non-parametric Wilcoxon Rank Sum test was performed to compare one single fertilized soil with the unfertilized one, and to determine which taxa changed significantly in each case. With this test, it was possible to recognize the fact that the differences that were evident from previous analysis and reported in the table 2.3.8.5, did not always result to be statistically significant. This fact could be due to deviations in the presence percentage of each phylum in the 4 replicates and in the 2 sampling times, that made the differences between treated and untreated soils, although evident, statistically not significant.
In the table 2.3.8.6, the ratio between the presence percentage of each phylum in each treated soil and the presence percentage of the same phylum in the untreated soil is reported, as in the table 2.3.8.5. However, in this table only the changes that resulted statistically significant are highlighted with a colour: orange for phyla present at a lower level in the treated soil and green for phyla present at higher level in the treated soil.

It is noticeable that only few phyla changed significantly in the presence of the treatment, in the majority of the cases. For UTY and UTN, the two composite fertilizers containing urea, no significant changes were detected from this analysis. The fertilizer Yeast, on the other hand, caused the significant change of the abundance of 8 phyla. In the presence of the organic fertilizer THL and of the organic fertilizer Castor Cake, 3 phyla were present at a significantly lower level, when compared to the unfertilized soil. More in detail, THL caused the decrease in number of the candidate phylum BRC1, of Planctomycetes and of Verrucomicrobia, while Castor Cake of Actinobacteria, Planctomycetes and Spirochetes. In the case of Neem, two phyla were found to be significantly influenced by fertilization: Actinobacteria, such as with Castor Cake, and Firmicutes, which on the other hand, were present at a higher number in fertilized soil. In the presence of a peculiar fertilizer like Neem, it is likely that some particular groups of microorganisms grow better to the detriment of others, based on their capacity to decompose and metabolize certain kinds of molecules. In the presence of the oxy-amino-triazine fertilizer OATr, only the candidate phylum OP3 showed significant differences in respect to its abundance in the unfertilized soil. As stated before, in the case of the two composite fertilizers UTY and UTN, no phyla were selected by the statistical test as significant. This result means that the overall effect of these fertilizers, as this observation are made on the mean over the two sampling times, on the community at this taxonomic level, is not relevant. The presence of urea, which is a fast and easy source of nitrogen both for plants and for microorganisms, could be an explanation of the absence of the "selection" of determinate groups of bacteria in these soils. This effect is however visible only when considering together the two sampling times; indeed, some differences were detected considering the two sampling times separately (see below, paragraph 2.3.8.1.5). As already mentioned, Yeast was the fertilizer that caused significant changes in the highest number of phyla. Of the 8 phyla that resulted significantly influenced by this kind of fertilization, only Firmicutes raised in number thanks to fertilization. Even if yeast is normally used as a base for many cultural media for bacteria, in soil it seems to favour determined groups of bacteria, and in particular Firmicutes. The increase in number of these microorganisms could be the cause for the decrease of many other groups.

At the bottom of the table, the genera, comprised in the significantly different phyla, that were selected as significantly different in the treated soil in respect to the untreated soil with the same kind of statistical analysis, are reported. In this way, it is possible to have an idea of which genera majorly contributed to the prevalence or decrease of a particular phylum.

Phylum	THL/ Ctr		CC/ Ctr		N/ Ctr		OAT/ Ctr		UTY/ Ctr	UTN/ Ctr	Y/ Ctr	
Acidobacteria	0.83		0.68		0.79		1.07		0.97	1.03	0.52	Y1
Actinobacteria	0.78		0.70	CC1	0.71	N1	1.11		0.95	1.09	0.53	Y2
Armatimonadetes	1.03		0.93		1.05		1.08		0.92	0.96	0.62	Y3
Bacteroidetes	0.94		1.08		1.14		0.95		0.92	0.77	1.59	
BRC1	0.71	THL1	1.07		0.87		1.19		1.52	0.90	0.71	Y4
[Caldithrix]	DIV/0		DIV/0		DIV/0		DIV/0		DIV/0	DIV/0	DIV/0	
Chlorobi	0.27		0.56		0.43		0.68		0.41	0.42	0.49	
Chloroflexi	0.77		0.71		0.80		1.09		0.85	0.92	0.58	
Cyanobacteria	1.07		1.57		1.04		1.38		1.15	0.63	0.91	
Elusimicrobia	0.68		0.76		1.17		1.23		0.86	0.99	0.91	
FCPU426	DIV/0		DIV/0		DIV/0		DIV/0		DIV/0	DIV/0	DIV/0	
Fibrobacteres	0.67		0.87		0.67		1.07		0.66	0.66	0.39	
Firmicutes	1.56		2.01		1.86	N2	0.60		0.88	1.02	2.75	Y5
Fusobacteria	DIV/0		DIV/0		DIV/0		DIV/0		DIV/0	DIV/0	DIV/0	
GAL15	0.82		0.84		1.53		1.79		0.00	2.08	0.00	
Gemmatimonadetes	0.94		0.74		0.76		1.09		0.98	1.24	0.69	
GN02	0.76		0.84		1.04		1.34		1.42	0.97	0.63	
GN04	1.22		1.26		0.00		1.79		0.47	0.52	0.49	
Lentisphaerae	1.63		1.68		0.76		1.79		1.90	1.04	1.95	
MVP-21	DIV/0		DIV/0		DIV/0		DIV/0		DIV/0	DIV/0	DIV/0	
Nitrospirae	0.95		0.84		0.85		1.05		1.14	1.10	0.65	
NKB19	1.09		0.56		1.53		2.09		0.95	1.39	2.27	
OD1	0.65		0.69		0.57		1.05		0.55	0.64	0.47	
OP11	2.10		0.48		0.76		2.69		0.27	0.74	0.14	
OP3	0.82		1.58		0.76		1.79	OATr 1	1.78	1.04	1.95	
Other	0.81		0.87		0.95		0.84		0.79	0.90	0.66	Y6
Planctomycetes	0.71	THL2	0.70	CC2	0.78		1.06		0.92	0.98	0.57	Y7
Proteobacteria	1.08		0.96		0.96		1.04		1.14	1.07	0.87	
Spirochaetes	0.73		0.47	CC3	1.36		2.39		2.32	0.58	0.22	
SR1	3.26		0.84		0.76		3.58		3.80	4.17	2.92	
Synergistetes	0.00		0.00		0.00		0.00		0.00	0.00	0.00	
Tenericutes	0.54		0.70		0.51		0.52		0.79	0.95	0.32	
Thermi	1.44		1.34		0.94		0.63		0.89	0.92	0.63	
TM6	0.66		1.07		0.94		1.38		0.58	0.92	0.41	
TM7	0.77		1.40		0.62		1.21		0.96	0.67	0.82	
Unclassified	0.00		0.42		0.38		0.45		0.47	0.52	0.00	
Unclassi_Other	0.92		0.74		1.10		0.67		0.89	1.30	0.67	
Verrucomicrobia	0.75	THL3	0.94		0.78		0.90		0.87	0.77	0.59	Y8
WPS-2	2.45		5.05		0.00		5.37		0.00	0.00	1.95	
WS2	DIV/0		DIV/0		DIV/0		DIV/0		DIV/0	DIV/0	DIV/0	
WS3	0.88		0.74		0.96		1.71		1.25	1.18	0.36	
WS4	0.00		0.00		0.00		1.34		0.47	1.04	0.00	
WYO	1.84		1.05		3.05		4.48		2.14	1.30	0.97	

Table 2.3.8.6

In the table, phyla, which resulted to be significantly different between soils fertilized with each of the tested fertilizers and unfertilized soils are highlighted with a colour. The ratios between the presence percentage of each of these phyla in the fertilized soil and in the unfertilized soil are reported. Boxes containing the ratios' values are coloured depending on the resulting value (green, when the percentage of the phylum is higher in the treated soil and therefore when fertilization increases the growth of those organisms, and orange, when the percentage of the phylum is lower in the treated soil than in the control). The taxa responsible for the changes at phylum level, detected with the Wilcoxon Rank Sum test performed at lower taxonomical levels (see paragraph 2.3.8.2.2) are reported: THL1 c_PRR-11; THL2 f_Phycisphaeraceae; THL3

f_Opitutaceae; CC1 g_Mycobacterium, g_Pimelobacter, g_Streptomyces, o_Micrococcales, o_0319-7L14; CC2 o_Phycisphaerales, f_Gemmataceae, f_Pirellulaceae; N1 g_Microlunatus, g_Sporichthya, g_Streptomyces, o_Actinomycetales, o_Micrococcales, c_MB-A2-108, o_Solirubrobacterales; N2 g_Brevibacillus, g_Clostridium, g_Sedimentibacter, f_Lachnospiraceae, g_Desulfitobacterium, g_Symbiobacterium, f_Symbiobacteriaceae; Y1 o_iii1-15, f_mb2424, c_EC1113, o_Holophagales, c_MVS-40; Y2 g_Virgisporangium, g_Streptomyces, o_Actinomycetales, g_Rubrobacter, f_Gaiellaceae, o_Solirubrobacterales; Y4 c_PRR-11; Y5 g_Aneurinibacillus, g_Brevibacillus, f_Paenibacillaceae, c_Bacilli, g_Clostridium, f_Clostridiaceae, f_Lachnospiraceae, g_Desulfitobacterium, g_Clostridium; Y6 k_Bacteria; Y7 c_BD7-11, g_Pirellula; Y8 g_Prosthecobacter

2.3.8.1.5 Detection and analysis of significant differences at Phylum level between fertilized and unfertilized soils at sampling day 9 and sampling day 58 considered separately

The Wilcoxon Rank Sum test was performed separately on soils sampled after 9 days and 58 days from fertilization. In this way, it was possible to appreciate the effects that different fertilizers had on soil bacterial community after a short time or after about 2 months. In the table 2.3.8.7, the ratio between the presence percentage of each phylum in each treated soil and the presence percentage of the same phylum in the untreated soil at day 9 is reported. As for the previous table, only the changes that resulted statistically significant are highlighted with a colour: orange for phyla present at a lower level in the treated soil, green for phyla present at higher level in the treated soil, yellow when the ratio resulted in a number around 1, and blue when the phylum was not detected in the control soil, and the ratio could not be calculated.

At day 9, for the fertilizer THL, the significant decrease in number of the candidate phylum BRC1, that was detected also with the analysis made on mean values between sampling times (Table 2.3.8.6), was described by a ratio of 0.28, indicating that these bacteria are rapidly negatively influenced by the presence of the fertilizer. Indeed, this significant change was not found at day 58, where, on the other hand, the ratio resulted higher than 1. Elusimicrobia were also present at a lower number after 9 days of fertilization, with significant difference with the unfertilized soil, while Verrucomicrobia were negatively influenced to a lower degree only after about two months, and, instead, the candidate phylum GN04 raised in abundance. The organic fertilizer Castor Cake did not induce fast significant changes at phylum level, as it is evident from the table 2.3.8.7. After 58 days, Chloroflexi and Planctomycetes significantly decreased in number (Table 2.3.8.8). Therefore, the decrease in the abundance of the phylum Planctomycetes, that was detected also considering the two sampling times together, happened only after a quite long time from the application of the fertilization.

Phylum	Day 9 THL/ Ctr		Day 9 CC/ Ctr	Day9 N/ Ctr		Day 9 OATr /Ctr		Day 9 UTY/ Ctr	Day 9 UTN/ Ctr		Day 9 Y/Ctr	
Acidobacteria	0.60		0.56	0.55		1.18		0.95	0.98		0.30	Y1
Actinobacteria	0.62		0.54	0.52	N1	1.25		0.87	1.04		0.27	Y2
Armatimonadetes	0.66		0.89	0.66		1.16		0.84	0.98		0.41	Y3
Bacteroidetes	0.78		1.07	1.09		0.85		0.76	0.63	UTN1	1.68	Y4
BRC1	0.28	THL1	0.91	0.18		1.21		1.58	0.57		0.32	
[Caldithrix]	DIV/0		DIV/0	DIV/0		DIV/0		DIV/0	DIV/0		DIV/0	
Chlorobi	0.07		0.46	0.40		0.33		0.35	0.28		0.32	
Chloroflexi	0.59		0.61	0.65		1.26		0.87	0.98		0.34	Y5
Cyanobacteria	0.87		0.92	1.25		1.18		1.21	0.59		0.38	
Elusimicrobia	0.44	THL2	1.42	1.82		1.49		1.19	1.11		0.72	
FCPU426	DIV/0		DIV/0	DIV/0		DIV/0		DIV/0	DIV/0		DIV/0	
Fibrobacteres	0.59		0.92	0.52		1.25		1.03	0.71		0.23	
Firmicutes	1.75		2.12	1.99		0.55		0.89	1.05		3.08	Y6
Fusobacteria	DIV/0		DIV/0	DIV/0		DIV/0		DIV/0	DIV/0		DIV/0	
GAL15	DIV/0		DIV/0	DIV/0		DIV/0		DIV/0	DIV/0		DIV/0	
Gemmatimonadetes	0.55		0.53	0.43		1.12		0.85	1.26		0.22	Y7
GN02	0.69		0.70	0.54		1.74		1.26	0.17		0.53	
GN04	0.00		0.96	0.00		1.30		0.49	0.00		0.46	
Lentisphaerae	DIV/0		DIV/0	DIV/0		DIV/0		DIV/0	DIV/0		DIV/0	
MVP-21	DIV/0		DIV/0	DIV/0		DIV/0		DIV/0	DIV/0		DIV/0	
Nitrospirae	0.85		0.82	0.84		1.11		1.34	1.11		0.37	
NKB19	DIV/0		DIV/0	DIV/0	N2	DIV/0		DIV/0	DIV/0		DIV/0	
OD1	0.47		0.82	0.68		1.82		0.71	1.08		0.52	
OP11	DIV/0		DIV/0	DIV/0		DIV/0		DIV/0	DIV/0		DIV/0	
OP3	DIV/0		DIV/0	DIV/0		DIV/0	OATr1	DIV/0	DIV/0		DIV/0	
Other	0.78		0.87	1.08	N3	0.74		0.70	0.85		0.63	
Planctomycetes	0.59		0.62	0.56	N4	1.13		0.90	0.91		0.38	Y8
Proteobacteria	1.19		0.93	0.95		1.07		1.24	UTY1	1.15	0.67	
Spirochaetes	0.39		0.00	1.08		1.18		1.30	1.40		0.47	
SR1	DIV/0		DIV/0	DIV/0		DIV/0		DIV/0	DIV/0		DIV/0	
Synergistetes	0.00		0.00	0.00		0.00		0.00	0.00		0.00	
Tenericutes	DIV/0		DIV/0	DIV/0		DIV/0		DIV/0	DIV/0		DIV/0	
Thermi	0.37		0.59	0.42		0.53		0.69	0.78		0.33	
TM6	0.66		0.36	0.59		1.50		0.59	0.67		0.38	
TM7	0.55		0.60	0.39		1.74		0.69	0.70		0.38	
Unclassified	0.00		0.00	0.00		0.36		0.00	0.47		0.00	
Unclassified;Other	2.06		1.25	0.76		1.43		0.67	3.29		1.26	
Verrucomicrobia	0.65		0.64	0.81		0.92		0.94	0.80		0.34	Y9
WPS-2	0.86		0.70	0.00		4.10		0.00	0.00		1.02	
WS2	DIV/0		DIV/0	DIV/0		DIV/0		DIV/0	DIV/0		DIV/0	
WS3	0.93		1.29	0.77		2.65	OATr2	2.24	1.65		0.56	
WS4	DIV/0		DIV/0	DIV/0		DIV/0		DIV/0	DIV/0		DIV/0	
WYO	1.12		0.80	2.64		5.40		1.83	1.66		0.35	

Table 2.3.8.7

In the table, phyla which resulted to be significantly different between soils fertilized with each of the tested fertilizers and unfertilized soils, at sampling day 9, are highlighted with a colour. The ratios between the presence percentage of each of these phyla in the fertilized soil and in the unfertilized soil are reported. Boxes containing the ratios' values are coloured depending on the resulting value (green, when the percentage of the phylum is higher in the treated soil and therefore when fertilization increases the growth of those organisms, yellow, when the percentage of the phylum is similar in the treated soil and in the control, with a ratio value between 0.8 and 1.2, orange, when the percentage of the phylum is lower in the treated soil than

in the control, and blue when the phylum is not present in the control soil, and therefore the ratio could not be calculated). The taxa responsible for the changes at phylum level, detected with the Wilcoxon Rank Sum test performed at lower taxonomical levels (see paragraph 2.3.8.2.2) are reported: THL1 c_PRR-11; N1 g_Mycobacterium, g_Pseudonocardia, f_Streptomycetaceae, o_0319-7L14, o_Solirubrobacterales; N2 p_NKB19; N4 f_Gemmataceae, f_Pirellulaceae; OATr1 f_kpj58rc; OATr2 f_PRR-10; UTY1 g_Bosea, g_Devosia, f_Methylocystaceae, g_Sinorhizobium, o_Rhodospirillales, c_Betaproteobacteria, g_Methylibium, g_Polaromonas, g_Rubrivivax, f_Rhodocyclaceae, f_Pelobacteraceae, g_Haliangium, f_Polyangiaceae, g_Trabulsiella, g_Methylomonas; UTN1 g_Dyadobacter; Y1 o_iii1-15, f_mb2424, c_Chloracidobacteria, o_Holophagales, c_MVS-40; Y2 o_Actinomycetales, g_Phycicoccus, g_Virgisporangium, f_Nocardioidaceae, g_Actinomadura, g_Rubrobacter, f_Gaiellaceae, o_Solirubrobacterales, o_Solirubrobacterales; Y4 g_Dysgonomonas, g_Parabacteroides, c_Flavobacteriia, g_Riemerella, g_Adhaeribacter, g_Pontibacter; Y5 f_Caldilineaceae, g_Caldilinea; Y6 f_Paenibacillaceae, g_Aneurinibacillus, g_Cohnella, g_Lysinibacillus, g_Clostridium, f_Lachnospiraceae, g_Desulfitobacterium, f_Veillonellaceae; Y7 c_Gemm-1, c_JL-ETNP-Z39; Y8 c_BD7-11, f_Gemmataceae; Y9 g_Candidatus Xiphinematobacter, g_Chthoniobacter, g_DA101, g_OR-59.

For the slow-release fertilizer Neem, only two significant changes were detected when considering the two sampling times together. From the tables reported in this paragraph it is although evident that some phyla were influenced from this kind of fertilization, but differently at the beginning and at the end of the two months period. In fact, after 9 days from the addition of the fertilizer, Actinobacteria and Planctomycetes were impaired, while this effect was not evident after 58 days. Conversely, the candidate phyla OD1, OP11 and the phylum Verrucomicrobia, were selected as significantly different from the test only with the last sampling time. At day 58, Spirochaetes resulted to be present at a significantly higher number in respect to the unfertilized sample, and this result indicates a delayed effect of Neem on this group of bacteria. The slow-release oxy-amino-triazine fertilizer OATr did not present any significant effect after two months, while it caused the significant change in abundance of the candidate phyla WS3 and OP3, which was not present in the unfertilized soil, after just 9 days. The significant positive effect on the abundance of the phylum OP3 was also detected when considering day 9 and day 58 together, and this last analysis explains that this change happened short after fertilization.

Soils treated with composite fertilizer UTY did not present any phylum that resulted to be significantly different in the unfertilized soil, considering the two sampling times together (paragraph 2.3.8.1.4, Table 2.3.8.6). However, when observing the two sampling times separately, interesting evaluations could be done.

Phylum	Day 58 THL/ Ctr	Day 58 CC/ Ctr	Day 58 N/ Ctr	Day 58 OAT/ Ctr	Day 58 UTY/ Ctr	Day 58 UTN/ Ctr	Day 58 Y/ Ctr
Acidobacteria	1.01	0.75	0.98	0.98	1.01	1.10	0.75
Actinobacteria	0.96	0.86	0.92	0.98	1.06	1.18 UTN1	0.82 Y1
Armatimonadetes	1.29	0.91	1.33	0.98	0.98	0.92	0.73
Bacteroidetes	1.22	1.08	1.22	1.11	1.18	1.00	1.21
BRC1	1.33	1.31	1.86	1.23	1.38	1.42	1.70 Y2
[Caldithrix]	DIV/0	DIV/0	DIV/0	DIV/0	DIV/0	DIV/0	DIV/0
Chlorobi	0.65	0.66	0.40	1.37	0.45	0.65	1.11
Chloroflexi	0.87	0.77 CC1	0.88	0.99	0.84 UTY1	0.90	0.73 Y3
Cyanobacteria	1.25	2.28	0.91	1.64	1.09	0.70	1.37
Elusimicrobia	0.75	0.46	0.89	1.16	0.71	0.98	1.01
FCPU426	DIV/0	DIV/0	DIV/0	DIV/0	DIV/0	DIV/0	DIV/0
Fibrobacteres	0.65	0.80	0.70	0.99	0.49	0.65	0.54
Firmicutes	1.16	1.90	1.57	0.65	0.84	0.86	1.90
Fusobacteria	DIV/0	DIV/0	DIV/0	DIV/0	DIV/0	DIV/0	DIV/0
GAL15	0.80	0.35	0.68	1.33	0.00	0.93	0.00
Gemmatimonadet	1.24	0.90	1.01	1.11	1.11	1.28	1.14
GN02	0.75	0.91	1.26	1.02	1.43	1.53	0.50
GN04	DIV/0 THL1	DIV/0	DIV/0	DIV/0	DIV/0	DIV/0	DIV/0
Lentisphaerae	0.00	0.76	0.00	1.07	2.04	0.00	0.83
MVP-21	DIV/0	DIV/0	DIV/0	DIV/0	DIV/0	DIV/0	DIV/0
Nitrospirae	1.02	0.84	0.86	1.00	1.01	1.10	0.98
NKB19	0.74	0.49	0.51	1.18	0.58	0.66	1.31
OD1	0.66	0.63	0.53 N1	0.95	0.51	0.58	0.56
OP11	1.85	0.38	0.58 N2	2.28	0.26	0.82	0.11
OP3	0.66	0.84	0.48	1.13	0.67	0.62	1.17
Other	0.84	0.85	0.78 N3	0.95	0.89	0.94	0.65 Y4
Planctomycetes	0.83	0.77 CC2	0.97	1.00	0.95	1.06	0.77 Y5
Proteobacteria	0.99	0.96	0.98	1.03	1.04	0.99	1.06
Spirochaetes	0.76	0.56	1.28 N4	2.86	2.47 UTY2	0.30	0.11
SR1	2.07	0.00	0.00	3.48	2.46	1.10	8.87
Synergistetes	DIV/0	DIV/0	DIV/0	DIV/0	DIV/0	DIV/0	DIV/0
Tenericutes	0.52	0.60	0.06	0.59	0.71	0.78	0.14
Thermi	2.93	2.27	1.81	0.80	1.24	1.15	1.44
TM6	0.72	2.13	1.40	1.33	0.61	1.36	0.38
TM7	0.90	2.02	0.75	0.83	1.11	0.64	1.13
Unclassified	DIV/0	DIV/0	DIV/0	DIV/0	DIV/0	DIV/0	DIV/0
Unclassified;Other	0.52	0.54	1.19	0.39	0.91	0.55	0.41
Verrucomicrobia	0.82 THL2	1.14	0.77 N5	0.89	0.79 UTY3	0.74	0.83
WPS-2	DIV/0	DIV/0	DIV/0	DIV/0	DIV/0	DIV/0	DIV/0
WS2	DIV/0	DIV/0	DIV/0	DIV/0	DIV/0	DIV/0	DIV/0
WS3	0.83	0.49	0.96	1.27	0.82	1.00	0.21
WS4	0.00	0.00	0.00	0.78	0.42	0.55	0.00
WYO	3.51	1.45	4.21	0.00	2.62	0.00	2.11

Table 2.3.8.8

In the table, phyla which resulted to be significantly different between soils fertilized with each of the tested fertilizers and unfertilized soils, at sampling day 58, are highlighted with a colour. The ratios between the presence percentage of each of these phyla in the fertilized soil and in the unfertilized soil are reported. Boxes containing the ratios' values are coloured depending on the resulting value (green, when the percentage of the phylum is higher in the treated soil and therefore when fertilization increases the growth of those organisms, yellow, when the percentage of the phylum is similar in the treated soil and in the control, with a ratio value

between 0.8 and 1.2, orange, when the percentage of the phylum is lower in the treated soil than in the control, and blue when the phylum is not present in the control soil, and therefore the ratio could not be calculated). The taxa responsible for the changes at phylum level, detected with the Wilcoxon Rank Sum test performed at lower taxonomical levels (see paragraph 2.3.8.2.2) are reported: THL2 f_Opitutaceae, g_Luteolibacter, f_Ellin515; CC1 f_Anaerolinaceae, g_Oscillochloris, f_Kouleothrixaceae, f_Thermobaculaceae, c_Thermomicrobia; CC2 o_Phycisphaerales, f_Pirellulaceae, g_A17, g_Pirellula; N1 c_ABY1; N2 c_OP11-4; N3 k_Bacteria; UTY1 c_Anaerolineae, o_envOPS12, f_Kouleothrixaceae, c_Ellin6529, c_Thermomicrobia; UTY3 f_Ellin515; UTN1 p_Actinobacteria, g_Micromonospora, g_Streptomyces, f_Gaiellaceae, o_Solirubrobacterales; Y1 o_Actinomycetales, f_Geodermatophilaceae, f_Micromonosporaceae, g_Micromonospora; Y2 c_PRR-11; Y3 f_Kouleothrixaceae, c_SAR202, o_AKYG1722; Y4 k_Bacteria; Y5 p_Planctomycetes, g_Planctomyces.

After 9 days, the presence of UTY in soil caused the significant increase in number of Proteobacteria. This phylum, which was observed to be stable in its abundance in almost all the treated soils, and that was not detected to have changed significantly in other cases, seemed to be rapidly positively influenced by this treatment. After 58 days, instead, Chloroflexi and Verrucomicrobia decreased in number, while Spirochaetes increased of about 2.5 times. The fertilizer UTN negatively influenced, after 9 days, the number of Bacteroidetes, while, after 58 days, it induced a slight increase in the number of Actinobacteria.

As with combined sampling times, also considering single sampling days the fertilizer Yeast induced changes in a high number of phyla. Acidobacteria, Actinobacteria, Armatimonadetes, Chloroflexi, Gemmatimonadetes, Planctomycetes and Verrucomicrobia were present in a lower number in the presence of the fertilizer after 9 days. This decrease in number was confirmed for Actinobacteria, Chloroflexi and Planctomycetes, also after two months, indicating that the effect of this fertilizer on these microorganisms remained in time. Bacteria that were positively influenced by Yeast fertilizer after 9 days were Bacteroidetes and Firmicutes, which were present in the Yeast-fertilized soil at a number which was 1.7 and 3 times higher, respectively, than in the unfertilized soil. The candidate phylum BRC1, instead, was positively influenced by the fertilization only after 2 months.

Also for the tables 2.3.8.7 and 2.3.8.8, the genera, comprised in the significantly different phyla, that were selected as significantly different in the treated soil in respect to the untreated soil with the same kind of statistical analysis, are reported at the bottom of the table.

2.3.8.1.6 Statistical analysis on diversity indices

Diversity indices were calculated, and statistical analyses were performed considering the number of taxa (S), Simpson index (1-D) and Evenness (e^H/S). In the table 2.3.8.9, mean values for these three indices calculated considering the treatments are shown. As it is evident from the Anova tests, the differences in diversity indices were not significant, and this result indicates that the presence of the different fertilizers did not cause an evident change in the number and abundance of the phyla, considering the total effect during the 2 month of the trial (Table 2.3.8.10). Results obtained by Fierer *et al*. (2012) are in line with these observations. In fact, they detected no significant effects of N fertilization on bacterial diversity, but significant shifts in community composition. In contrast, Campbell *et al*., (2010) found a decrease in bacterial diversity with N additions. These two contrasting visions suggest that the effects of N amendments on bacterial diversity levels are variable and likely site-dependent.

Treatment		Taxa	Simpson 1-D	Evenness e^H/S
THL	Mean	27.7500	.7798	.2666
	N	8	8	8
	Std. Deviation	3.41216	.05126	.03814
CC	Mean	27.2500	.7830	.2800
	N	8	8	8
	Std. Deviation	2.91548	.07885	.05486
N	Mean	27.5000	.7952	.2791
	N	8	8	8
	Std. Deviation	3.16228	.04793	.04756
OATr	Mean	29.2500	.8125	.2947
	N	8	8	8
	Std. Deviation	1.75255	.01710	.01783
UTY	Mean	26.5000	.7903	.2979
	N	8	8	8
	Std. Deviation	3.50510	.03976	.03205
UTN	Mean	27.3750	.7981	.2892
	N	8	8	8
	Std. Deviation	1.99553	.02739	.02981
Y	Mean	24.3750	.7367	.2632
	N	8	8	8
	Std. Deviation	4.30739	.11713	.07061
Ctr	Mean	27.6250	.8142	.3072
	N	8	8	8
	Std. Deviation	2.50357	.01462	.02927
Total	Mean	27.2031	.7887	.2847
	N	64	64	64
	Std. Deviation	3.15313	.06006	.04305

Table 2.3.8.9

Mean values of the three considered diversity indices (taxa number, Simpson's index 1-D, Evenness index e^H/S) obtained considering the different fertilization treatments. In the table, apart from the mean values, the standard deviation and the number of samples considered are also reported.

			Sum of Squares	df	Mean Square	F	Sig.
Taxa * Treatment	Between Groups	(Combined)	106.234	7	15.176	1.634	.145
	Within Groups		520.125	56	9.288		
	Total		626.359	63			
Simpson 1-D * Treatment	Between Groups	(Combined)	.033	7	.005	1.377	.233
	Within Groups		.194	56	.003		
	Total		.227	63			
Evenness e^H/S * Treatment	Between Groups	(Combined)	.013	7	.002	1.015	.431
	Within Groups		.104	56	.002		
	Total		.117	63			

Table 2.3.8.10

The table shows the results obtained performing the test Anova on the three diversity indices considered in respect to the fertilization used.

Day		Taxa	Simpson	Evenness e^H/S
9	Mean	25.6875	.7654	.2680
	N	32	32	32
	Std. Deviation	3.06318	.07726	.04954
58	Mean	28.7188	.8121	.3014
	N	32	32	32
	Std. Deviation	2.46569	.01531	.02715
Total	Mean	27.2031	.7887	.2847
	N	64	64	64
	Std. Deviation	3.15313	.06006	.04305

Table 2.3.8.11

Mean values of the three considered diversity indices (Taxa number, Simpson's index 1-D, Evenness index e^H/S) obtained considering the sampling day. In the table, the standard deviation and the number of samples considered are also reported.

			Sum of Squares	df	Mean Square	F	Sig.
Taxa * Day	Between Groups	(Combined)	147.016	1	147.016	19.016	.000
	Within Groups		479.344	62	7.731		
	Total		626.359	63			
Simpson * Day	Between Groups	(Combined)	.035	1	.035	11.275	.001
	Within Groups		.192	62	.003		
	Total		.227	63			
Evenness e^H/S * Day	Between Groups	(Combined)	.018	1	.018	11.164	.001
	Within Groups		.099	62	.002		
	Total		.117	63			

Table 2.3.8.12

The table shows the results obtained performing the test Anova on the three considered diversity indices in respect to the sampling day.

The same statistical analyses (mean and Anova test) were made considering the sampling day. As stated before, treated soils sampled at day 9 and at day 58 were sequenced.

The table 2.3.8.11 shows the results obtained calculating the mean and the standard deviation of the three considered diversity indices taking all the soils sampled at a certain day. In this case, significant differences (Table 2.3.8.12) were found for all the

three indices, and in particular it can be seen that at day 58 both diversity and evenness raise, confirming the observations done previously (Figure 2.3.8.3).

2.3.8.2 Analysis of soil bacterial communities at Genus level

To have a more detailed insight into the soil bacterial communities of the treated pots, similar analysis were made considering the lowest taxonomic level possible in this study: the genus.
900 different genera were detected among all the 64 soil samples analysed (see table 2.3.8.13).

Phylum	Number of Genera	Phylum	Number of Genera	Phylum	Number of Genera
Proteobacteria	296	Elusimicrobia	6	Fusobacteria	1
Actinobacteria	123	OD1	6	GAL15	1
Firmicutes	110	Spirochaetes	6	Lentisphaerae	1
Chloroflexi	66	GN02	5	MVP-21	1
Bacteroidetes	52	GN04	3	Other	1
Acidobacteria	34	NKB19	3	SR1	1
Verrucomicrobia	34	OP3	3	Synergistetes	1
Planctomycetes	33	TM6	3	Unclassified	1
Cyanobacteria	29	Fibrobacteres	2	Unclassified;Other	1
Gemmatimonadetes	16	OP11	2	WPS-2	1
Armatimonadetes	13	Tenericutes	2	WS2	1
Chlorobi	11	Thermi	2	WS4	1
TM7	9	BRC1	1	WYO	1
WS3	8	[Caldithrix]	1		
Nitrospirae	7	FCPU426	1		

Table 2.3.8.13
In the table, the number of genera that were detected for each single phylum, in all the soil samples analysed, are reported.

2.3.8.2.1 Analysis of the changes in soil microbial communities over time at Genus level

Similar analyses to those performed for the highest level of taxonomy (phylum), were done also for the lower taxonomic level, the genus. Tamames *et al*. (2010) indicate that environmental specificity is not very common at phylum level, but emerges at lower taxonomic levels, such as genus and species. This observation confirms the importance of this kind of analysis.

One way Anova test, performed with a particular software adapted to deal with this kind of data, highlighted the taxa which were changing in a significant way among soil communities present in all the treatments and sampling days (the 4 replicates of each conditions were taken into account separately as groups). From this analysis, 109 taxa were selected for further analysis, as they resulted to have significantly different percentages in the samples.

In the table 2.3.8.14, the presence percentage of each significant taxon at day 58 was divided by the presence percentage of the same taxon at day 9. As for the analysis made with the phyla, this calculation was made to stress how some particular genera changed in number over time. From this table, it can be observed that, in general, Clostridia decreased in number over time (red and orange boxes), and in the same way Burkholderiales. This results could be explained in two ways: the addition of the fertilizers to the soil could have caused a fast positive response on the growth of these two genera of bacteria, which then decreased in two months to the basal growth, or fertilization could have caused the decrease of these organisms from the beginning of the trial.

Among Clostridia, the number of organisms belonging to the genus *Symbiobacterium* was around 150 times higher at day 9 in the presence of the fertilizer THL, and between 30 and 40 times higher in the presence of Neem, UTY and with no fertilization (in the table, the ratio between the presence percentage of this taxon at day 58 and at day 9 is reported, so the resulting values are very low, between 0.03 and 0.01, and coloured in red).

The nitrogen-fixing organism *Azospirillum* was 30 times more abundant at day 9 with UTN, and around 10 times more abundant with UTY and THL. In the case of soil treated with Castor Cake, OATr and Yeast, instead, the number of these organisms was higher at day 58 than at day 9. The soil treated with Neem gave a similar response as the unfertilized soil on the growth of these organisms: they were slightly more abundant at day 9.

Another order of organisms involved in the nitrogen cycle, the Nitrospirales, grew in time in all the cases, and in particular in the presence of Neem and Yeast. Only in the presence of UTY, the number of Nitrospirales remained stable over time.

Taxon	THL D58/ D9	CC D58/ D9	N D58/ D9	OATr D58/ D9	UTY D58/ D9	UTN D58/ D9	Y D58/ D9	Ctr D58/ D9
Acidobacteria_c_Acidobacteria-6_o_iii1-15_f_g_	1.82	1.43	2.19	1.07	1.31	1.42	3.00	1.17
Acidobacteria_c_Acidobacteria-6_o_iii1-15_f_mb2424_g_	2.23	2.11	3.24	1.09	1.70	1.76	2.80	1.28
Acidobacteria_c_Chloracidobacteria_o_f_g_	2.62	1.66	2.30	1.11	1.51	1.56	3.33	1.41
Acidobacteria_c_iii1-8_o_DS-18_f_g_	2.77	3.64	1.89	0.93	1.52	1.19	5.29	1.67
Acidobacteria_c_o_f_g_	4.86	1.89	3.43	1.26	1.59	1.90	2.66	3.58
Acidobacteria_c_S035_o_f_g_	2.42	1.14	2.25	0.91	1.24	0.89	1.47	0.77
Actinobacteria_c_Acidimicrobiia_o_Acidimicrobiales_f_EB1017_g_	2.59	3.47	1.72	1.41	1.24	0.86	6.35	1.16
Actinobacteria_c_Actinobacteria_o_Actinomycetales_f_Geodermatophilaceae_g_Geodermatophilus	4.08	0.0003	0.0001	3.06	2.55	0.0005	0.0001	0.0003
Actinobacteria_c_Actinobacteria_o_Actinomycetales_f_Intrasporangiaceae_g_Phycicoccus	1.00	1.43	1.01	0.26	0.48	0.86	1.75	2.58
Actinobacteria_c_Actinobacteria_o_Actinomycetales_f_Intrasporangiaceae_Other	1.12	1.14	1.30	0.36	1.30	0.91	3.23	1.34
Actinobacteria_c_Actinobacteria_o_Actinomycetales_f_Micromonosporaceae_g_	0.00	2.74	12.27	0.43	2.13	0.00	4.86	0.19
Actinobacteria_c_Actinobacteria_o_Actinomycetales_f_Micromonosporaceae_Other	1.48	4.01	2.23	0.86	3.51	0.78	8.12	0.79
Actinobacteria_c_Actinobacteria_o_Actinomycetales_f_Mycobacteriaceae_g_Mycobacterium	1.10	1.68	1.48	0.65	0.82	0.95	7.73	1.01
Actinobacteria_c_Actinobacteria_o_Actinomycetales_f_Nocardioidaceae_Other	0.46	0.60	0.68	0.16	0.69	0.46	1.47	0.52
Actinobacteria_c_Actinobacteria_o_Actinomycetales_f_Pseudonocardiaceae_g_Pseudonocardia	2.41	1.49	0.0003	3.69	1.20	1.77	14.02	1.39
Actinobacteria_c_Actinobacteria_o_Actinomycetales_f_Sporichthyaceae_g_Sporichthya	0.00	0.0002	3.98	0.00	0.0001	1.99	0.0000	0.00
Actinobacteria_c_Actinobacteria_o_Actinomycetales_f_Thermomonosporaceae_g_	0.0000	0.0000	0.0000	0.0001	0.0000	0.0000	0.0000	0.0000
Actinobacteria_c_Actinobacteria_o_Actinomycetales_Other_Other	1.41	1.30	1.18	0.60	1.04	1.08	2.08	0.90
Actinobacteria_c_MB-A2-108_o_0319-7L14_f_g_	3.34	4.97	6.65	2.19	2.65	2.09	11.09	1.28
Actinobacteria_c_Rubrobacteria_o_Rubrobacterales_f_Rubrobacteraceae_g_Rubrobacter	1.87	0.94	1.30	0.37	0.83	0.78	2.31	0.83
Actinobacteria_c_Thermoleophilia_o_Gaiellales_f_Gaiellaceae_g_	2.24	2.49	4.14	1.12	1.38	1.36	4.64	1.09
Actinobacteria_c_Thermoleophilia_o_Solirubrobacterales_f_g_	2.60	2.63	2.88	0.90	1.64	1.59	4.29	0.99
Actinobacteria_c_Thermoleophilia_o_Solirubrobacterales_Other_Other	1.98	1.34	1.94	1.54	1.55	1.51	2.37	1.22
Actinobacteria_Other_Other_Other_Other	1.77	1.46	1.76	1.06	1.78	1.32	1.41	0.76
Armatimonadetes_c_[Fimbriimonadetes]_o_[Fimbriimonadales]_f_[Fimbriimonadaceae]_Other	7.78	0.36	2.67	0.38	0.52	0.22	0.00	1.81
Armatimonadetes_Other_Other_Other_Other	2.64	0.79	2.37	1.25	1.36	1.90	7.84	1.13
Bacteroidetes_c_Bacteroidia_o_Bacteroidales_f_g_	0.05	0.01	0.02	0.93	0.02	0.00	0.01	0.02
Bacteroidetes_c_Bacteroidia_o_Bacteroidales_f_Porphyromonadaceae_g_Dysgonomonas	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.00	0.0000
Bacteroidetes_c_Bacteroidia_o_Bacteroidales_f_Porphyromonadaceae_g_Parabacteroides	0.00	0.00	0.00	0.0000	0.0000	0.0000	0.00	0.0000
Bacteroidetes_c_Bacteroidia_o_Bacteroidales_f_Porphyromonadaceae_Other	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.00	0.0000
Bacteroidetes_c_Bacteroidia_o_Bacteroidales_Other_Other	0.00	0.00	0.00	0.0000	0.0000	0.98	0.00	0.00
Bacteroidetes_c_Flavobacteriia_o_Flavobacteriales_f_Flavobacteriaceae_g_Riemerella	0.00	0.00	0.00	0.0000	0.00	0.0000	0.00	0.0000
Bacteroidetes_c_Flavobacteriia_o_Flavobacteriales_f_Flavobacteriaceae_Other	0.00	0.00	0.00	0.67	0.00	0.00	0.00	0.00
Bacteroidetes_c_Sphingobacteriia_o_Sphingobacteriales_f_Chitinophagaceae_g_Flavisolibacter	0.74	0.61	0.38	0.47	0.52	0.55	0.63	0.35
Bacteroidetes_Other_Other_Other_Other	1.51	1.53	0.65	2.40	0.93	1.66	0.42	0.71
Chloroflexi_c_Anaerolineae_o_Caldilineales_f_Caldilineaceae_g_	1.85	1.27	1.94	0.86	1.05	1.19	3.07	1.14
Chloroflexi_c_Bljii12_o_f_g_	1.63	0.0005	3.07	1.29	3.30	1.67	2.15	0.94
Chloroflexi_c_Chloroflexi_o_Roseiflexales_f_g_	0.0000	0.0000	0.0001	0.00	0.0001	0.0000	0.0000	0.0000
Chloroflexi_c_Thermomicrobia_o_f_g_	0.0000	0.0002	0.0002	0.0001	0.0002	0.0000	0.0003	0.00
Cyanobacteria_c_4C0d-2_o_SM1D11_f_g_	24.26	10.49	16.81	0.63	16.39	0.0007	12.95	6.51
Cyanobacteria_c_Nostocophycideae_o_Nostocales_f_Nostocaceae_g_Nostoc	0.00	2.35	0.76	0.11	6.98	0.00	0.0005	0.24
Cyanobacteria_c_Synechococcophycideae_o_Pseudanabaenales_f_Pseudanabaenaceae_g_Leptolyngbya	0.00	0.00	0.29	0.00	3.30	0.0000	0.0001	0.37
Firmicutes_c_Bacilli_o_Bacillales_f_Bacillaceae_Other	0.59	0.18	0.29	0.71	0.98	0.33	0.41	1.07
Firmicutes_c_Bacilli_o_Bacillales_f_Planococcaceae_g_Sporosarcina	0.0002	1.13	0.32	1.02	1.51	0.0001	0.00	2.25
Firmicutes_c_Bacilli_o_Bacillales_f_Thermoactinomycetaceae_g_Planifilum	0.00	0.0000	3.69	4.13	0.0001	0.60	0.0001	0.00
Firmicutes_c_Bacilli_Other_Other_Other	0.91	0.32	0.91	0.49	1.21	0.43	0.00	0.60
Firmicutes_c_Clostridia_o_Clostridiales_f_Catabacteriaceae_g_	0.0000	0.00	0.00	0.0000	0.00	0.0000	0.00	0.00
Firmicutes_c_Clostridia_o_Clostridiales_f_Clostridiaceae_g_	0.08	0.00	0.21	0.00	0.00	0.15	0.03	0.10
Firmicutes_c_Clostridia_o_Clostridiales_f_Clostridiaceae_g_Caloramator	0.07	0.13	0.27	0.00	0.00	0.00	0.08	0.38
Firmicutes_c_Clostridia_o_Clostridiales_f_Clostridiaceae_g_Clostridium	0.25	0.84	0.63	0.48	0.35	0.17	0.67	0.46
Firmicutes_c_Clostridia_o_Clostridiales_f_Clostridiaceae_Other	0.06	0.18	0.22	0.19	0.13	0.09	0.09	0.16
Firmicutes_c_Clostridia_o_Clostridiales_f_Dehalobacteriaceae_g_	0.40	0.87	0.49	0.00	0.09	0.00	1.07	0.40
Firmicutes_c_Clostridia_o_Clostridiales_f_Dehalobacteriaceae_g_Dehalobacterium	0.00	0.0000	0.00	0.0001	0.0001	0.00	0.0000	0.00
Firmicutes_c_Clostridia_o_Clostridiales_f_Peptococcaceae_g_Desulfitobacterium	2.01	0.22	0.33	0.0000	0.00	0.00	0.47	0.0000
Firmicutes_c_Clostridia_o_Clostridiales_f_Peptococcaceae_g_WCHB1-84	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes_c_Clostridia_o_Clostridiales_f_Peptococcaceae_Other	0.29	0.31	0.33	0.07	0.00	0.04	0.26	0.20
Firmicutes_c_Clostridia_o_Clostridiales_f_Ruminococcaceae_g_Oscillospira	0.25	0.00	0.69	0.0000	0.00	0.00	2.06	0.0000
Firmicutes_c_Clostridia_o_Clostridiales_f_Ruminococcaceae_Other	0.00	0.00	0.00	0.00	0.00	0.81	0.00	0.00
Firmicutes_c_Clostridia_o_Clostridiales_f_Symbiobacteriaceae_g_Symbiobacterium	0.01	0.11	0.03	0.00	0.03	0.00	0.12	0.03
Firmicutes_c_Clostridia_o_Clostridiales_f_Symbiobacteriaceae_Other	0.08	0.11	0.10	0.00	0.10	1.16	0.00	0.00
Firmicutes_c_Clostridia_o_Clostridiales_f_Veillonellaceae_g_Desulfosporomusa	0.05	0.69	0.25	0.00	0.07	0.03	0.17	0.07
Firmicutes_c_Clostridia_o_Clostridiales_f_Veillonellaceae_g_G07	0.60	0.20	0.11	0.00	0.27	0.00	0.15	0.33
Firmicutes_c_Clostridia_o_Clostridiales_f_Veillonellaceae_g_Propionispora	0.0000	0.0000	0.00	0.0000	0.0000	0.0000	0.0000	0.0000
Firmicutes_c_Clostridia_o_Clostridiales_f_Veillonellaceae_g_Thermosinus	0.04	0.00	0.00	0.33	0.12	0.18	0.00	0.21
Firmicutes_c_Clostridia_o_Clostridiales_Other_Other	0.16	0.20	0.18	0.11	0.16	0.12	0.15	0.22
Firmicutes_c_Clostridia_Other_Other_Other	0.24	0.19	0.10	0.23	0.23	0.23	0.05	0.29
Firmicutes_c_Erysipelotrichi_o_Erysipelotrichales_f_Erysipelotrichaceae_Other	0.0000	0.00	0.00	0.0000	0.0000	0.00	0.00	0.00
Firmicutes_c_o_f_g_	0.11	0.00	0.00	0.0000	0.00	0.00	0.20	0.00
Firmicutes_Other_Other_Other_Other	0.11	0.07	0.09	0.39	0.05	0.06	0.05	0.16
Gemmatimonadetes_c_Gemm-1_o_f_g_	3.68	2.65	2.60	1.27	1.32	1.41	8.94	1.21
Gemmatimonadetes_c_Gemm-5_o_f_g_	4.79	2.64	3.45	1.51	1.61	2.05	6.50	1.64
Gemmatimonadetes_c_Gemmatimonadetes_o_C114_f_g_	0.0000	0.0000	0.69	0.00	4.34	0.00	0.0000	0.50
Gemmatimonadetes_c_Gemmatimonadetes_o_N1423WL_f_g_	2.09	0.56	1.86	0.84	1.05	1.12	3.26	1.24
MVP-21_c_o_f_g_	0.0000	0.0000	0.0000	0.0000	0.0002	0.0002	0.0000	0.0000
Nitrospirae_c_Nitrospira_o_Nitrospirales_f_g_	1.83	1.38	2.15	1.78	1.02	1.84	3.08	1.63
OP11_c_OP11-4_o_f_g_	0.0010	3.19	0.0003	18.88	0.0001	0.0004	0.0001	0.0005
Planctomycetes_c_C6_o_d113_f_g_	0.0000	0.0000	0.0000	0.0001	0.0000	0.0000	0.0000	0.0000
Planctomycetes_c_Planctomycetia_o_Gemmatales_f_Gemmataceae_g_	2.22	1.54	4.08	1.64	1.44	1.99	5.37	1.37
Proteobacteria_c_Alphaproteobacteria_o_Rhizobiales_f_Hyphomicrobiaceae_g_	2.95	1.68	1.73	1.55	0.98	0.95	2.08	1.56
Proteobacteria_c_Alphaproteobacteria_o_Rhizobiales_f_Hyphomicrobiaceae_g_Rhodoplanes	2.39	2.00	1.98	1.49	1.57	1.42	4.43	1.10
Proteobacteria_c_Alphaproteobacteria_o_Rhizobiales_f_Hyphomicrobiaceae_Other	2.02	2.04	1.77	1.24	1.22	1.18	2.95	0.99
Proteobacteria_c_Alphaproteobacteria_o_Rhizobiales_f_Rhodobiaceae_g_	4.83	2.20	6.51	0.88	0.84	0.97	3.82	1.32
Proteobacteria_c_Alphaproteobacteria_o_Rhizobiales_Other_Other	1.34	1.54	1.46	0.92	1.34	1.21	1.69	1.13
Proteobacteria_c_Alphaproteobacteria_o_Rhodobacterales_f_Rhodobacteraceae_Other	0.87	8.68	1.04	3.17	2.24	1.57	0.0008	0.94
Proteobacteria_c_Alphaproteobacteria_o_Rhodospirillales_f_Rhodospirillaceae_g_Azospirillum	0.10	1.67	0.60	0.0001	0.12	0.03	2.08	0.63
Proteobacteria_c_Alphaproteobacteria_o_Rhodospirillales_f_Rhodospirillaceae_g_Skermanella	1.82	1.37	1.30	0.89	1.28	1.39	5.05	1.24
Proteobacteria_c_Alphaproteobacteria_o_Rickettsiales_f_mitochondria_g_	3.06	2.72	1.56	2.19	1.01	1.62	3.61	1.91
Proteobacteria_c_Alphaproteobacteria_o_Sphingomonadales_f_Erythrobacteraceae_g_	1.54	1.15	0.92	0.46	0.54	0.74	3.21	2.12
Proteobacteria_c_Alphaproteobacteria_Other_Other_Other	2.53	2.91	2.53	1.85	2.04	1.23	3.77	1.67
Proteobacteria_c_Betaproteobacteria_o_Burkholderiales_f_Comamonadaceae_g_Pelomonas	0.00	0.04	0.00	0.18	0.00	0.34	0.00	0.22
Proteobacteria_c_Betaproteobacteria_o_Burkholderiales_f_Comamonadaceae_g_Polaromonas	0.77	0.00	0.00	0.32	0.00	0.00	0.00	0.65
Proteobacteria_c_Betaproteobacteria_o_Burkholderiales_f_Oxalobacteraceae_Other	0.25	0.31	0.35	0.32	0.24	0.29	0.40	0.37

Taxon	THL D58/ D9	CC D58/ D9	N D58/ D9	OATr D58/ D9	UTY D58/ D9	UTN D58/ D9	Y D58/ D9	Ctr D58/ D9
Proteobacteria_c_Betaproteobacteria_o_MND1_f_g_	4.26	2.42	4.13	2.81	2.83	3.03	6.69	2.32
Proteobacteria_c_Betaproteobacteria_o_Rhodocyclales_f_Rhodocyclaceae_g_Zoogloea	0.00	0.00	0.00	0.0000	0.00	0.00	0.00	0.51
Proteobacteria_c_Deltaproteobacteria_o_[Entotheonellales]_f_[Entotheonellaceae]_g_	2.20	1.36	1.85	1.05	0.99	1.13	2.05	1.39
Proteobacteria_c_Deltaproteobacteria_o_Myxococcales_f_g_	2.02	1.56	2.26	1.38	1.24	1.49	2.21	1.28
Proteobacteria_c_Deltaproteobacteria_o_Myxococcales_f_Haliangiaceae_g_Haliangium	4.51	7.66	4.74	21.74	3.34	0.0036	0.0032	0.0039
Proteobacteria_c_Deltaproteobacteria_o_Syntrophobacterales_f_Syntrophaceae_g_Smithella	0.0002	0.0000	2.90	4.12	0.0004	0.0002	0.0004	0.0001
Proteobacteria_c_Deltaproteobacteria_o_Syntrophobacterales_f_Syntrophobacteraceae_g_	2.41	1.35	2.35	0.96	1.22	1.06	3.57	1.09
Proteobacteria_c_Deltaproteobacteria_Other_Other_Other	1.65	1.55	1.50	0.89	1.30	1.09	2.42	1.22
Proteobacteria_c_Epsilonproteobacteria_o_Campylobacterales_f_Campylobacteraceae_g_Sulfurospirillum	0.00	0.00	0.00	0.0000	0.0000	0.0000	0.00	0.00
Proteobacteria_c_Gammaproteobacteria_o_Enterobacteriales_f_Enterobacteriaceae_g_Enterobacter	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Proteobacteria_c_Gammaproteobacteria_o_Legionellales_f_Legionellaceae_g_Legionella	0.0006	11.35	0.0006	3.23	9.78	0.0001	2.15	5.13
Proteobacteria_c_Gammaproteobacteria_o_Xanthomonadales_f_Sinobacteraceae_g_Hydrocarboniphaga	0.0005	5.78	0.0003	0.0004	0.0003	0.0002	0.0008	0.0002
Proteobacteria_c_Gammaproteobacteria_o_Xanthomonadales_f_Sinobacteraceae_g_Steroidobacter	2.21	2.15	2.13	1.26	1.42	1.29	3.20	1.24
Proteobacteria_Other_Other_Other_Other	1.41	1.41	1.37	1.59	1.37	1.64	2.23	1.43
TM7_c_TM7-3_o_I025_f_g_	1.46	0.00	0.0002	0.00	0.0000	0.30	0.0000	0.00
Verrucomicrobia_c_[Spartobacteria]_o_[Chthoniobacterales]_f_[Chthoniobacteraceae]_g_DA101	2.30	1.95	0.73	0.63	0.20	0.00	0.0006	1.80
Verrucomicrobia_c_Verrucomicrobiae_o_Verrucomicrobiales_f_Verrucomicrobiaceae_g_Prosthecobacter	1.27	4.23	3.36	0.00	7.88	2.17	0.0000	0.0006
L6: Day58/Day9								
	51	49	49	56	50	57	57	53
	4	6	8	7	7	6	2	10
	5	5	4	18	9	13	1	13
	17	25	19	16	28	29	5	26
	31	24	29	11	15	4	44	7
	1	0	0	1	0	0	0	0

Legend	x<0.5	0.5<=x<0.85	0.85=<x<1.15	1.15<=x<2	2<=x<20	x>020
	Taxon absent at day9. In these boxes, the % of the taxon in the sample at day58 is shown, not the ratio.					

Table 2.3.8.14

In the table, the ratios between the presence percentage of each genus at day 58 and at day 9 are reported, and each box is coloured depending on the resulting value (from green, when the percentage of the genus is higher at day 58 than at day 9 and therefore when it has raised over time, to red, when the percentage of the genus is lower at day 58 and therefore when it has decreased over time). At the bottom of the table, the number of genera changed to a certain extent (and coloured in a certain way) in each treated soil is reported.

The order of Rhizobiales, which comprehends some genera of nitrogen-fixing, legume nodulating microsymbiotic bacteria, tended to be more abundant at day 58, and therefore to grow over time, apart for some cases where it remained stable. In almost no cases the number of Rhizobiales was higher at day 9 than at day 58, indicating that these organisms tended to grow gradually in the two months of the trial.

Looking at the summarizing section at the bottom of table 2.3.8.14, it could be observed that Yeast caused the increase over time of a high number of taxa. This result was obtained also at the phylum level (Table 2.3.8.5). Instead, the conditions that promoted the decrease of a higher number of microorganisms over time, or the rapid increase after around 10 days and then the diminution to a basal level, were the unfertilized soil and the soil fertilized with the oxy-amino-triazine treatment OATr, together with soil treated with UTN. These results also confirm the analysis done at phylum level.

2.3.8.2.2 Changes induced by fertilization treatments on soil bacterial community at Genus level

To evaluate the differences between the treated soil samples and the unfertilized soils, two analyses were made and are reported in the following tables.

Taxon	THL/ Ctr	CC/ Ctr	N/ Ctr	OATr/ Ctr	UTY/ Ctr	UTN/ Ctr	Y/ Ctr
Acidobacteria_c__o__f__g_	0.87	0.56	0.91	1.27	1.02	1.22	0.58
Acidobacteria_c_Acidobacteria-6_o_iii1-15_f__g_	0.85	0.62	0.80	1.18	0.99	1.10	0.46
Acidobacteria_c_Acidobacteria-6_o_iii1-15_f_mb2424_g_	0.76	0.73	0.63	1.15	1.09	1.06	0.44
Acidobacteria_c_Chloracidobacteria_o__f__g_	0.85	0.63	0.80	0.89	0.98	1.00	0.56
Acidobacteria_c_iii1-8_o_DS-18_f__g_	0.85	0.96	0.90	0.80	0.87	0.94	0.81
Acidobacteria_c_S035_o__f__g_	0.99	0.68	0.71	1.38	1.05	1.06	0.53
Actinobacteria_c_Acidimicrobiia_o_Acidimicrobiales_f_EB1017_g_	0.50	0.47	0.61	0.82	0.78	1.29	0.49
Actinobacteria_c_Actinobacteria_o_Actinomycetales_f_Geodermatophilaceae_g_Geodermatophilus	1.02	1.05	0.38	1.57	2.37	1.56	0.49
Actinobacteria_c_Actinobacteria_o_Actinomycetales_f_Intrasporangiaceae_g_Phycicoccus	1.25	1.04	0.80	1.06	1.15	1.45	0.74
Actinobacteria_c_Actinobacteria_o_Actinomycetales_f_Intrasporangiaceae_Other	1.53	0.82	0.80	1.64	0.86	1.22	0.46
Actinobacteria_c_Actinobacteria_o_Actinomycetales_f_Micromonosporaceae_g_	0.07	0.14	0.41	0.45	0.36	0.13	0.37
Actinobacteria_c_Actinobacteria_o_Actinomycetales_f_Micromonosporaceae_Other	0.51	1.37	0.91	1.23	1.19	0.95	0.61
Actinobacteria_c_Actinobacteria_o_Actinomycetales_f_Mycobacteriaceae_g_Mycobacterium	0.61	0.49	0.62	0.95	0.64	0.82	0.38
Actinobacteria_c_Actinobacteria_o_Actinomycetales_f_Nocardioidaceae_Other	0.66	0.87	0.70	1.34	1.22	1.05	0.49
Actinobacteria_c_Actinobacteria_o_Actinomycetales_f_Pseudonocardiaceae_g_Pseudonocardia	0.37	0.19	0.21	0.65	0.73	0.80	0.40
Actinobacteria_c_Actinobacteria_o_Actinomycetales_f_Sporichthyaceae_g_Sporichthya	0.82	2.52	3.82	1.79	0.95	3.13	0.00
Actinobacteria_c_Actinobacteria_o_Actinomycetales_f_Thermomonosporaceae_g_	0.0000	0.0000	0.0000	0.0065	0.0000	0.0000	0.0000
Actinobacteria_c_Actinobacteria_o_Actinomycetales_Other_Other	0.67	0.73	0.72	1.05	0.94	0.96	0.47
Actinobacteria_c_MB-A2-108_o_0319-7L14_f__g_	0.51	0.37	0.50	0.88	0.71	0.91	0.34
Actinobacteria_c_Rubrobacteria_o_Rubrobacterales_f_Rubrobacteraceae_g_Rubrobacter	0.83	0.65	0.66	1.32	1.06	1.05	0.44
Actinobacteria_c_Thermoleophilia_o_Gaiellales_f_Gaiellaceae_g_	0.91	0.70	0.78	1.28	1.02	1.37	0.53
Actinobacteria_c_Thermoleophilia_o_Solirubrobacterales_f__g_	0.87	0.91	1.09	1.25	1.22	1.56	0.95
Actinobacteria_c_Thermoleophilia_o_Solirubrobacterales_Other_Other	0.73	0.70	0.65	0.91	0.87	1.12	0.55
Actinobacteria_Other_Other_Other_Other	1.28	0.83	1.01	1.55	1.12	1.60	0.51
Armatimonadetes_c_[Fimbriimonadetes]_o_[Fimbriimonadales]_f_[Fimbriimonadaceae]_Other	0.73	0.67	0.61	1.07	0.28	0.73	0.10
Armatimonadetes_Other_Other_Other_Other	1.12	1.26	1.57	1.51	0.95	1.50	0.49
Bacteroidetes_c_Bacteroidia_o_Bacteroidales_f__g_	0.49	1.28	1.32	0.01	0.14	0.19	3.12
Bacteroidetes_c_Bacteroidia_o_Bacteroidales_f_Porphyromonadaceae_g_Dysgonomonas	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0105
Bacteroidetes_c_Bacteroidia_o_Bacteroidales_f_Porphyromonadaceae_g_Parabacteroides	0.0059	0.0182	0.2013	0.0000	0.0000	0.0000	1.5046
Bacteroidetes_c_Bacteroidia_o_Bacteroidales_f_Porphyromonadaceae_Other	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0070
Bacteroidetes_c_Bacteroidia_o_Bacteroidales_Other_Other	0.41	2.10	3.44	0.00	0.00	1.04	7.30
Bacteroidetes_c_Flavobacteriia_o_Flavobacteriales_f_Flavobacteriaceae_g_Riemerella	0.0177	0.0061	0.0055	0.0000	0.0617	0.0000	0.0844
Bacteroidetes_c_Flavobacteriia_o_Flavobacteriales_f_Flavobacteriaceae_Other	182.83	11.78	29.79	5.37	33.26	5.21	582.33
Bacteroidetes_c_Sphingobacteriia_o_Sphingobacteriales_f_Chitinophagaceae_g_Flavisolibacter	1.37	1.49	1.86	0.74	1.03	0.86	1.17
Bacteroidetes_Other_Other_Other_Other	1.00	2.90	2.27	1.85	0.86	0.97	2.92
Chloroflexi_c_Anaerolineae_o_Caldilineales_f_Caldilineaceae_g_	0.77	0.65	0.74	0.94	0.76	0.80	0.53
Chloroflexi_c_Bljii12_o__f__g_	0.69	0.35	0.68	0.99	1.00	0.93	0.51
Chloroflexi_c_Chloroflexi_o_Roseiflexales_f__g_	0.0000	0.0000	0.0055	0.0097	0.0069	0.0000	0.0000
Chloroflexi_c_Thermomicrobia_o__f__g_	0.00	3.36	3.05	0.89	2.85	0.00	4.87
Cyanobacteria_c_4C0d-2_o_SM1D11_f__g_	0.89	1.76	1.16	0.78	0.66	0.41	1.61
Cyanobacteria_c_Nostocophycideae_o_Nostocales_f_Nostocaceae_g_Nostoc	0.14	0.33	0.38	0.60	0.42	0.06	0.22
Cyanobacteria_c_Synechococcophycideae_o_Pseudanabaenales_f_Pseudanabaenaceae_g_Leptolyngbya	0.41	0.21	1.34	1.34	0.47	0.00	0.12
Firmicutes_c__o__f__g_	2.72	2.24	1.27	0.00	0.16	1.39	0.81
Firmicutes_c_Bacilli_o_Bacillales_f_Bacillaceae_Other	1.88	2.66	1.48	1.22	1.48	1.50	1.60
Firmicutes_c_Bacilli_o_Bacillales_f_Planococcaceae_g_Sporosarcina	0.31	0.84	0.76	1.45	1.19	0.13	0.24
Firmicutes_c_Bacilli_o_Bacillales_f_Thermoactinomycetaceae_g_Planifilum	1.22	0.00	1.91	4.03	0.95	3.13	0.49
Firmicutes_c_Bacilli_Other_Other_Other	1.34	1.14	1.58	2.24	1.02	1.41	1.25
Firmicutes_c_Clostridia_o_Clostridiales_f_Catabacteriaceae_g_	0.00	0.84	0.38	0.00	0.95	0.00	4.87
Firmicutes_c_Clostridia_o_Clostridiales_f_Clostridiaceae_g_	1.49	0.88	0.80	0.16	0.25	0.41	3.51
Firmicutes_c_Clostridia_o_Clostridiales_f_Clostridiaceae_g_Caloramator	1.87	14.90	5.13	0.13	0.27	0.30	16.41
Firmicutes_c_Clostridia_o_Clostridiales_f_Clostridiaceae_g_Clostridium	1.61	3.99	3.21	0.19	0.63	0.85	5.91
Firmicutes_c_Clostridia_o_Clostridiales_f_Clostridiaceae_Other	1.82	2.22	1.61	0.17	0.63	0.90	3.18
Firmicutes_c_Clostridia_o_Clostridiales_f_Dehalobacteriaceae_g_	4.93	1.41	1.53	0.16	4.36	1.52	1.68
Firmicutes_c_Clostridia_o_Clostridiales_f_Dehalobacteriaceae_g_Dehalobacterium	0.41	0.00	2.29	0.45	0.48	0.52	0.00
Firmicutes_c_Clostridia_o_Clostridiales_f_Peptococcaceae_g_Desulfitobacterium	0.0088	0.0759	0.0607	0.0000	0.0034	0.0038	0.1266
Firmicutes_c_Clostridia_o_Clostridiales_f_Peptococcaceae_g_WCHB1-84	0.52	1.99	0.80	0.04	1.25	0.99	1.42
Firmicutes_c_Clostridia_o_Clostridiales_f_Peptococcaceae_Other	0.45	1.88	1.19	0.25	0.31	0.96	2.05
Firmicutes_c_Clostridia_o_Clostridiales_f_Ruminococcaceae_g_Oscillospira	0.0854	0.0121	0.0138	0.0000	0.0858	0.0451	0.0141
Firmicutes_c_Clostridia_o_Clostridiales_f_Ruminococcaceae_Other	6.53	5.89	6.87	2.69	3.80	5.21	22.40
Firmicutes_c_Clostridia_o_Clostridiales_f_Symbiobacteriaceae_g_Symbiobacterium	4.48	1.92	2.79	0.15	0.62	0.37	0.69
Firmicutes_c_Clostridia_o_Clostridiales_f_Symbiobacteriaceae_Other	6.45	2.37	1.67	0.08	0.95	0.57	0.71
Firmicutes_c_Clostridia_o_Clostridiales_f_Veillonellaceae_g_Desulfosporomusa	3.21	5.39	4.21	0.09	1.08	1.83	5.27
Firmicutes_c_Clostridia_o_Clostridiales_f_Veillonellaceae_g_G07	1.82	0.91	1.76	0.14	1.46	0.56	0.52
Firmicutes_c_Clostridia_o_Clostridiales_f_Veillonellaceae_g_Propionispora	0.0000	0.0000	0.0165	0.0000	0.0000	0.0000	0.0000
Firmicutes_c_Clostridia_o_Clostridiales_f_Veillonellaceae_g_Thermosinus	0.76	0.81	1.02	0.50	0.63	0.96	0.43
Firmicutes_c_Clostridia_o_Clostridiales_Other_Other	1.39	1.59	1.39	0.37	0.63	0.87	1.60
Firmicutes_c_Clostridia_Other_Other_Other	0.95	2.21	2.67	0.21	0.67	1.04	3.63
Firmicutes_c_Erysipelotrichi_o_Erysipelotrichales_f_Erysipelotrichaceae_Other	0.00	1.68	1.27	0.00	0.00	0.69	0.97
Firmicutes_Other_Other_Other_Other	1.00	1.70	1.69	0.18	0.38	0.91	1.68
Gemmatimonadetes_c_Gemm-1_o__f__g_	0.96	0.66	1.02	1.36	1.06	1.48	0.64
Gemmatimonadetes_c_Gemm-5_o__f__g_	1.23	0.91	0.87	1.10	1.09	1.36	0.94
Gemmatimonadetes_c_Gemmatimonadetes_o_C114_f__g_	0.00	0.00	1.27	0.60	1.58	1.04	0.00
Gemmatimonadetes_c_Gemmatimonadetes_o_N1423WL_f__g_	0.95	0.55	0.67	1.31	1.13	1.38	0.38
MVP-21_c__o__f__g_	0.0000	0.0000	0.0000	0.0000	0.0103	0.0075	0.0000
Nitrospirae_c_Nitrospira_o_Nitrospirales_f__g_	1.03	0.82	0.95	1.41	1.40	1.55	0.74
OP11_c_OP11-4_o__f__g_	1.87	0.48	0.65	2.05	0.27	0.60	0.14
Planctomycetes_c_C6_o_d113_f__g_	0.0000	0.0000	0.0000	0.0065	0.0000	0.0000	0.0000
Planctomycetes_c_Planctomycetia_o_Gemmatales_f_Gemmataceae_g_	0.64	0.71	0.77	1.19	0.90	1.07	0.57
Proteobacteria_c_Alphaproteobacteria_o_Rhizobiales_f_Hyphomicrobiaceae_g_	0.80	0.68	0.72	1.08	0.96	1.16	0.57

Taxon	THL/ Ctr	CC/ Ctr	N/ Ctr	OATr/ Ctr	UTY/ Ctr	UTN/ Ctr	Y/ Ctr
Proteobacteria_c_Alphaproteobacteria_o_Rhizobiales_f_Hyphomicrobiaceae_g_Rhodoplanes	0.82	0.74	0.76	0.93	0.96	1.07	0.59
Proteobacteria_c_Alphaproteobacteria_o_Rhizobiales_f_Hyphomicrobiaceae_Other	0.61	0.61	0.76	0.93	0.87	1.09	0.30
Proteobacteria_c_Alphaproteobacteria_o_Rhizobiales_f_Rhodobiaceae_g_	0.68	0.64	0.61	1.11	0.96	1.19	0.33
Proteobacteria_c_Alphaproteobacteria_o_Rhizobiales_Other_Other	0.93	0.89	0.84	1.16	1.02	1.12	0.64
Proteobacteria_c_Alphaproteobacteria_o_Rhodobacterales_f_Rhodobacteraceae_Other	0.58	0.68	0.76	1.24	0.72	0.94	0.37
Proteobacteria_c_Alphaproteobacteria_o_Rhodospirillales_f_Rhodospirillaceae_g_Azospirillum	7.55	4.54	4.70	0.02	1.19	0.86	4.77
Proteobacteria_c_Alphaproteobacteria_o_Rhodospirillales_f_Rhodospirillaceae_g_Skermanella	0.63	0.75	0.73	1.08	0.93	0.93	0.67
Proteobacteria_c_Alphaproteobacteria_o_Rickettsiales_f_mitochondria_g_	1.10	1.45	1.23	1.33	1.24	0.77	1.16
Proteobacteria_c_Alphaproteobacteria_o_Sphingomonadales_f_Erythrobacteraceae_g_	0.73	1.05	1.07	1.92	0.71	1.51	1.17
Proteobacteria_c_Alphaproteobacteria_Other_Other_Other	0.82	1.13	0.88	1.12	0.86	0.92	0.97
Proteobacteria_c_Betaproteobacteria_o_Burkholderiales_f_Comamonadaceae_g_Pelomonas	1.29	1.89	1.02	1.72	1.90	1.04	0.73
Proteobacteria_c_Betaproteobacteria_o_Burkholderiales_f_Comamonadaceae_g_Polaromonas	0.71	0.32	0.67	1.01	1.19	0.78	0.24
Proteobacteria_c_Betaproteobacteria_o_Burkholderiales_f_Oxalobacteraceae_Other	2.63	0.91	1.13	0.68	1.25	1.24	1.17
Proteobacteria_c_Betaproteobacteria_o_MND1_f__g_	0.91	0.73	1.00	1.34	1.20	1.35	0.78
Proteobacteria_c_Betaproteobacteria_o_Rhodocyclales_f_Rhodocyclaceae_g_Zoogloea	12.24	0.56	1.27	0.00	0.32	2.43	4.22
Proteobacteria_c_Deltaproteobacteria_o_[Entotheonellales]_f_[Entotheonellaceae]_g_	0.82	0.51	0.79	1.22	1.22	1.28	0.49
Proteobacteria_c_Deltaproteobacteria_o_Myxococcales_f__g_	0.70	0.75	0.91	1.46	0.87	0.96	0.65
Proteobacteria_c_Deltaproteobacteria_o_Myxococcales_f_Haliangiaceae_g_Haliangium	0.17	0.31	0.44	1.01	0.93	0.91	0.96
Proteobacteria_c_Deltaproteobacteria_o_Syntrophobacterales_f_Syntrophaceae_g_Smithella	3.26	0.00	3.05	4.48	4.75	2.08	5.84
Proteobacteria_c_Deltaproteobacteria_o_Syntrophobacterales_f_Syntrophobacteraceae_g_	0.80	0.48	0.72	1.04	0.88	1.11	0.52
Proteobacteria_c_Deltaproteobacteria_Other_Other_Other	1.11	0.95	1.08	1.31	1.37	1.72	0.76
Proteobacteria_c_Epsilonproteobacteria_o_Campylobacterales_f_Campylobacteraceae_g_Sulfurospirillum	11.43	5.89	5.35	0.00	0.00	0.00	8.76
Proteobacteria_c_Gammaproteobacteria_o_Enterobacteriales_f_Enterobacteriaceae_g_Enterobacter	0.41	4.63	6.49	0.45	0.48	3.13	1.95
Proteobacteria_c_Gammaproteobacteria_o_Legionellales_f_Legionellaceae_g_Legionella	1.80	1.68	1.53	1.97	1.90	0.21	0.58
Proteobacteria_c_Gammaproteobacteria_o_Xanthomonadales_f_Sinobacteraceae_g_Hydrocarboniphaga	3.26	2.52	2.29	2.24	1.90	1.04	4.38
Proteobacteria_c_Gammaproteobacteria_o_Xanthomonadales_f_Sinobacteraceae_g_Steroidobacter	0.85	1.00	0.97	1.15	1.10	1.13	0.90
Proteobacteria_Other_Other_Other_Other	0.71	0.88	0.79	1.04	0.89	1.02	0.61
TM7_c_TM7-3_o_I025_f__g_	4.08	1.68	2.29	5.37	0.00	5.21	0.00
Verrucomicrobia_c_[Spartobacteria]_o_[Chthoniobacterales]_f_[Chthoniobacteraceae]_g_DA101	1.02	0.47	0.91	1.40	0.71	0.72	0.37
Verrucomicrobia_c_Verrucomicrobiae_o_Verrucomicrobiales_f_Verrucomicrobiaceae_g_Prosthecobacter	0.92	0.42	0.38	0.11	1.07	0.39	0.00
L6: Treatment/Ctr							
	15	18	8	27	18	14	32
	30	30	33	8	15	12	29
	22	16	18	21	40	39	6
	17.000	16	21	34	19	25	12
	13	18	17	8	5	8	17
	1	0	1	0	1	0	2

Legend	x<0.5	0.5<=x<0.85	0.85=<x<1.15	1.15<=x<2	2<=x<20	x>020
	Taxon absent in Ctr (not fertilized). In these boxes, the % of the taxon in the sample is shown, not the ratio.					

Table 2.3.8.15

In the table, the ratios between the average presence percentage of each genus in each treated soil with the average presence percentage of the same genus in the unfertilized soil are reported, and each box is coloured depending on the resulting value (from green, when the percentage of the genus is higher in the treated soil and therefore when fertilization increases the growth of those organisms, to red, when the percentage of the genus is lower in the treated soil than in the control. At the bottom of the table, the number of genera changed to a certain extent (and coloured in a certain way) in each treated soil is reported.

Firstly, significant taxa used before were taken into account and the mean of the presence percentage of each taxon for a certain treatment (considering together the two sampling times), weas divided by the presence percentage in the unfertilized soil (Table 2.3.8.15), as done for the analysis at phylum level. From the observation of these results, it was possible to appreciate some interesting differences.

Some taxa were always present at higher levels (green) in the treated samples, such as Flavobacteriaceae and Ruminococcaceae, while other were always present at lower levels (red, orange), such as Micromonosporaceae, Pseudonocardiaceae, Nostocaceae.

For some taxa, it was possible to appreciate a similar trend in soils treated with urea-containing fertilizers (UTY, UTN) and with OATr, in comparison with single-matrix organic fertilizers (THL, Castor Cake, Neem and Yeast); for example, the genus

Geodermatophilus, of the order Actinomycetales, was present at higher levels (1.5 to 2.5 times more abundant than in the unfertilized soil) in the presence of the urea-containing fertilizers and OATr, while in the presence of the organic fertilizers it decreased (with Neem and Yeast) or remained stable. In the case of the family Hyphomicrobiaceae, of the order Rhizobiales, the addition of OATr, UTY or UTN did not cause a change in the number of these microorganisms, while in the presence of THL, Castor Cake, Neem and Yeast they decresed. The same happened with *Skermanella*, of the family Rhodospirillaceae and with the class Bljii12 of the phylum Chloroflexi. N-cycle bacteria of the order Nitrospirales were more abundant in the presence of OATr, UTY and UTN, while they were found at a similar or lower level in the single-matrix fertilizers, when compared to the unfertilized soil: the same situation could be found for the order MND1, for one family of the order Solirubrobacterales. *Sulfuspirillum*, of the order Campylobacterales, on the other hand, was completely impaired by the presence of the urea-containing fertilizers, while it was favoured by the single-matrix organic fertilization. Indeed, in soils treated with these last fertilizers, the number of *Sulfuspirillum* organisms increases in comparison to the unfertilized soil. All these analogies indicate a similar response of some bacterial taxa to fertilizers that have some characteristics in common.
The fertilizer Yeast seemed to have a negative effect on the growth of many microorganisms: 62% of the considered taxa were present at lower level than in the untreated sample. This result could be explained by the advantage of some bacterial groups in the presence of this fertilizer, that caused the decrease in number of some other groups. The fertilizer OATr had a strong negative effect on the growth of Clostridia.

Another kind of analysis was done to have a more precise idea of the changes caused by fertilization at genus level.
Non-parametric Wilcoxon Rank Sum test was performed to compare one single fertilized soil with the unfertilized one, and to determine which taxa changed significantly in each case. This test was used instead of the one-way Anova, because it permitted to compare two groups of samples. Results obtained for each single treatment are reported in the following tables, together with the p-value indicating the significance of the differences between treated and untreated soils.
Addition to soils of the fertilizers THL, Castor Cake and Yeast caused the change in the abundance of more than 40 different taxa, Neem and UTN of about 30 taxa, while OATr and UTY of only around 20 taxa. This observation gives a first idea of the major effects of the different fertilizers on bacterial communities. The three organic fertilizers, which gave also a major effect on plant growth, had influences on a higher number of genera, followed by the slow-release fertilizer Neem and the composite fertilizer UTN (which

contains also Neem), and at last by the composite fertilizer UTY and the oxy-amino-triazine fertilizer OATr. These last two fertilizers seemed to cause very little alterations in soil communities.

Taxon	THL %	Ctr %	THL/ Ctr	P value
k_Bacteria_p_Acidobacteria_c_Holophagae_o_Holophagales_f_Holophagaceae_g_Geothrix	0.0118	0.0397	0.30	3.47E-02
k_Bacteria_p_Acidobacteria_c_MVS-40_o__f__g_	0.0619	0.0433	1.43	2.74E-02
k_Bacteria_p_Actinobacteria_c_Actinobacteria_o__f__g_	0.0383	0.0506	0.76	1.42E-02
k_Bacteria_p_Actinobacteria_c_Actinobacteria_o_Actinomycetales_f_Geodermatophilaceae_g_Geodermatophilus	0.0147	0.0144	1.02	4.91E-02
k_Bacteria_p_Actinobacteria_c_Actinobacteria_o_Actinomycetales_f_Mycobacteriaceae_g_Mycobacterium	0.1856	0.3033	0.61	4.60E-02
k_Bacteria_p_Actinobacteria_c_Actinobacteria_o_Actinomycetales_Other_Other	1.1786	1.7513	0.67	8.65E-03
k_Bacteria_p_Actinobacteria_c_MB-A2-108_o_0319-7L14_f__g_	0.2652	0.5200	0.51	3.57E-02
k_Bacteria_p_Armatimonadetes_c_Chthonomonadetes_o_Chthonomonadales_f_Chthonomonadaceae_g_Chthonomonas	0.0471	0.0794	0.59	2.08E-02
k_Bacteria_p_Bacteroidetes_c_Sphingobacteriia_o_Sphingobacteriales_f_Sphingobacteriaceae_g_	0.1002	0.1769	0.57	3.56E-02
k_Bacteria_p_BRC1_c_PRR-11_o__f__g_	0.0383	0.0542	0.71	1.99E-02
k_Bacteria_p_Chloroflexi_c_Bljii12_o_B07_WMSP1_f_FFCH4570_g_	0.0177	0.0108	1.63	1.36E-02
k_Bacteria_p_Chloroflexi_c_Bljii12_o_B07_WMSP1_Other_Other	0.0354	0.0578	0.61	1.17E-02
k_Bacteria_p_Chloroflexi_c_Ellin6529_o__f__g_	0.0825	0.1986	0.42	1.17E-02
k_Bacteria_p_Chloroflexi_c_Ktedonobacteria_o_TK10_f__g_	0.0560	0.0036	15.51	3.72E-02
k_Bacteria_p_Chloroflexi_c_SAR202_o__f__g_	0.0059	0.0361	0.16	4.23E-03
k_Bacteria_p_Cyanobacteria_c_Chloroplast_o_Chlorophyta_f__g_	0.0471	0.0831	0.57	2.08E-02
k_Bacteria_p_Cyanobacteria_c_Chloroplast_o_Chlorophyta_f_Trebouxiophyceae_g_	0.0000	0.0867	0.00	3.78E-03
k_Bacteria_p_Cyanobacteria_c_S15B-MN24_o__f__g_	0.2298	0.0903	2.55	6.28E-03
k_Bacteria_p_Firmicutes_c_Bacilli_o_Bacillales_f_Bacillaceae_Other	0.2446	0.1300	1.88	4.60E-02
k_Bacteria_p_Firmicutes_c_Bacilli_o_Bacillales_f_Paenibacillaceae_g_Brevibacillus	0.6217	0.1083	5.74	1.57E-02
k_Bacteria_p_Firmicutes_c_Bacilli_o_Bacillales_f_Paenibacillaceae_g_Paenibacillus	0.5186	0.7294	0.71	4.60E-02
k_Bacteria_p_Firmicutes_c_Clostridia_o_Clostridiales_f_Clostridiaceae_g_Sedimentibacter	0.2711	0.0036	75.09	1.59E-02
k_Bacteria_p_Firmicutes_c_Clostridia_o_Clostridiales_f_Peptostreptococcaceae_g_Clostridium	0.6659	0.1264	5.27	2.09E-02
k_Bacteria_p_Firmicutes_c_Clostridia_o_Clostridiales_f_Ruminococcaceae_g_Oscillospira	0.0854	0.0000	0.0854	1.19E-03
k_Bacteria_p_Firmicutes_c_Clostridia_o_Clostridiales_f_Symbiobacteriaceae_Other	0.2563	0.0397	6.45	2.13E-02
k_Bacteria_p_OD1_c__o__f__g_	0.0354	0.0289	1.22	3.73E-02
k_Bacteria_p_Planctomycetes_c_Phycisphaerae_o_Phycisphaerales_f_Phycisphaeraceae_g_	0.0000	0.0289	0.00	2.73E-02
k_Bacteria_p_Proteobacteria_c_Alphaproteobacteria_o_Rhizobiales_f_Brucellaceae_g_Ochrobactrum	0.0265	0.0000	0.0265	2.73E-02
k_Bacteria_p_Proteobacteria_c_Alphaproteobacteria_o_Rhizobiales_f_Hyphomicrobiaceae_Other	0.2328	0.3828	0.61	1.57E-02
k_Bacteria_p_Proteobacteria_c_Betaproteobacteria_o_Burkholderiales_f_Comamonadaceae_g_Acidovorax	0.0383	0.0000	0.0383	1.07E-02
k_Bacteria_p_Proteobacteria_c_Betaproteobacteria_o_Burkholderiales_f_Oxalobacteraceae_g_Cupriavidus	0.0236	0.0000	0.0236	2.73E-02
k_Bacteria_p_Proteobacteria_c_Betaproteobacteria_o_Neisseriales_f_Neisseriaceae_g_Vogesella	0.1621	0.0000	0.1621	3.78E-03
k_Bacteria_p_Proteobacteria_c_Betaproteobacteria_o_Nitrosomonadales_f_Nitrosomonadaceae_g_	0.0147	0.0000	0.0147	1.07E-02
k_Bacteria_p_Proteobacteria_c_Betaproteobacteria_o_Rhodocyclales_f_Rhodocyclaceae_g_Azospira	0.0678	0.0036	18.77	3.47E-02
k_Bacteria_p_Proteobacteria_c_Betaproteobacteria_o_Rhodocyclales_f_Rhodocyclaceae_Other	1.1197	0.2311	4.84	4.60E-02
k_Bacteria_p_Proteobacteria_c_Deltaproteobacteria_o_Myxococcales_f_0319-6G20_g_	0.0265	0.0181	1.47	4.85E-02
k_Bacteria_p_Proteobacteria_c_Deltaproteobacteria_o_Myxococcales_f_Cystobacteraceae_g_	0.4037	0.6536	0.62	3.57E-02
k_Bacteria_p_Proteobacteria_c_Deltaproteobacteria_o_Myxococcales_f_Cystobacterineae_g_	0.0177	0.0000	0.0177	2.73E-02
k_Bacteria_p_Proteobacteria_c_Deltaproteobacteria_o_Myxococcales_Other_Other	0.6217	1.0508	0.59	6.32E-03
k_Bacteria_p_Proteobacteria_c_Gammaproteobacteria_o_Xanthomonadales_f_Xanthomonadaceae_g_Lysobacter	0.0383	0.0108	3.54	1.09E-02
k_Bacteria_p_Proteobacteria_c_Gammaproteobacteria_o_Xanthomonadales_f_Xanthomonadaceae_g_Thermomonas	0.1355	0.1517	0.89	4.54E-02
k_Bacteria_p_Proteobacteria_c_Gammaproteobacteria_o_Xanthomonadales_f_Xanthomonadaceae_Other	0.6453	0.3755	1.72	3.57E-02
k_Bacteria_p_Spirochaetes_c_[Leptospirae]_o_[Leptospirales]_f_Leptospiraceae_g_	0.0000	0.0181	0.00	2.73E-02
k_Bacteria_p_Verrucomicrobia_c_Opitutae_o_Opitutales_f_Opitutaceae_g_	0.3418	0.6500	0.53	3.57E-02
k_Bacteria_p_WS3_c_PRR-12_o_LD1-PA13_f__g_	0.0147	0.0036	4.08	2.56E-02
k_Bacteria_p_WS3_c_PRR-12_o_Sediment-1_f_CV106_g_	0.0147	0.0072	2.04	4.91E-02

Legend	x>=1.2	0.8<=x<1.2	x<0.8
	Taxon absent in Z (not fertilized). In these boxes, the % of the taxon in the sample is shown, not the ratio.		

Table 2.3.8.16

In the table, genera which resulted to be significantly different between soils fertilized with THL and unfertilized soils are shown. The average presence percentage of each of these genera in the fertilized soil and in the unfertilized soil, together with their ratio, are reported. Boxes containing the ratios' values are coloured depending on the resulting value (from green, when the percentage of the genus is higher in the treated soil and therefore when fertilization increases the growth of those organisms, to orange, when the percentage of the genus is lower in the treated soil than in the control).

From these tables it could be noticed that bacteria of the class Clostridia were found at higher number in the soils treated with the organic fertilizers THL, Castor Cake, Yeast, which are the fertilizers with a major effect on plant growth, and with the fourth single-matrix organic fertilizer Neem. OATr, instead, caused the decrease of 2 genera of Clostridia, while UTY and UTN did not cause a significant change in the abundance of these microorganisms. It could be hypothesized, from these observations, that Clostridia have a positive influence on plant growth. These organisms often have nitrogen-fixing activity, and this could be a possible reason why their presence in soil could enhance plant growth.

Going more into detail, the results obtained with the non-parametric Wilcoxon Rank Sum test for each fertilizer were considered separately.

Fertilization of soil with the organic fertilizer THL caused the increase and the decrease of a similar number of microorganisms (Table 2.3.8.16). In particular, seven genera were detected in the fertilized soil, that were not present in the control soil: *Oscillospira*, *Ochrobactrum*, *Acidovorax*, *Cupriavidus*, *Vogesella*, an unknown genus of Nitrosomonadaceae, and an unknown genus of Cystobacterineae. As mentioned above, this kind of fertilization led to the growth of some bacteria of the class Clostridia; more in detail, the genera *Sedimentibacter*, *Clostridium*, *Oscillospira* and an unknown genus of the family Symbiobacteriaceae were found at a higher number in the THL-fertilized pots than in the unfertilized ones. Interestingly, the genus *Sedimentibacter*, which is an anaerobic bacterium of which not much is known yet (Breitenstein *et al.*, 2002), is strongly influenced by the presence of THL, and raises of about 75 times in the presence of this organic fertilizer. Of the phylum Firmicutes, which comprehends also the class Clostridia, only one genus decreased in the presence of fertilization: *Paenibacillus*. However, several species belonging to this genus are known to be Plant Growth Promoting Rhizobacteria (PGPRs), and therefore to give advantages to the plants, when present, through several mechanisms that make them biofertilizers and/or antagonists to plant pathogens (Bloemberg & Lugtenberg, 2001). Because of that, the diminution of these organisms in fertilized soils could not be considered a positive effect. On the other hand, THL has a positive impact on the growth of a root bacterial genus, which has been shown to fix nitrogen: *Azospira* is present in the fertilized pots at a number which is almost 19 times higher than in the unfertilized pots (Bae *et al.*, 2007).

The class of bacteria Ktedonobacteria of the order TK10 also raised significantly with fertilization.

Taxon	CC %	Ctr %	CC/Ctr	P value
k_Bacteria_p_Acidobacteria_c_MVS-40_o__f__g_	0.0273	0.0433	0.63	4.44E-02
k_Bacteria_p_Actinobacteria_c_Actinobacteria_o_Actinomycetales_f_Mycobacteriaceae_g_Mycobacterium	0.1488	0.3033	0.49	1.57E-02
k_Bacteria_p_Actinobacteria_c_Actinobacteria_o_Actinomycetales_f_Nocardioidaceae_g_Pimelobacter	0.0152	0.0000	0.0152	2.73E-02
k_Bacteria_p_Actinobacteria_c_Actinobacteria_o_Actinomycetales_f_Streptomycetaceae_g_Streptomyces	0.0759	0.1697	0.45	2.38E-02
k_Bacteria_p_Actinobacteria_c_Actinobacteria_o_Micrococcales_f__g_	0.2672	0.6500	0.41	3.12E-02
k_Bacteria_p_Actinobacteria_c_MB-A2-108_o_0319-7L14_f__g_	0.1913	0.5200	0.37	4.57E-03
k_Bacteria_p_Armatimonadetes_c_Armatimonadia_o_Armatimonadales_f_Armatimonadaceae_g_	0.0304	0.0072	4.21	1.59E-02
k_Bacteria_p_Bacteroidetes_c_Sphingobacteriia_o_Sphingobacteriales_f__g_	0.2733	0.4442	0.62	2.74E-02
k_Bacteria_p_Bacteroidetes_Other_Other_Other_Other	0.3249	0.1119	2.90	1.17E-02
k_Bacteria_p_Chlorobi_c_BSV26_o_PK329_f__g_	0.0000	0.0144	0.00	2.73E-02
k_Bacteria_p_Chloroflexi_c_Anaerolineae_o_Anaerolineales_f_Anaerolinaceae_Other	0.0304	0.0831	0.37	3.19E-03
k_Bacteria_p_Chloroflexi_c_Anaerolineae_o_Anaerolineales_Other_Other	0.0000	0.0217	0.00	2.73E-02
k_Bacteria_p_Chloroflexi_c_Blji12_o_AKYG885_f_Dolo_23_g_	0.0972	0.1192	0.82	2.73E-02
k_Bacteria_p_Chloroflexi_c_Chloroflexi_o_Roseiflexales_f_Kouleothrixaceae_Other	0.0000	0.0144	0.00	2.73E-02
k_Bacteria_p_Chloroflexi_c_Ktedonobacteria_o_TK10_f__g_	0.0213	0.0036	5.89	4.65E-02
k_Bacteria_p_Chloroflexi_c_Thermobacula_o_Thermobaculales_f_Thermobaculaceae_g_	0.0425	0.0578	0.74	1.90E-02
k_Bacteria_p_Elusimicrobia_c_Elusimicrobia_o_FAC88_f__g_	0.0091	0.0361	0.25	2.83E-02
k_Bacteria_p_Firmicutes_c_Bacilli_o_Bacillales_f_Paenibacillaceae_g_Brevibacillus	0.9687	0.1083	8.94	1.13E-03
k_Bacteria_p_Firmicutes_c_Clostridia_o_Clostridiales_f_Clostridiaceae_g_Clostridium	4.7282	1.1844	3.99	4.57E-03
k_Bacteria_p_Firmicutes_c_Clostridia_o_Clostridiales_f_Clostridiaceae_g_Sedimentibacter	0.0820	0.0036	22.71	4.91E-02
k_Bacteria_p_Firmicutes_c_Clostridia_o_Clostridiales_f_Peptococcaceae_g_Desulfitobacterium	0.0759	0.0000	0.0759	2.73E-02
k_Bacteria_p_Firmicutes_c_Clostridia_o_Clostridiales_f_Symbiobacteriaceae_Other	0.0941	0.0397	2.37	3.31E-02
k_Bacteria_p_Firmicutes_c_Clostridia_o_OPB54_f__g_	0.2369	0.0794	2.98	3.19E-03
k_Bacteria_p_Planctomycetes_c_Phycisphaerae_o_Phycisphaerales_f__g_	0.0941	0.1697	0.55	3.57E-02
k_Bacteria_p_Planctomycetes_c_Planctomycetia_o_Gemmatales_f_Gemmataceae_g_	0.2551	0.3611	0.71	4.60E-02
k_Bacteria_p_Planctomycetes_c_Planctomycetia_o_Pirellulales_f_Pirellulaceae_g_	0.2733	0.4586	0.60	4.57E-03
k_Bacteria_p_Proteobacteria_c_Alphaproteobacteria_o_Rhizobiales_f__g_	0.0000	0.0253	0.00	2.73E-02
k_Bacteria_p_Proteobacteria_c_Alphaproteobacteria_o_Rhizobiales_f_Hyphomicrobiaceae_g_Pedomicrobium	0.1367	0.2419	0.56	4.60E-02
k_Bacteria_p_Proteobacteria_c_Alphaproteobacteria_o_Rhizobiales_f_Hyphomicrobiaceae_Other	0.2338	0.3828	0.61	1.57E-02
k_Bacteria_p_Proteobacteria_c_Alphaproteobacteria_o_Rhizobiales_f_Rhizobiaceae_g_Sinorhizobium	0.0364	0.0469	0.78	1.05E-02
k_Bacteria_p_Proteobacteria_c_Alphaproteobacteria_o_Rhizobiales_f_Rhodobiaceae_g_	0.1974	0.3069	0.64	2.09E-02
k_Bacteria_p_Proteobacteria_c_Alphaproteobacteria_o_Sphingomonadales_f_Sphingomonadaceae_g_Sphingobium	0.0213	0.0397	0.54	3.47E-02
k_Bacteria_p_Proteobacteria_c_Betaproteobacteria_o_Burkholderiales_f_Comamonadaceae_g_Polaromonas	0.0091	0.0289	0.32	1.59E-02
k_Bacteria_p_Proteobacteria_c_Deltaproteobacteria_o_[Entotheonellales]_f_[Entotheonellaceae]_g_	0.3857	0.7511	0.51	6.32E-03
k_Bacteria_p_Proteobacteria_c_Deltaproteobacteria_o_MIZ46_f__g_	0.2217	0.3792	0.58	1.57E-02
k_Bacteria_p_Proteobacteria_c_Deltaproteobacteria_o_Myxococcales_f_Cystobacteraceae_g_	0.4160	0.6536	0.64	2.09E-02
k_Bacteria_p_Proteobacteria_c_Deltaproteobacteria_o_Myxococcales_f_Nannocystaceae_g_Plesiocystis	0.0395	0.0289	1.37	1.53E-02
k_Bacteria_p_Proteobacteria_c_Deltaproteobacteria_o_NB1-j_f_NB1-i_g_	0.0000	0.0217	0.00	2.73E-02
k_Bacteria_p_Proteobacteria_c_Deltaproteobacteria_o_Syntrophobacterales_f_Syntrophobacteraceae_g_	0.6195	1.2927	0.48	3.28E-03
k_Bacteria_p_Proteobacteria_c_Gammaproteobacteria_o_Xanthomonadales_f_Xanthomonadaceae_g_Lysobacter	0.0334	0.0108	3.08	3.47E-02
k_Bacteria_p_Verrucomicrobia_c_Verrucomicrobiae_o_Verrucomicrobiales_f_Verrucomicrobiaceae_g_Luteolibacter	0.0304	0.0433	0.70	3.01E-02
k_Bacteria_p_WS3_c_PRR-12_o_Sediment-1_f__g_	0.1245	0.2167	0.57	2.74E-02

Legend	x>=1.2	0.8<=x<1.2	x<0.8
	Taxon absent in Z (not fertilized). In these boxes, the % of the taxon in the sample is shown, not the ratio.		

Table 2.3.8.17

In the table, genera which resulted to be significantly different between soils fertilized with Castor Cake and unfertilized soils are shown. The average presence percentage of each of these genera in the fertilized soil and in the unfertilized soil, together with their ratio, are reported. Boxes containing the ratios' values are coloured depending on the resulting value (from green, when the percentage of the genus is higher in the treated soil and therefore when fertilization increases the growth of those organisms, to orange, when the percentage of the genus is lower in the treated soil than in the control).

At a first observation of table 2.3.8.17, it can be easily observed that most of the microorganisms which significantly changed in number in the presence of the organic fertilizer Castor Cake decreased, while just 12 genera were found at a higher number in fertilized pots, 5 of which are part of the class Clostridia. Only two genera, which were not present in the unfertilized soil, appeared after fertilization: *Pimelobacter* and *Desulfitobacterium*.

Taxon	N %	Ctr %	N/Ctr	P value
k_Bacteria_p_Actinobacteria_c_Actinobacteria_o_Actinomycetales_f_Propionibacteriaceae_g_Microlunatus	0.0303	0.0506	0.60	3.52E-02
k_Bacteria_p_Actinobacteria_c_Actinobacteria_o_Actinomycetales_f_Sporichthyaceae_g_Sporichthya	0.0138	0.0036	3.82	2.56E-02
k_Bacteria_p_Actinobacteria_c_Actinobacteria_o_Actinomycetales_f_Streptomycetaceae_g_Streptomyces	0.0744	0.1697	0.44	2.38E-02
k_Bacteria_p_Actinobacteria_c_Actinobacteria_o_Actinomycetales_Other_Other	1.2655	1.7513	0.72	2.09E-02
k_Bacteria_p_Actinobacteria_c_Actinobacteria_o_Micrococcales_f__g_	0.2923	0.6500	0.45	4.04E-02
k_Bacteria_p_Actinobacteria_c_MB-A2-108_o__f__g_	0.0000	0.0036	0.00	4.60E-02
k_Bacteria_p_Actinobacteria_c_Thermoleophilia_o_Solirubrobacterales_Other_Other	0.4687	0.7222	0.65	3.57E-02
k_Bacteria_p_Bacteroidetes_c_Sphingobacteriia_o_Sphingobacteriales_f_Chitinophagaceae_g_Niastella	0.0551	0.0831	0.66	2.08E-02
k_Bacteria_p_Bacteroidetes_Other_Other_Other_Other	0.2537	0.1119	2.27	4.60E-02
k_Bacteria_p_Chloroflexi_c_Bljii12_o__f__g_	0.0469	0.0686	0.68	3.43E-02
k_Bacteria_p_Chloroflexi_c_Chloroflexi_o_Chloroflexales_f_Oscillochloridaceae_g_Oscillochloris	0.0000	0.0217	0.00	1.07E-02
k_Bacteria_p_Chloroflexi_c_Ktedonobacteria_o_TK10_f__g_	0.0358	0.0036	9.93	2.06E-02
k_Bacteria_p_Chloroflexi_c_Thermomicrobia_o_JG30-KF-CM45_f__g_	0.0248	0.0397	0.62	4.54E-02
k_Bacteria_p_Chloroflexi_Other_Other_Other_Other	0.0414	0.0650	0.64	4.60E-02
k_Bacteria_p_Cyanobacteria_c_Chloroplast_o_Chlorophyta_f__g_	0.0551	0.0831	0.66	4.58E-02
k_Bacteria_p_Firmicutes_c_Bacilli_o_Bacillales_f_Paenibacillaceae_g_Brevibacillus	1.8445	0.1083	17.03	7.78E-04
k_Bacteria_p_Firmicutes_c_Clostridia_o_Clostridiales_f_Clostridiaceae_g_Clostridium	3.7993	1.1844	3.21	1.17E-02
k_Bacteria_p_Firmicutes_c_Clostridia_o_Clostridiales_f_Clostridiaceae_g_Sedimentibacter	0.0634	0.0036	17.57	4.65E-02
k_Bacteria_p_Firmicutes_c_Clostridia_o_Clostridiales_f_Lachnospiraceae_g_	0.5762	0.1083	5.32	3.57E-02
k_Bacteria_p_Firmicutes_c_Clostridia_o_Clostridiales_f_Peptococcaceae_g_Desulfitobacterium	0.0607	0.0000	0.0607	3.78E-03
k_Bacteria_p_Firmicutes_c_Clostridia_o_Clostridiales_f_Symbiobacteriaceae_g_Symbiobacterium	1.8417	0.6608	2.79	1.17E-02
k_Bacteria_p_Firmicutes_c_Clostridia_o_Clostridiales_f_Symbiobacteriaceae_Other	0.0662	0.0397	1.67	2.49E-02
k_Bacteria_p_Proteobacteria_c_Alphaproteobacteria_o_Rhizobiales_f_Rhizobiaceae_g_Sinorhizobium	0.0469	0.0469	1.00	1.90E-02
k_Bacteria_p_Proteobacteria_c_Alphaproteobacteria_o_Rhizobiales_f_Xanthobacteraceae_g_	0.0303	0.0036	8.40	4.23E-03
k_Bacteria_p_Proteobacteria_c_Alphaproteobacteria_o_Rhodobacterales_f_Rhodobacteraceae_g_Amaricoccus	0.0772	0.1300	0.59	3.57E-02
k_Bacteria_p_Proteobacteria_c_Betaproteobacteria_o_Burkholderiales_f_Comamonadaceae_g_Polaromonas	0.0193	0.0289	0.67	4.61E-02
k_Bacteria_p_Proteobacteria_c_Betaproteobacteria_o_Methylophilales_f_Methylophilaceae_g_Methylobacillus	0.0331	0.0506	0.65	3.91E-02
k_Bacteria_p_Proteobacteria_c_Deltaproteobacteria_o_Myxococcales_f_Cystobacteraceae_g_	0.3612	0.6536	0.55	1.57E-02
k_Bacteria_p_Proteobacteria_c_Deltaproteobacteria_o_Myxococcales_f_Cystobacterineae_g_	0.0248	0.0000	0.0248	2.73E-02
k_Bacteria_p_Proteobacteria_c_Deltaproteobacteria_o_Myxococcales_f_Haliangiaceae_g_Haliangium	0.0855	0.1950	0.44	4.00E-03
k_Bacteria_p_Verrucomicrobia_c_[Spartobacteria]_o_[Chthoniobacterales]_f_[Chthoniobacteraceae]_Other	0.0165	0.0289	0.57	2.13E-02

Legend	x>=1.2	0.8<=x<1.2	x<0.8
	Taxon absent in Z (not fertilized). In these boxes, the % of the taxon in the sample is shown, not the ratio.		

Table 2.3.8.18

In the table, genera which resulted to be significantly different between soils fertilized with Neem and unfertilized soils are shown. The average presence percentage of each of these genera in the fertilized soil and in the unfertilized soil, together with their ratio, are reported. Boxes containing the ratios' values are coloured depending on the resulting value (from green, when the percentage of the genus is higher in the treated soil and therefore when fertilization increases the growth of those organisms, to orange, when the percentage of the genus is lower in the treated soil than in the control).

As for the fertilizer THL, the organisms that raised most were part of the genus *Sedimentibacter*. The genus *Brevibacillus* was detected in fertilized pots with a percentage, which was around 9 times higher than in the unfertilized control. Some species of this genus were described as PGPRs, and their abundance in agricultural soil is favourable (Kadyan *et al.*, 2013; Ratón, Teresa de los Milagros Orberá *et al.*, 2012). Also the increase in the number of *Lysobacter* can be considered a positive effect of Castor Cake on soil community, as several species belonging to this genus are known to produce bioactive natural products and to be antagonists of important plant diseases (Hayward *et al.*, 2010; Xie *et al.*, 2012). As for the fertilizer THL, an increase of the number of organisms of the class Ktedonobacteria could be detected.

The slow-release organic fertilizer Neem caused significant changes in about 30 of the more than 900 genera of bacteria present in the soil (Table 2.3.8.18). Some analogies could be detected with the effects induced by Castor Cake. Indeed, also in this case a considerable increase in the number of the genera *Brevibacillus*, *Sedimentibacter*, and *Ktedonobacteria* could be identified. Also with Neem, the genus *Desulfitobacterium* grew in the fertilized soil, while it was not detected in the unfertilized control, together with a genus of the family Cystobacterineae. Six genera belonging to the class Clostridia significantly grew in number with fertilization, together with the genus *Sporichthya* of the phylum Actinobacteria and with an unknown genus of the family Xanthobacteraceae.

Taxon	OATr %	Ctr %	OATr/Ctr	P value
k_Bacteria_p_Acidobacteria_c_MVS-40_o__f__g_	0.0841	0.0433	1.94	1.57E-02
k_Bacteria_p_Actinobacteria_c_Actinobacteria_o_Actinomycetales_f_Actinosynnemataceae_Other	0.0065	0.0289	0.22	1.09E-02
k_Bacteria_p_Actinobacteria_c_Actinobacteria_o_Actinomycetales_f_Streptomycetaceae_Other	0.0323	0.0253	1.28	4.28E-02
k_Bacteria_p_Actinobacteria_Other_Other_Other_Other	0.4914	0.3178	1.55	3.57E-02
k_Bacteria_p_Chloroflexi_c_Anaerolineae_Other_Other_Other	0.1875	0.1228	1.53	2.74E-02
k_Bacteria_p_Chloroflexi_c_Bljii12_o_AKYG885_f_5B-12_g_	0.0194	0.0000	0.0194	2.73E-02
k_Bacteria_p_Chloroflexi_c_Ktedonobacteria_o_TK10_f__g_	0.0388	0.0036	10.75	5.99E-03
k_Bacteria_p_Elusimicrobia_c_Elusimicrobia_o_MVP-88_f__g_	0.0582	0.0361	1.61	2.04E-02
k_Bacteria_p_Firmicutes_c_Bacilli_o_Bacillales_f_Planococcaceae_g_Sporosarcina	0.0420	0.0289	1.45	1.90E-02
k_Bacteria_p_Firmicutes_c_Clostridia_o_Clostridiales_f_Clostridiaceae_g_Clostridium	0.2231	1.1844	0.19	2.32E-03
k_Bacteria_p_Firmicutes_c_Clostridia_o_Clostridiales_f_Veillonellaceae_g_G07	0.0065	0.0469	0.14	2.06E-02
k_Bacteria_p_Gemmatimonadetes_c_Gemm-3_o__f__g_	0.1778	0.2925	0.61	4.60E-02
k_Bacteria_p_Gemmatimonadetes_Other_Other_Other_Other	0.0259	0.0000	0.0259	2.73E-02
k_Bacteria_p_OP11_Other_Other_Other_Other	0.0162	0.0000	0.0162	1.07E-02
k_Bacteria_p_Planctomycetes_c_028H05-P-BN-P5_o__f__g_	0.0259	0.0036	7.16	1.51E-02
k_Bacteria_p_Planctomycetes_c_Planctomycetia_o_Pirellulales_f_Pirellulaceae_g_A17	0.0808	0.0506	1.60	1.99E-02
k_Bacteria_p_Proteobacteria_c_Alphaproteobacteria_o_Rhodospirillales_f_Acetobacteraceae_Other	0.0259	0.0361	0.72	4.91E-02
k_Bacteria_p_Proteobacteria_c_Alphaproteobacteria_o_Rhodospirillales_f_Rhodospirillaceae_g_Azospirillum	0.0032	0.1444	0.02	4.91E-02
k_Bacteria_p_Proteobacteria_c_Alphaproteobacteria_o_Sphingomonadales_f_Erythrobacteraceae_Other	0.0194	0.0108	1.79	2.49E-02
k_Bacteria_p_Proteobacteria_c_Betaproteobacteria_o_Hydrogenophilales_f__g_	0.0000	0.0469	0.00	2.73E-02
k_Bacteria_p_Proteobacteria_c_Deltaproteobacteria_o_Myxococcales_f_Cystobacteraceae_g_	0.4526	0.6536	0.69	3.57E-02
k_Bacteria_p_Proteobacteria_c_Deltaproteobacteria_o_Myxococcales_f_Nannocystaceae_g_Nannocystis	0.0323	0.0289	1.12	3.08E-03
k_Bacteria_p_Proteobacteria_c_Gammaproteobacteria_o_Xanthomonadales_f_Xanthomonadaceae_g_Lysobacter	0.0291	0.0108	2.69	3.47E-02
k_Bacteria_p_WS3_c_PRR-12_o_LD1-PA13_f__g_	0.0388	0.0036	10.75	3.72E-02

Legend	x>=1.2	0.8<=x<1.2	x<0.8
	Taxon absent in Z (not fertilized). In these boxes, the % of the taxon in the sample is shown, not the ratio.		

Table 2.3.8.19

In the table, genera which resulted to be significantly different between soils fertilized with OATr and unfertilized soils are shown. The average presence percentage of each of these genera in the fertilized soil and in the unfertilized soil, together with their ratio, are reported. Boxes containing the ratios' values are coloured depending on the resulting value (from green, when the percentage of the genus is higher in the treated soil and therefore when fertilization increases the growth of those organisms, to orange, when the percentage of the genus is lower in the treated soil than in the control).

From table 2.3.8.19, it can be noticed that the slow-release oxy-amino-triazine fertilizer OATr induced significant changes in the abundance of few genera of microorganisms. Indeed, only 24 genera are reported in this table. 15 of these genera were found at a higher level after fertilization, while other 8 were reduced by the treatment.

Acidobacteria and Actinobacteria, which were found to be impaired by the three organic fertilizers taken into consideration before, are prevalently favoured in the presence of this fertilizer. Their number is indeed higher in fertilized pots than in the unfertilized control (apart from an unknown genus of the family Actinosynnemataceae). Another difference found with OATr is the positive growth effect on the phylum Planctomycetes, which was instead impaired or had no significant changes in the three soils treated with THL, Castor Cake and Neem.

Taxon	UTY %	Ctr %	UTY/Ctr	P value
k_Bacteria_p_Acidobacteria_c_EC1113_o__f__g_	0.0000	0.0144	0.00	2.73E-02
k_Bacteria_p_Acidobacteria_c_Holophagae_o_Holophagales_Other_Other	0.0000	0.1083	0.00	2.73E-02
k_Bacteria_p_Actinobacteria_c_Actinobacteria_o_Actinomycetales_f_Geodermatophilaceae_g_Geodermatophilus	0.0343	0.0144	2.37	1.36E-02
k_Bacteria_p_Actinobacteria_c_Actinobacteria_o_Actinomycetales_f_Micrococcaceae_Other	0.2058	0.0939	2.19	1.57E-02
k_Bacteria_p_Actinobacteria_c_Actinobacteria_o_Micrococcales_f__g_	0.3396	0.6500	0.52	8.65E-03
k_Bacteria_p_Chlorobi_c_Ignavibacteria_o_Ignavibacteriales_f_Ignavibacteriaceae_g_Ignavibacterium	0.0412	0.0000	0.0412	2.73E-02
k_Bacteria_p_Chloroflexi_c_Bljii12_o__f__g_	0.0686	0.0686	1.00	3.56E-02
k_Bacteria_p_Chloroflexi_c_Bljii12_o_AKYG885_f_Dolo_23_g_	0.0720	0.1192	0.60	8.60E-03
k_Bacteria_p_Cyanobacteria_c_S15B-MN24_o__f__g_	0.1612	0.0903	1.79	6.28E-03
k_Bacteria_p_Elusimicrobia_c_Elusimicrobia_o_FAC88_f__g_	0.0206	0.0361	0.57	4.61E-02
k_Bacteria_p_Firmicutes_c_Bacilli_o_Bacillales_f_Alicyclobacillaceae_g_Alicyclobacillus	0.1304	0.0939	1.39	3.56E-02
k_Bacteria_p_OD1_c__o__f__g_	0.0034	0.0289	0.12	3.72E-02
k_Bacteria_p_Proteobacteria_c_Alphaproteobacteria_o_Caulobacterales_f_Caulobacteraceae_g_Mycoplana	0.0137	0.0000	0.0137	2.73E-02
k_Bacteria_p_Proteobacteria_c_Alphaproteobacteria_o_Rhizobiales_f_Bradyrhizobiaceae_g_	0.0926	0.1228	0.75	4.60E-02
k_Bacteria_p_Proteobacteria_c_Alphaproteobacteria_o_Rhizobiales_f_Rhizobiaceae_g_Sinorhizobium	0.0446	0.0469	0.95	1.90E-02
k_Bacteria_p_Proteobacteria_c_Alphaproteobacteria_o_Rickettsiales_Other_Other	0.0274	0.0108	2.53	3.31E-02
k_Bacteria_p_Proteobacteria_c_Betaproteobacteria_o_Burkholderiales_f_Comamonadaceae_g_Polaromonas	0.0343	0.0289	1.19	5.96E-03
k_Bacteria_p_Proteobacteria_c_Deltaproteobacteria_o_Myxococcales_f_Cystobacterineae_g_	0.0172	0.0000	0.0172	1.07E-02
k_Bacteria_p_Proteobacteria_c_Deltaproteobacteria_o_Myxococcales_f_Haliangiaceae_g_Haliangium	0.1818	0.1950	0.93	4.61E-02
k_Bacteria_p_Proteobacteria_c_Gammaproteobacteria_o_Methylococcales_f_Methylococcaceae_g_Methylomonas	0.0000	0.0325	0.00	2.73E-02
k_Bacteria_p_Proteobacteria_c_Gammaproteobacteria_o_Xanthomonadales_f_Xanthomonadaceae_g_Lysobacter	0.0172	0.0108	1.58	2.56E-02
k_Bacteria_p_WS3_c_PRR-12_o_LD1-PA13_f__g_	0.0206	0.0036	5.70	4.91E-02

Legend	x>=1.2	0.8<=x<1.2	x<0.8
	Taxon absent in Z (not fertilized). In these boxes, the % of the taxon in the sample is shown, not the ratio.		

Table 2.3.8.20

In the table, genera which resulted to be significantly different between soils fertilized with UTY and unfertilized soils are shown. The average presence percentage of each of these genera in the fertilized soil and in the unfertilized soil, together with their ratio, are reported. Boxes containing the ratios' values are coloured depending on the resulting value (from green, when the percentage of the genus is higher in the treated soil and therefore when fertilization increases the growth of those organisms, to orange, when the percentage of the genus is lower in the treated soil than in the control).

Also with this kind of fertilization, as with the previously considered organic ones, the class Ktedonobacteria had a benefit. The organisms belonging to the genus *Lydobacter* were present at a number, which was around 3 times higher than in the unfertilized soil, a similar result as with the fertilizer Castor Cake.

As stated before in this chapter, an important difference found at this level is the decrease in the number of Clostridia in the presence of this fertilizer.

Only 22 genera, out of more than 900 found in these soils, changed in number in the presence of the composite fertilizer UTY (Table 2.3.8.20). The genus *Ignavibacterium*

belonging to the phylum Clorobi, the genus *Mycoplana* and an unknown genus of the family Cystobacterineae, both belonging to the phylum Proteobacteria, were detected only in the fertilized pots. Bacteria belonging to the candidate division WS3 were found, in the presence of UTY, at a percentage that was almost 6 times higher than in the control soil.

Taxon	UTN %	Ctr %	UTN/Ctr	P value
k_Bacteria_p_Acidobacteria_c_MVS-40_o__f__g_	0.0339	0.0433	0.78	4.54E-02
k_Bacteria_p_Actinobacteria_c_Actinobacteria_o_Actinomycetales_f_Intrasporangiaceae_g_Phycicoccus	0.1994	0.1372	1.45	4.60E-02
k_Bacteria_p_Actinobacteria_c_Actinobacteria_o_Actinomycetales_f_Micromonosporaceae_g_Actinoplanes	0.0000	0.0289	0.00	2.73E-02
k_Bacteria_p_Actinobacteria_c_Actinobacteria_o_Micrococcales_f__g_	0.3912	0.6500	0.60	3.57E-02
k_Bacteria_p_Actinobacteria_c_Thermoleophilia_o_Solirubrobacterales_f__g_	0.5116	0.3286	1.56	3.57E-02
k_Bacteria_p_Actinobacteria_Other_Other_Other_Other	0.5078	0.3178	1.60	2.09E-02
k_Bacteria_p_Bacteroidetes_c_Sphingobacteriia_o_Sphingobacteriales_f_Chitinophagaceae_g_Niastella	0.0715	0.0831	0.86	4.58E-02
k_Bacteria_p_Bacteroidetes_c_Sphingobacteriia_o_Sphingobacteriales_f_Flexibacteraceae_g_Dyadobacter	0.0000	0.0325	0.00	2.73E-02
k_Bacteria_p_Chloroflexi_c_Bljii12_o_AKYG885_f_Dolo_23_g_	0.0865	0.1192	0.73	1.56E-02
k_Bacteria_p_Chloroflexi_c_TK17_o__f__g_	0.0301	0.0036	8.34	3.72E-02
k_Bacteria_p_Cyanobacteria_c_Chloroplast_o_Chlorophyta_f_Trebouxiophyceae_g_	0.0038	0.0867	0.04	4.91E-02
k_Bacteria_p_Cyanobacteria_c_Nostocophycideae_o_Nostocales_f_Nostocaceae_g_Nostoc	0.0038	0.0650	0.06	3.72E-02
k_Bacteria_p_Cyanobacteria_c_Synechococcophycideae_o_Pseudanabaenales_f_Pseudanabaenaceae_g_Leptolyngbya	0.0000	0.0289	0.00	2.73E-02
k_Bacteria_p_Elusimicrobia_c_Elusimicrobia_o_FAC88_f__g_	0.0301	0.0361	0.83	4.28E-02
k_Bacteria_p_Firmicutes_c_Bacilli_Other_Other_Other	0.0715	0.0506	1.41	2.29E-02
k_Bacteria_p_Firmicutes_c_Clostridia_o_Clostridiales_f_Symbiobacteriaceae_g_Symbiobacterium	0.2445	0.6608	0.37	2.63E-02
k_Bacteria_p_Nitrospirae_c_Nitrospira_o_Nitrospirales_f__g_	0.4589	0.2961	1.55	4.60E-02
k_Bacteria_p_Nitrospirae_c_Nitrospira_o_Nitrospirales_f_Nitrospiraceae_g_JG37-AG-70	0.0113	0.0181	0.63	4.91E-02
k_Bacteria_p_Planctomycetes_c_BD7-11_o__f__g_	0.0226	0.0325	0.69	4.28E-02
k_Bacteria_p_Planctomycetes_c_Planctomycetia_o_Gemmatales_f_Gemmataceae_Other	0.0715	0.0506	1.41	8.60E-03
k_Bacteria_p_Proteobacteria_c_Alphaproteobacteria_o_BD7-3_f__g_	0.0451	0.0578	0.78	3.08E-03
k_Bacteria_p_Proteobacteria_c_Alphaproteobacteria_o_Rhizobiales_f_Hyphomicrobiaceae_g_Devosia	0.1768	0.3105	0.57	3.57E-02
k_Bacteria_p_Proteobacteria_c_Alphaproteobacteria_o_Rhizobiales_f_Methylobacteriaceae_g_Methylobacterium	0.0188	0.0614	0.31	3.47E-02
k_Bacteria_p_Proteobacteria_c_Betaproteobacteria_o_Burkholderiales_f_Comamonadaceae_g_Polaromonas	0.0226	0.0289	0.78	3.08E-03
k_Bacteria_p_Proteobacteria_c_Betaproteobacteria_o_Burkholderiales_f_Oxalobacteraceae_g_Janthinobacterium	0.0527	0.0361	1.46	4.61E-02
k_Bacteria_p_Proteobacteria_c_Deltaproteobacteria_o_Myxococcales_f_Cystobacterineae_g_	0.0301	0.0000	0.0301	2.73E-02
k_Bacteria_p_Proteobacteria_c_Deltaproteobacteria_o_Myxococcales_f_Haliangiaceae_g_	0.2144	0.1661	1.29	1.57E-02
k_Bacteria_p_Proteobacteria_c_Deltaproteobacteria_Other_Other_Other	1.3279	0.7728	1.72	1.57E-02

Legend	x>=1.2	0.8<=x<1.2	x<0.8
	Taxon absent in Z (not fertilized). In these boxes, the % of the taxon in the sample is shown, not the ratio.		

Table 2.3.8.21

In the table, genera which resulted to be significantly different between soils fertilized with UTN and unfertilized soils are shown. The average presence percentage of each of these genera in the fertilized soil and in the unfertilized soil, together with their ratio, are reported. Boxes containing the ratios' values are coloured depending on the resulting value (from green, when the percentage of the genus is higher in the treated soil and therefore when fertilization increases the growth of those organisms, to orange, when the percentage of the genus is lower in the treated soil than in the control).

The composite fertilizer UTN, containing Urea, THL and Neem, caused a significant change in the number of bacteria belonging to about 30 genera (Table 2.3.8.21). Only one genus was detected in the fertilized but not in the unfertilized soil: an unknown genus of the family Cystobacterineae. This evidence was detected for the single-matrix fertilizer Neem, as well.

An evident decrease in the number of Cyanobacteria could be detected, together with the decrease in the number of bacteria belonging to two families of the order

Rhizobiales. The taxon which increased the most in number, thanks to fertilization, belonged to the phylum Chloroflexi, class TK17 (a candidate division). Three classes belonging to the phylum Cyanobacteria, on the other hand, similarly as in soils fertilized with UTY, decreased in number.

The genera *Devosia* and *Methylobacterium*, both belonging to the order Rhizobiales, decreased in number after fertilization, together with bacteria of the genus *Polaromonas*, of the order Burkholderiales. These three genera all comprehend species, which could have beneficial effects on plant growth. It was shown that some species belonging to the first two genera, *Devosia* and *Methylobacterium*, can form root nodules with specific plants and fix nitrogen (Rivas *et al.*, 2002; Joe *et al.*, 2013), while some species of the genus *Polaromonas* are able to degrade some contaminant substances, such as naphthalene (Jeon *et al.*, 2003).

The single-matrix organic fertilizer Yeast, with THL, was the treatment, which caused significant changes to the highest number of genera. Indeed, 46 taxa are reported in the table 2.3.8.22. Four genera were detected in the soil only after the addition of the fertilizer: *Parabacteroides*, *Riemerella*, *Aneurinibacillus*, and *Desulfitobacterium*.

It could be noticed from the results, that the changes caused by Yeast fertilization are mostly negative, with the majority of microorganisms belonging to several genera present at a lower number when compared to the unfertilized soil. In particular, the decrease in number of bacteria belonging to several genera of Acidobacteria and Actinobacteria occurred, together with Planctomycetes and Proteobacteria. Also the taxon JG37-AG-70, belonging to the family Nitrospiraceae, decreased in number with fertilization. Bacteria belonging to this family (Nitrospira) are known to be nitrifying organisms.

Five genera of the class Clostridia significantly raised in number. In particular, bacteria of the family Lachnospiraceae were detected at a number, which was more than 14 times higher in the Yeast-fertilized soil than in the unfertilized control.

Taxon	Y %	Ctr %	Y/Ctr	P value
k_Bacteria_Other_Other_Other_Other_Other	2.5874	3.9360	0.66	4.57E-03
k_Bacteria_p_Acidobacteria_c_Acidobacteria-6_o_iii1-15_f__g_	2.3061	5.0627	0.46	3.57E-02
k_Bacteria_p_Acidobacteria_c_Acidobacteria-6_o_iii1-15_f_mb2424_g_	0.4500	1.0291	0.44	6.32E-03
k_Bacteria_p_Acidobacteria_c_EC1113_o__f__g_	0.0000	0.0144	0.00	2.73E-02
k_Bacteria_p_Acidobacteria_c_Holophagae_o_Holophagales_Other_Other	0.0000	0.1083	0.00	2.73E-02
k_Bacteria_p_Acidobacteria_c_MVS-40_o__f__g_	0.0387	0.0433	0.89	8.17E-03
k_Bacteria_p_Actinobacteria_c_Actinobacteria_o_Actinomycetales_f_Micromonosporaceae_g_Virgisporangium	0.0070	0.0469	0.15	5.99E-03
k_Bacteria_p_Actinobacteria_c_Actinobacteria_o_Actinomycetales_f_Streptomycetaceae_g_Streptomyces	0.0703	0.1697	0.41	4.04E-02
k_Bacteria_p_Actinobacteria_c_Actinobacteria_o_Actinomycetales_Other_Other	0.8226	1.7513	0.47	1.63E-03
k_Bacteria_p_Actinobacteria_c_Rubrobacteria_o_Rubrobacterales_f_Rubrobacteraceae_g_Rubrobacter	0.1266	0.2889	0.44	1.57E-02
k_Bacteria_p_Actinobacteria_c_Thermoleophilia_o_Gaiellales_f_Gaiellaceae_g_	0.8367	1.5816	0.53	2.74E-02
k_Bacteria_p_Actinobacteria_c_Thermoleophilia_o_Solirubrobacterales_Other_Other	0.3972	0.7222	0.55	2.74E-02
k_Bacteria_p_Bacteroidetes_c_Bacteroidia_o_Bacteroidales_f_Porphyromonadaceae_g_Parabacteroides	1.5046	0.0000	1.5046	2.73E-02
k_Bacteria_p_Bacteroidetes_c_Flavobacteriia_o_Flavobacteriales_f_Flavobacteriaceae_g_Riemerella	0.0844	0.0000	0.0844	2.73E-02
k_Bacteria_p_Bacteroidetes_c_Sphingobacteriia_o_Sphingobacteriales_f_Flexibacteraceae_g_Adhaeribacter	0.0633	0.0650	0.97	1.15E-02
k_Bacteria_p_Bacteroidetes_Other_Other_Other_Other	0.3269	0.1119	2.92	1.17E-02
k_Bacteria_p_BRC1_c_PRR-11_o__f__g_	0.0387	0.0542	0.71	4.54E-02
k_Bacteria_p_Chlorobi_c_BSV26_o_PK329_f__g_	0.0000	0.0144	0.00	2.73E-02
k_Bacteria_p_Chloroflexi_c_Anaerolineae_o_Caldilineales_f_Caldilineaceae_g_	0.2144	0.4080	0.53	1.57E-02
k_Bacteria_p_Chloroflexi_c_Chloroflexi_o_Chloroflexales_f_Oscillochloridaceae_g_Oscillochloris	0.0246	0.0217	1.14	4.91E-02
k_Bacteria_p_Firmicutes_c_Bacilli_o_Bacillales_f_Paenibacillaceae_g_Aneurinibacillus	0.5414	0.0000	0.5414	1.07E-02
k_Bacteria_p_Firmicutes_c_Bacilli_o_Bacillales_f_Paenibacillaceae_g_Brevibacillus	0.3972	0.1083	3.67	1.17E-02
k_Bacteria_p_Firmicutes_c_Bacilli_o_Bacillales_f_Paenibacillaceae_Other	0.0527	0.1372	0.38	3.57E-02
k_Bacteria_p_Firmicutes_c_Bacilli_Other_Other_Other	0.0633	0.0506	1.25	8.51E-03
k_Bacteria_p_Firmicutes_c_Clostridia_o_Clostridiales_f_Clostridiaceae_g_Clostridium	6.9957	1.1844	5.91	1.13E-03
k_Bacteria_p_Firmicutes_c_Clostridia_o_Clostridiales_f_Clostridiaceae_Other	2.2499	0.7078	3.18	2.74E-02
k_Bacteria_p_Firmicutes_c_Clostridia_o_Clostridiales_f_Lachnospiraceae_g_	1.5292	0.1083	14.12	3.28E-03
k_Bacteria_p_Firmicutes_c_Clostridia_o_Clostridiales_f_Peptococcaceae_g_Desulfitobacterium	0.1266	0.0000	0.1266	1.19E-03
k_Bacteria_p_Firmicutes_c_Clostridia_o_Clostridiales_f_Peptostreptococcaceae_g_Clostridium	0.4886	0.1264	3.87	3.57E-02
k_Bacteria_p_Gemmatimonadetes_c_JL-ETNP-Z39_o__f__g_	0.0281	0.0506	0.56	2.71E-02
k_Bacteria_p_GN02_c_GKS2-174_o__f__g_	0.0176	0.0469	0.37	4.35E-02
k_Bacteria_p_Nitrospirae_c_Nitrospira_o_Nitrospirales_f_Nitrospiraceae_g_JG37-AG-70	0.0000	0.0181	0.00	1.07E-02
k_Bacteria_p_OD1_c__o__f__g_	0.0035	0.0289	0.12	7.77E-03
k_Bacteria_p_Planctomycetes_c_BD7-11_o__f__g_	0.0141	0.0325	0.43	2.83E-02
k_Bacteria_p_Planctomycetes_c_Planctomycetia_o_Pirellulales_f_Pirellulaceae_g_Pirellula	0.1090	0.2311	0.47	4.60E-02
k_Bacteria_p_Proteobacteria_c_Alphaproteobacteria_o_Rhizobiales_f__g_	0.0000	0.0253	0.00	2.73E-02
k_Bacteria_p_Proteobacteria_c_Alphaproteobacteria_o_Rhizobiales_f_Hyphomicrobiaceae_Other	0.1160	0.3828	0.30	2.32E-03
k_Bacteria_p_Proteobacteria_c_Alphaproteobacteria_o_Rhizobiales_Other_Other	0.6152	0.9605	0.64	8.65E-03
k_Bacteria_p_Proteobacteria_c_Alphaproteobacteria_o_Rhodobacterales_f_Rhodobacteraceae_Other	0.0281	0.0758	0.37	1.22E-03
k_Bacteria_p_Proteobacteria_c_Alphaproteobacteria_o_Sphingomonadales_f_Erythrobacteraceae_g_	0.0844	0.0722	1.17	3.19E-03
k_Bacteria_p_Proteobacteria_c_Deltaproteobacteria_o_[Entotheonellales]_f_[Entotheonellaceae]_g_	0.3691	0.7511	0.49	4.60E-02
k_Bacteria_p_Proteobacteria_c_Deltaproteobacteria_o_Desulfuromonadales_f_Pelobacteraceae_g_	0.0000	0.0181	0.00	2.73E-02
k_Bacteria_p_Proteobacteria_c_Deltaproteobacteria_o_Syntrophobacterales_f_Syntrophobacteraceae_g_	0.6679	1.2927	0.52	4.60E-02
k_Bacteria_p_Proteobacteria_c_Gammaproteobacteria_o_Methylococcales_f_Methylococcaceae_g_Methylomonas	0.0000	0.0325	0.00	2.73E-02
k_Bacteria_p_Proteobacteria_Other_Other_Other_Other	0.5343	0.8739	0.61	2.09E-02
k_Bacteria_p_Verrucomicrobia_c_Verrucomicrobiae_o_Verrucomicrobiales_f_Verrucomicrobiaceae_g_Prosthecobacter	0.0000	0.0289	0.00	2.73E-02

Legend	x>=1.2	0.8<=x<1.2	x<0.8
	Taxon absent in Z (not fertilized). In these boxes, the % of the taxon in the sample is shown, not the ratio.		

Table 2.3.8.22

In the table, genera which resulted to be significantly different between soils fertilized with Yeast and unfertilized soils are shown. The average presence percentage of each of these genera in the fertilized soil and in the unfertilized soil, together with their ratio, are reported. Boxes containing the ratios' values are coloured depending on the resulting value (from green, when the percentage of the genus is higher in the treated soil and therefore when fertilization increases the growth of those organisms, to orange, when the percentage of the genus is lower in the treated soil than in the control).

2.3.8.3 Analysis of the correlation among major bacterial groups detected in agricultural soil subject to different fertilization treatments

In order to determine whether the changes in the abundance of some microorganisms were linked to the growth or to the decrease in number of other specific groups of bacteria, Pearson's correlation index was calculated among all the bacterial phyla. In most natural environments, indeed, individual organisms do not live in isolation but rather form complex systems of inter-species interactions. Recent researches demonstrated that these kinds of social behaviours are commonly observed also in bacterial species, and not only in animals, even if microorganisms were traditionally thought to be independent free-swimming organisms (Freilich *et al*., 2010). Moreover, Allen and Banfield (2005) highlighted that species composition and interactions, in a microbial community, change over time and in response to environmental stimuli, and that the presence of a microbial species might depend upon the presence (or absence) of another species. In 2009, Fuhrman stressed the importance of co-occurrence patterns of microorganisms from environmental samples, in order to examine the potential interactions between organisms and aspects of the niches of microorganisms within extremely complex and dynamic natural communities.

Few scientific works researched correlations or similarities between the behaviour of various soil bacterial groups. For example, in the work by Fierer *et al*. (2007), correlations of the bacterial groups with carbon availability were calculated, and indicated an opposite trend between microorganisms belonging to the phylum Acidobacteria and those belonging to the phyla Bacteroidetes and Betaptoteobacteria.

In this study, the Pearson's coefficient was calculated among all the bacterial phyla that were detected in the investigated agricultural soil. The presence percentage of each phylum in each soil sample was taken into account for this statistical analysis, in order to avoid any possible bias caused by the use of average values. In the tables 2.3.8.23 (A, B) and 2.3.8.24 (A, B), the results obtained for the major phyla in the two considered sampling times (day 9 and day 58) are reported.

		Acidobacteria	Actinobacteria	Armatimonadetes	Chloroflexi	Firmicutes	Gemmatimonadetes
Acidobacteria	Pearson Correlation	1.000	0.943**	0.784**	0.929**	-0.821**	0.867**
	p-value		0.0000	0.0000	0.0000	0.0000	0.0000
Actinobacteria	Pearson Correlation	0.943**	1.000	0.775**	0.925**	-0.841**	0.907**
	p-value	0.0000		0.0000	0.0000	0.0000	0.0000
Armatimonadetes	Pearson Correlation	0.784**	0.775**	1.000	0.770**	-0.721**	0.690**
	p-value	0.0000	0.0000		0.0000	0.0000	0.0000
Bacteroidetes	Pearson Correlation	-0.598**	-0.590**	-0.411*	-0.478**	0.373*	-0.546**
	p-value	0.0003	0.0004	0.0193	0.0057	0.0355	0.0012
Chloroflexi	Pearson Correlation	0.929**	0.925**	0.770**	1.000	-0.854**	0.841**
	p-value	0.0000	0.0000	0.0000		0.0000	0.0000
Cyanobacteria	Pearson Correlation	0.305	0.340	0.374*	0.348	-0.612**	0.315
	p-value	0.0902	0.0568	0.0350	0.0512	0.0002	0.0786
Firmicutes	Pearson Correlation	-0.821**	-0.841**	-0.721**	-0.854**	1.000	-0.804**
	p-value	0.0000	0.0000	0.0000	0.0000		0.0000
Gemmatimonadetes	Pearson Correlation	0.867**	0.907**	0.690**	0.841**	-0.804**	1.000
	p-value	0.0000	0.0000	0.0000	0.0000	0.0000	
Nitrospirae	Pearson Correlation	0.834**	0.728**	0.661**	0.761**	-0.769**	0.770**
	p-value	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Planctomycetes	Pearson Correlation	0.918**	0.911**	0.752**	0.912**	-0.826**	0.799**
	p-value	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
TM6	Pearson Correlation	0.747**	0.691**	0.595**	0.730**	-0.546**	0.540**
	p-value	0.0000	0.0000	0.0003	0.0000	0.0012	0.0014
TM7	Pearson Correlation	0.366*	0.511**	0.362*	0.551**	-0.520**	0.464**
	p-value	0.0392	0.0028	0.0420	0.0011	0.0023	0.0075
Thermi	Pearson Correlation	0.401*	0.400*	0.503**	0.411*	-0.356*	0.468**
	p-value	0.0229	0.0233	0.0034	0.0194	0.0458	0.0070
Verrucomicrobia	Pearson Correlation	0.557**	0.571**	0.536**	0.659**	-0.799**	0.590**
	p-value	0.0009	0.0006	0.0016	0.0000	0.0000	0.0004
WS3	Pearson Correlation	0.703**	0.640**	0.507**	0.634**	-0.567**	0.507**
	p-value	0.0000	0.0001	0.0031	0.0001	0.0007	0.0030
Alphaproteobacteria	Pearson Correlation	0.042	0.084	0.010	-0.030	-0.218	0.120
	p-value	0.8186	0.6466	0.9577	0.8700	0.2298	0.5132
Betaproteobacteria	Pearson Correlation	-0.221	-0.185	-0.247	-0.279	0.063	-0.192
	p-value	0.2244	0.3096	0.1727	0.1222	0.7316	0.2923
Gammaproteobacteria	Pearson Correlation	0.070	0.073	0.099	0.094	-0.421*	0.127
	p-value	0.7053	0.6929	0.5900	0.6070	0.0165	0.4868
Deltaproteobacteria	Pearson Correlation	0.123	0.057	-0.038	0.038	-0.056	0.025
	p-value	0.5008	0.7571	0.8354	0.8349	0.7592	0.8923
Epsilonproteobacteria	Pearson Correlation	-0.164	-0.165	-0.183	-0.203	0.168	-0.091
	p-value	0.3707	0.3657	0.3153	0.2645	0.3589	0.6190

Table 2.3.8.23 A

Pearson's correlation coefficient among major phyla, at sampling day 9. The presence percentage of each phylum in each soil sample from day 9 was taken into account for this statistical analysis, in order to avoid any possible bias caused by the use of average values. In the table, significant coefficients with a p-value < 0.01 are coloured in light green, and those with p-value < 0.05 in yellow. At the bottom of each correlation coefficient, the p-value is reported. In this table (A) the results for the phyla Acidobacteria, Actinobacteria, Armatimonadetes, Chloroflexi, Firmicutes, and Gemmatimonadetes are reported.

		Nitrospirae	Planctomycetes	Alphaproteobacteria	Betaproteobacteria	Deltaproteobacteria	Epsilonproteobacteria	Gammaproteobacteria
Acidobacteria	Pearson Correlation	0.834**	0.918**	0.042	-0.221	0.123	-0.164	0.070
	p-value	0.0000	0.0000	0.8186	0.2244	0.5008	0.3707	0.7053
Actinobacteria	Pearson Correlation	0.728**	0.911**	0.084	-0.185	0.057	-0.165	0.073
	p-value	0.0000	0.0000	0.6466	0.3096	0.7571	0.3657	0.6929
Armatimonadetes	Pearson Correlation	0.661**	0.752**	0.010	-0.247	-0.038	-0.183	0.099
	p-value	0.0000	0.0000	0.9577	0.1727	0.8354	0.3153	0.5900
Bacteroidetes	Pearson Correlation	-0.548**	-0.496**	0.047	-0.088	-0.012	-0.008	0.006
	p-value	0.0012	0.0039	0.7982	0.6316	0.9497	0.9638	0.9740
Chloroflexi	Pearson Correlation	0.761**	0.912**	-0.030	-0.279	0.038	-0.203	0.094
	p-value	0.0000	0.0000	0.8700	0.1222	0.8349	0.2645	0.6070
Cyanobacteria	Pearson Correlation	0.441*	0.345	0.275	-0.061	0.017	0.020	0.380*
	p-value	0.0116	0.0531	0.1280	0.7407	0.9271	0.9129	0.0321
Firmicutes	Pearson Correlation	-0.769**	-0.826**	-0.218	0.063	-0.056	0.168	-0.421*
	p-value	0.0000	0.0000	0.2298	0.7316	0.7592	0.3589	0.0165
Gemmatimonadetes	Pearson Correlation	0.770**	0.799**	0.120	-0.192	0.025	-0.091	0.127
	p-value	0.0000	0.0000	0.5132	0.2923	0.8923	0.6190	0.4868
Nitrospirae	Pearson Correlation	1.000	0.762**	0.072	-0.093	0.220	-0.049	0.101
	p-value		0.0000	0.6944	0.6111	0.2269	0.7880	0.5808
Planctomycetes	Pearson Correlation	0.762**	1.000	0.014	-0.265	0.135	-0.277	0.050
	p-value	0.0000		0.9373	0.1429	0.4608	0.1244	0.7846
TM6	Pearson Correlation	0.626**	0.732**	0.039	-0.093	-0.039	-0.059	-0.103
	p-value	0.0001	0.0000	0.8323	0.6109	0.8340	0.7470	0.5758
TM7	Pearson Correlation	0.220	0.468**	0.086	-0.178	-0.028	-0.122	0.149
	p-value	0.2273	0.0070	0.6402	0.3305	0.8774	0.5044	0.4162
Thermi	Pearson Correlation	0.422*	0.437*	-0.064	-0.318	-0.104	0.004	0.053
	p-value	0.0160	0.0124	0.7286	0.0764	0.5719	0.9829	0.7751
Verrucomicrobia	Pearson Correlation	0.596**	0.637**	0.065	-0.156	0.005	-0.123	0.275
	p-value	0.0003	0.0001	0.7219	0.3935	0.9801	0.5035	0.1281
WS3	Pearson Correlation	0.600**	0.622**	0.232	0.030	0.500**	-0.184	0.126
	p-value	0.0003	0.0001	0.2019	0.8691	0.0036	0.3127	0.4910
Alphaproteobacteria	Pearson Correlation	0.072	0.014	1.000	0.423*	0.343	-0.329	0.162
	p-value	0.6944	0.9373		0.0159	0.0547	0.0656	0.3762
Betaproteobacteria	Pearson Correlation	-0.093	-0.265	0.423*	1.000	0.050	0.107	0.568**
	p-value	0.6111	0.1429	0.0159		0.7877	0.5617	0.0007
Gammaproteobacteria	Pearson Correlation	0.101	0.050	0.162	0.568**	0.039	-0.096	1.000
	p-value	0.5808	0.7846	0.3762	0.0007	0.8311	0.5999	0.039
Deltaproteobacteria	Pearson Correlation	0.220	0.135	0.343	0.050	1.000	-0.438*	0.8311
	p-value	0.2269	0.4608	0.0547	0.7877		0.0121	-0.096
Epsilonproteobacteria	Pearson Correlation	-0.049	-0.277	-0.329	0.107	-0.438*	1.000	0.5999
	p-value	0.7880	0.1244	0.0656	0.5617	0.0121		0.279

Table 2.3.8.23 B

Pearson's correlation coefficient among major phyla, at sampling day 9. The presence percentage of each phylum in each soil sample from day 9 was taken into account for this statistical analysis, in order to avoid any possible bias caused by the use of average values. In the table, significant coefficients with a p-value < 0.01 are coloured in light green, and those with p-value < 0.05 in yellow. At the bottom of each correlation coefficient, the p-value is reported. In this table (B) the results for the phyla Nitrospirae, Planctomycetes, Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, and Epsilonproteobacteria are reported.

The phyla that were reported in the tables, apart from being the most abundant, are those, which gave higher values of Pearson's coefficient, with lower p-value, in one or both the sampling times, or which could be most interesting.

Considering the correlation values obtained at the first sampling time, after 9 days from the addition of the different treatments (Table 2.3.8.23 A, B), it is evident that the major bacterial phyla changed in abundance in parallel, or in opposition, to other abundant or scarce bacterial species. In particular, Acidobacteria and Actinobacteria presented a correlation coefficient of about 0.95, with a p-value of less than 0.00001, and also showed similar correlation coefficients with the other considered bacterial groups (Table 2.3.8.23 A). This result could indicate a strict interaction between these two important and abundant groups of soil microorganisms, which seem to have a similar response in the presence of outer stimuli, such as the addition of a fertilizer, and to relate to the other bacterial phyla likewise. Another very interesting observation is the correlations of the phylum Firmicutes with the other phyla, which in the majority of the cases is strong and negative (Table 2.3.8.23 A). In particular, the most marked negative correlations were detected with Acidobacteria, Actinobacteria, Cyanobacteria, Gemmatimonadetes, Planctomycetes, Verrucomicrobia. Firmicutes seem to respond in a peculiar way to the addition of organic fertilizers, and to grow in abundance rapidly after their addition, apparently to the detriment of other important soil bacterial groups. Negative correlations with many other bacterial groups were detected also for Bacteroidetes, even if the correlations were less strong than those found for Firmicutes. Moreover, one of the few positive correlations detected for Firmicutes was detected with this bacterial phylum, the Bacteroidetes, indicating a possible positive interaction between these two groups, or a similar trend in the presence of the same environmental changes. The phylum Nitrospirae is also reported in the table (Table 2.3.8.23 B), not only because it showed various strong correlations with other bacterial groups, but also because it is important as it comprehends many species, which were demonstrated to be involved in the nitrification process. Evidently, the abundance of this bacterial group, in this kind of agricultural soil and in the presence of fertilization treatments, was strictly related to the increase or decrease in number of Acidobacteria, Actinobacteria, Chloroflexi and Planctomycetes, while, as mentioned before, a negative correlation was detected with the abundance of the bacterial phyla Firmicutes and Bacteroidetes.

Armatimonadetes, Chloroflexi, Gemmatimonadetes and Planctomycetes, as partially mentioned before, presented several very significant correlations with many of the other bacterial groups.

		Acidobacteria	Actinobacteria	Armatimonadetes	Chloroflexi	Firmicutes	Gemmatimonadetes
Acidobacteria	Pearson Correlation	1.000	0.592**	0.329	0.248	-0.253	0.662**
	p-value		0.0004	0.0656	0.1719	0.1626	0.0000
Actinobacteria	Pearson Correlation	0.592**	1.000	0.158	0.332	-0.549**	0.437*
	p-value	0.0004		0.3867	0.0631	0.0011	0.0124
Armatimonadetes	Pearson Correlation	0.329	0.158	1.000	0.072	0.014	0.099
	p-value	0.0656	0.3867		0.6970	0.9377	0.5901
Bacteroidetes	Pearson Correlation	-0.646**	-0.320	-0.071	-0.098	-0.260	-0.546**
	p-value	0.0001	0.0738	0.6986	0.5940	0.1513	0.0012
Chloroflexi	Pearson Correlation	0.248	0.332	0.072	1.000	-0.522**	0.098
	p-value	0.1719	0.0631	0.6970		0.0022	0.5930
Cyanobacteria	Pearson Correlation	-0.418*	-0.221	-0.211	-0.006	-0.153	-0.317
	p-value	0.0172	0.2239	0.2460	0.9731	0.4039	0.0769
Firmicutes	Pearson Correlation	-0.253	-0.549**	0.014	-0.522**	1.000	-0.100
	p-value	0.1626	0.0011	0.9377	0.0022		0.5857
Gemmatimonadetes	Pearson Correlation	0.662**	0.437*	0.099	0.098	-0.100	1.000
	p-value	0.0000	0.0124	0.5901	0.5930	0.5857	
Nitrospirae	Pearson Correlation	0.742**	0.290	0.210	0.034	0.110	0.506**
	p-value	0.0000	0.1071	0.2479	0.8516	0.5500	0.0031
Planctomycetes	Pearson Correlation	0.409*	0.535**	0.199	0.565**	-0.469**	0.124
	p-value	0.0200	0.0016	0.2754	0.0007	0.0068	0.4996
TM6	Pearson Correlation	-0.180	-0.136	0.004	0.145	-0.083	-0.191
	p-value	0.3230	0.4566	0.9838	0.4296	0.6499	0.2962
TM7	Pearson Correlation	-0.293	0.044	-0.200	-0.279	-0.154	-0.381*
	p-value	0.1037	0.8098	0.2725	0.1221	0.3992	0.0314
Thermi	Pearson Correlation	-0.055	0.016	0.252	-0.360*	0.469**	0.183
	p-value	0.7661	0.9322	0.1647	0.0432	0.0068	0.3168
Verrucomicrobia	Pearson Correlation	-0.595**	-0.450**	-0.218	-0.243	0.110	-0.443*
	p-value	0.0003	0.0097	0.2310	0.1795	0.5476	0.0110
WS3	Pearson Correlation	0.800**	0.518**	0.393*	0.469**	-0.444*	0.341
	p-value	0.0000	0.0024	0.0262	0.0068	0.0110	0.0562
Alphaproteobacteria	Pearson Correlation	-0.599**	-0.285	0.052	-0.193	0.256	-0.181
	p-value	0.0003	0.1134	0.7791	0.2894	0.1569	0.3219
Betaproteobacteria	Pearson Correlation	0.080	0.153	-0.052	0.216	-0.495**	-0.046
	p-value	0.6639	0.4032	0.7795	0.2358	0.0040	0.8033
Deltaproteobacteria	Pearson Correlation	-0.010	0.157	-0.256	0.292	-0.213	-0.119
	p-value	0.9584	0.3902	0.1565	0.1051	0.2421	0.5165
Gammaproteobacteria	Pearson Correlation	-0.625**	-0.407*	-0.234	-0.206	-0.080	-0.466**
	p-value	0.0001	0.0208	0.1975	0.2576	0.6624	0.0072

Table 2.3.8.24 A

Pearson's correlation coefficient among major phyla, at sampling day 58. The presence percentage of each phylum in each soil sample from day 58 was taken into account for this statistical analysis, in order to avoid any possible bias caused by the use of average values. In the table, significant coefficients with a p-value < 0.01 are coloured in light green, and those with p-value < 0.05 in yellow. At the bottom of each correlation coefficient, the p-value is reported. In this table (A) the results for the phyla Acidobacteria, Actinobacteria, Armatimonadetes, Chloroflexi, Firmicutes, and Gemmatimonadetes are reported.

		Nitrospirae	Planctomycetes	Alphaproteobacteria	Betaproteobacteria	Gammaproteobacteria	Deltaproteobacteria
Acidobacteria	Pearson Correlation	0.742**	0.409*	-0.599**	0.080	-0.625**	-0.010
	p-value	0.0000	0.0200	0.0003	0.6639	0.0001	0.9584
Actinobacteria	Pearson Correlation	0.290	0.535**	-0.285	0.153	-0.407*	0.157
	p-value	0.1071	0.0016	0.1134	0.4032	0.0208	0.3902
Armatimonadetes	Pearson Correlation	0.210	0.199	0.052	-0.052	-0.234	-0.256
	p-value	0.2479	0.2754	0.7791	0.7795	0.1975	0.1565
Bacteroidetes	Pearson Correlation	-0.686**	-0.148	0.425*	0.171	0.711**	-0.108
	p-value	0.0000	0.4190	0.0152	0.3502	0.0000	0.5574
Chloroflexi	Pearson Correlation	0.034	0.565**	-0.193	0.216	-0.206	0.292
	p-value	0.8516	0.0007	0.2894	0.2358	0.2576	0.1051
Cyanobacteria	Pearson Correlation	-0.345	-0.278	-0.026	0.009	0.030	-0.049
	p-value	0.0529	0.1241	0.8888	0.9601	0.8721	0.7883
Firmicutes	Pearson Correlation	0.110	-0.469**	0.256	-0.495**	-0.080	-0.213
	p-value	0.5500	0.0068	0.1569	0.0040	0.6624	0.2421
Gemmatimonadetes	Pearson Correlation	0.506**	0.124	-0.181	-0.046	-0.466**	-0.119
	p-value	0.0031	0.4996	0.3219	0.8033	0.0072	0.5165
Nitrospirae	Pearson Correlation	1.000	0.210	-0.439*	-0.280	-0.580**	-0.065
	p-value		0.2492	0.0118	0.1213	0.0005	0.7257
Planctomycetes	Pearson Correlation	0.210	1.000	-0.400*	0.382*	0.005	0.204
	p-value	0.2492		0.0232	0.0308	0.9787	0.2620
TM6	Pearson Correlation	-0.255	-0.033	-0.172	-0.050	0.237	0.061
	p-value	0.1585	0.8556	0.3461	0.7878	0.1908	0.7401
TM7	Pearson Correlation	-0.360*	-0.165	0.110	0.109	0.290	-0.141
	p-value	0.0428	0.3682	0.5506	0.5526	0.1077	0.4418
Thermi	Pearson Correlation	-0.002	-0.394*	0.337	-0.479**	-0.233	-0.576**
	p-value	0.9912	0.0256	0.0592	0.0055	0.1989	0.0006
Verrucomicrobia	Pearson Correlation	-0.549**	-0.322	0.233	-0.003	0.436*	-0.036
	p-value	0.0011	0.0721	0.2000	0.9889	0.0126	0.8459
WS3	Pearson Correlation	0.493**	0.524**	-0.585**	0.216	-0.470**	0.187
	p-value	0.0041	0.0021	0.0004	0.2355	0.0066	0.3053
Alphaproteobacteria	Pearson Correlation	-0.439*	-0.400*	1.000	-0.318	0.531**	-0.197
	p-value	0.0118	0.0232		0.0759	0.0018	0.2789
Betaproteobacteria	Pearson Correlation	-0.280	0.382*	-0.318	1.000	-0.037	0.360*
	p-value	0.1213	0.0308	0.0759		0.8405	0.0429
Deltaproteobacteria	Pearson Correlation	-0.065	0.204	-0.197	0.360*	-0.036	1.000
	p-value	0.7257	0.2620	0.2789	0.0429	0.8451	
Gammaproteobacteria	Pearson Correlation	-0.580**	0.005	0.531**	-0.037	1.000	-0.036
	p-value	0.0005	0.9787	0.0018	0.8405		0.8451

Table 2.3.8.24 B

Pearson's correlation coefficient among major phyla, at sampling day 58. The presence percentage of each phylum in each soil sample from day 58 was taken into account for this statistical analysis, in order to avoid any possible bias caused by the use of average values. In the table, significant coefficients with a p-value < 0.01 are coloured in light green, and those with p-value < 0.05 in yellow. At the bottom of each correlation coefficient, the p-value is reported. In this table (B) the results for the phyla Nitrospirae, Planctomycetes, Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, and Epsilonproteobacteria are reported.

For the five classes of Proteobacteria (Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, Epsilonproteobacteria), which also represent a wide group of microorganisms, only relatively weak correlations were detected, and especially among each other. For example, a positive and significant correlation (p-value<0.01) was reported between Betaproteobacteria and Gammaproteobacteria.

A lower number of significant correlations were observed in soils sampled after 58 days from the addition of the fertilizer. An explanation to this result could be that major responses of the soil microbial community happened within few days from the addition of the treatment, and therefore, that the abundances of bacterial groups changed rapidly, though maintaining their relative proportions and following complex webs of interactions. For these reasons the correlations between different bacterial phyla could be more visible and strong in this first period. After about two months, the investigated soil bacterial communities seemed to have reached a certain equilibrium, and the rates of changes in the abundance of bacterial groups were lower. In this sense, the correlations found at this sampling time could have a different meaning of those detected at day 9, as the first group gives an indication of how bacterial groups behave, in a complex bacterial community, in the presence of an external important stimulus, while the second could show the final balances in the structure of the community. Moreover, the significance of the correlation coefficients detected at day 58 was in average lower.

For Acidobacteria (Table 2.3.8.24 A), significant (p-value<0.01) positive correlations were detected at day 58 with Actinobacteria, Gemmatimonadetes, Nitrospirae, and the candidate phylum WS3, and negative correlations with Firmicutes, Bacteroidetes, as found at day 9. However, some new negative correlations were established after a longer period from the addition of the fertilizers, such as the correlation of this group with Alphaproteobacteria and Gammaproteobacteria. Moreover, some interactions, which were detected as positive at day 9, changed in direction and became negative after two months, such as the correlation with Cyanobacteria and Verrucomicrobia.

Less significant correlations were detected for the phylum Actinobacteria, and for the group Armatimonadetes.

Chloroflexi maintained their correlations with Planctomycetes, WS3, and Firmicutes, while their correlation with the phylum Termi changed from being positive to being negative.

Also the significant correlations observed at day 58 for the phylum Firmicutes, though being again mainly negative, were less in number.

Nitrospirae showed and interesting, strong positive correlation with Acidobacteria, which was confirmed at both the sampling times. Their negative correlation with

Bacteroidetes was also strong, while with Firmicutes this negative correlation was not significant any more, after two months from the addition of the fertilizer.
Also for Planctomycetes, the correlation with the phylum Thermi changed in direction. Moreover, new correlations were established with Alphaproteobacteria (negative) and Betaproteobacteria (positive).
The classes of Proteobacteria (Epsilonproteobacteria is not shown), presented correlations at day 58, that were not detected at the first sampling time. This result could indicate that these classes of microorganisms need more time to respond to external inputs, and that they play an important role in the equilibrium of the structure of the final community.
The tables reporting the Pearson's correlation coefficients calculated considering together the two sampling times are reported in Table 2.3.8.25 A, B.

The results reported in this paragraph show that, in this kind of agricultural soil, specific balances between different groups of microorganisms determine the structure of soil community, and that the relative proportions of specific bacterial groups, in the presence of an important environmental change or in a more established situation, are maintained. Moreover, from these results positive or negative interaction among bacterial groups could be highlighted.

		Acidobacteria	Actinobacteria	Armatimonadetes	Chloroflexi	Firmicutes	Gemmatimonadetes	Nitrospirae
Acidobacteria	Pearson Correlation	1.000	0.857**	0.709**	0.812**	-0.770**	0.869**	0.834**
	p-value		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Actinobacteria	Pearson Correlation	0.857**	1.000	0.624**	0.766**	-0.834**	0.789**	0.645**
	p-value	0.0000		0.0000	0.0000	0.0000	0.0000	0.0000
Armatimonadetes	Pearson Correlation	0.709**	0.624**	1.000	0.670**	-0.617**	0.645**	0.568**
	p-value	0.0000	0.0000		0.0000	0.0000	0.0000	0.0000
Bacteroidetes	Pearson Correlation	-0.725**	-0.640**	-0.503**	-0.635**	0.503**	-0.711**	-0.655**
	p-value	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Chloroflexi	Pearson Correlation	0.812**	0.766**	0.670**	1.000	-0.813**	0.818**	0.611**
	p-value	0.0000	0.0000	0.0000		0.0000	0.0000	0.0000
Cyanobacteria	Pearson Correlation	0.113	0.190	0.170	0.332**	-0.426**	0.214	0.101
	p-value	0.3751	0.1330	0.1796	0.0073	0.0004	0.0901	0.4256
Firmicutes	Pearson Correlation	-0.770**	-0.834**	-0.617**	-0.813**	1.000	-0.758**	-0.628**
	p-value	0.0000	0.0000	0.0000	0.0000		0.0000	0.0000
Gemmatimonadetes	Pearson Correlation	0.869**	0.789**	0.645**	0.818**	-0.758**	1.000	0.728**
	p-value	0.0000	0.0000	0.0000	0.0000	0.0000		0.0000
Nitrospirae	Pearson Correlation	0.834**	0.645**	0.568**	0.611**	-0.628**	0.728**	1.000
	p-value	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	
Planctomycetes	Pearson Correlation	0.822**	0.856**	0.643**	0.830**	-0.811**	0.716**	0.649**
	p-value	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
TM6	Pearson Correlation	0.277*	0.329**	0.263*	0.364**	-0.335**	0.208	0.162
	p-value	0.0265	0.0080	0.0356	0.0031	0.0068	0.0989	0.2023
TM7	Pearson Correlation	0.177	0.333**	0.168	0.293*	-0.387**	0.226	0.024
	p-value	0.1627	0.0072	0.1839	0.0190	0.0016	0.0727	0.8525
Thermi	Pearson Correlation	0.337**	0.330**	0.476**	0.303*	-0.261*	0.468**	0.310*
	p-value	0.0065	0.0077	0.0001	0.0149	0.0373	0.0001	0.0126
Verrucomicrobia	Pearson Correlation	0.363**	0.429**	0.388**	0.538**	-0.688**	0.464**	0.300*
	p-value	0.0032	0.0004	0.0015	0.0000	0.0000	0.0001	0.0161
WS3	Pearson Correlation	0.729**	0.570**	0.510**	0.548**	-0.494**	0.479**	0.584**
	p-value	0.0000	0.0000	0.0000	0.0000	0.0000	0.0001	0.0000
Alphaproteobacteria	Pearson Correlation	0.122	0.173	0.290*	0.314*	-0.323**	0.346**	0.079
	p-value	0.3384	0.1726	0.0199	0.0115	0.0092	0.0051	0.5372
Betaproteobacteria	Pearson Correlation	-0.270*	-0.227	-0.279*	-0.308*	0.143	-0.290*	-0.220
	p-value	0.0311	0.0710	0.0254	0.0133	0.2591	0.0200	0.0810
Deltaproteobacteria	Pearson Correlation	0.269*	0.205	0.112	0.326**	-0.238	0.243	0.260*
	p-value	0.0314	0.1037	0.3801	0.0086	0.0578	0.0528	0.0380
Gammaproteobacteria	Pearson Correlation	-0.071	-0.002	-0.001	0.021	-0.330**	0.001	-0.070
	p-value	0.5780	0.9874	0.9947	0.8716	0.0077	0.9956	0.5837
Epsilonproteobacteria	Pearson Correlation	-0.294*	-0.264*	-0.281*	-0.355**	0.300*	-0.287*	-0.176
	p-value	0.0185	0.0354	0.0244	0.0040	0.0159	0.0214	0.1645

Table 2.3.8.25 A

Pearson's correlation coefficient among major phyla, considering both the sampling times (day 9 and day 58). The presence percentage of each phylum in each soil sample was taken into account for this statistical analysis, in order to avoid any possible bias caused by the use of average values. In the table, significant coefficients with a p-value < 0.01 are coloured in light green, and those with p-value < 0.05 in yellow. At the bottom of each correlation coefficient, the p-value is reported. In this table (A) the results for the phyla Acidobacteria, Actinobacteria, Armatimonadetes, Chloroflexi, Firmicutes, Gemmatimonadetes, and Nitrospirae are reported.

		Planctomycetes	Alphaproteobacteria	Betaproteobacteria	Deltaproteobacteria	Gammaproteobacteria	Epsilonproteobacteria
Acidobacteria	Pearson Correlation	0.822**	0.122	-0.270*	0.269*	-0.071	-0.294*
	p-value	0.0000	0.3384	0.0311	0.0314	0.5780	0.0185
Actinobacteria	Pearson Correlation	0.856**	0.173	-0.227	0.205	-0.002	-0.264*
	p-value	0.0000	0.1726	0.0710	0.1037	0.9874	0.0354
Armatimonadetes	Pearson Correlation	0.643**	0.290*	-0.279*	0.112	-0.001	-0.281*
	p-value	0.0000	0.0199	0.0254	0.3801	0.9947	0.0244
Bacteroidetes	Pearson Correlation	-0.579**	-0.167	0.098	-0.218	0.088	0.181
	p-value	0.0000	0.1859	0.4388	0.0838	0.4898	0.1531
Chloroflexi	Pearson Correlation	0.830**	0.314*	-0.308*	0.326**	0.021	-0.355**
	p-value	0.0000	0.0115	0.0133	0.0086	0.8716	0.0040
Cyanobacteria	Pearson Correlation	0.174	0.224	-0.107	0.105	0.169	-0.101
	p-value	0.1697	0.0754	0.3998	0.4101	0.1831	0.4291
Firmicutes	Pearson Correlation	-0.811**	-0.323**	0.143	-0.238	-0.330**	0.300*
	p-value	0.0000	0.0092	0.2591	0.0578	0.0077	0.0159
Gemmatimonadetes	Pearson Correlation	0.716**	0.346**	-0.290*	0.243	0.001	-0.287*
	p-value	0.0000	0.0051	0.0200	0.0528	0.9956	0.0214
Nitrospirae	Pearson Correlation	0.649**	0.079	-0.220	0.260*	-0.070	-0.176
	p-value	0.0000	0.5372	0.0810	0.0380	0.5837	0.1645
Planctomycetes	Pearson Correlation	1.000	0.133	-0.256*	0.292*	0.037	-0.358**
	p-value		0.2949	0.0414	0.0190	0.7700	0.0037
TM6	Pearson Correlation	0.358**	-0.007	-0.095	0.050	0.022	-0.076
	p-value	0.0037	0.9583	0.4568	0.6945	0.8635	0.5491
TM7	Pearson Correlation	0.252*	0.238	-0.128	0.056	0.153	-0.159
	p-value	0.0447	0.0586	0.3135	0.6604	0.2261	0.2089
Thermi	Pearson Correlation	0.220	0.322**	-0.359**	-0.090	-0.042	-0.125
	p-value	0.0810	0.0094	0.0036	0.4809	0.7439	0.3246
Verrucomicrobia	Pearson Correlation	0.482**	0.313*	-0.215	0.145	0.275*	-0.227
	p-value	0.0001	0.0117	0.0876	0.2517	0.0281	0.0710
WS3	Pearson Correlation	0.586**	-0.053	-0.010	0.397**	-0.072	-0.195
	p-value	0.0000	0.6796	0.9390	0.0011	0.5707	0.1225
Alphaproteobacteria	Pearson Correlation	0.133	1.000	0.062	0.302*	0.218	-0.356**
	p-value	0.2949		0.6289	0.0151	0.0834	0.0039
Betaproteobacteria	Pearson Correlation	-0.256*	0.062	1.000	-0.007	0.478**	0.177
	p-value	0.0414	0.6289		0.9554	0.0001	0.1613
Deltaproteobacteria	Pearson Correlation	0.292*	0.302*	-0.007	1.000	0.026	-0.466**
	p-value	0.0190	0.0151	0.9554		0.8366	0.0001
Gammaproteobacteria	Pearson Correlation	0.037	0.218	0.478**	0.026	1.000	-0.085
	p-value	0.7700	0.0834	0.0001	0.8366		0.5036
Epsilonproteobacteria	Pearson Correlation	-0.358**	-0.356**	0.177	-0.466**	-0.085	1.000
	p-value	0.0037	0.0039	0.1613	0.0001	0.5036	

Table 2.3.8.25 B

Pearson's correlation coefficient among major phyla, considering both the sampling times (day 9 and day 58). The presence percentage of each phylum in each soil sample was taken into account for this statistical analysis, in order to avoid any possible bias caused by the use of average values. In the table, significant coefficients with a p-value < 0.01 are coloured in light green, and those with p-value < 0.05 in yellow. At the bottom of each correlation coefficient, the p-value is reported. In this table (B) the results for the phyla Planctomycetes, Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, and Epsilonproteobacteria are reported.

2.3.8.4 Relative proportions of the strongly correlated phyla

Considering the results reported at paragraph 2.3.8.3, the phyla, which showed strong correlation (Pearson's correlation coefficient > 0.7) at sampling day 9, were taken into account for further analysis. In particular, the calculations of the ratios between the presence percentage of a determined phylum in each single soil sampled at day 9 and the presence percentage of the correlated phylum in the same soil sample were done, followed by the calculation, considering all the soils sampled in the same day, of the mean value, the standard deviation and the coefficient of variation. This kind of analysis was done in order to determine the mean relative proportions of the correlated phyla in soil bacterial communities, and to understand whether these proportions were constant in all the soil samples, even if treated differently (through the observation of the standard deviation and of the coefficient of variation). The results obtained considering positively correlated phyla are reported in the figure 2.3.8.4 and 2.3.8.5, while the results obtained considering the phyla, which were negatively correlated with the phylum Firmicutes, are reported in figure 2.3.8.6 to have a graphical perspective of this kind of correlation, as well.

Considering the ratios between the abundance of the phylum Acidobacteria and of phyla resulting to be strongly correlated to it (Figure 2.3.8.4), it can be noticed that no major differences were present in the abundance of this important phylum and Actinobacteria. Indeed, the ratio between these two phyla gave a value of about 1.3, with a low standard deviation and a low coefficient of variation (of 0.23, reported in the figure over the correspondent column).

The coefficient of variation was low, with values lower than 0.3, also in the cases of the ratios between Acidobacteria and Chloroflexi, Nitrospirae and Planctomycetes, indicating that the relative proportions of these phyla were constant in all the soil pots, while it had values of 0.3 in the cases of the ratios with Armatimonadetes and Gemmatimonadetes, indicating a slightly more variable situation. In the case of Firmicutes, the coefficient of variation was very high, a result which is obvious as this phylum has a negative correlation with Acidobacteria.

Similar results were obtained for the phylum Actinobacteria (Figure 2.3.8.4). The ratios calculated for these two phyla were reported in the same graph, as they gave similar values.

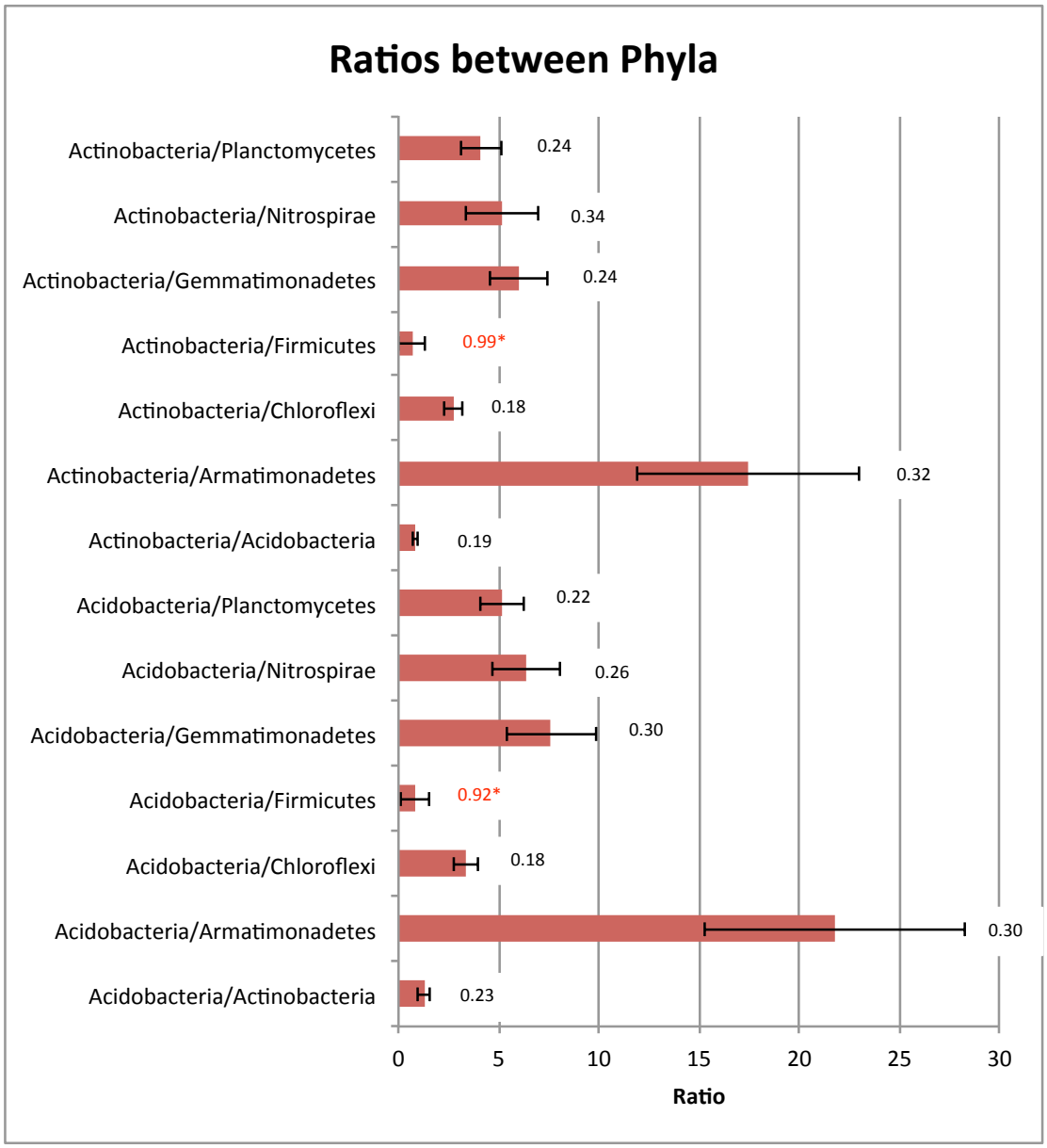

Figure 2.3.8.4

Mean values of the ratios between the presence percentage of a determined phylum in each single soil sampled at day 9 and the presence percentage of the correlated phylum in the same soil sample. The error bars represent the standard deviation of the mean. In this graph, the ratios between the phylum Acidobacteria and its correlated phyla (Pearson's correlation > 0.7) and the ratios between the phylum Actinobacteria and its correlated phyla (Pearson's correlation > 0.7) are shown. Over each column, the value of the coefficient of variation (CV) for each ratio is reported. *The CV corresponding to negative correlations is highlighted in red.

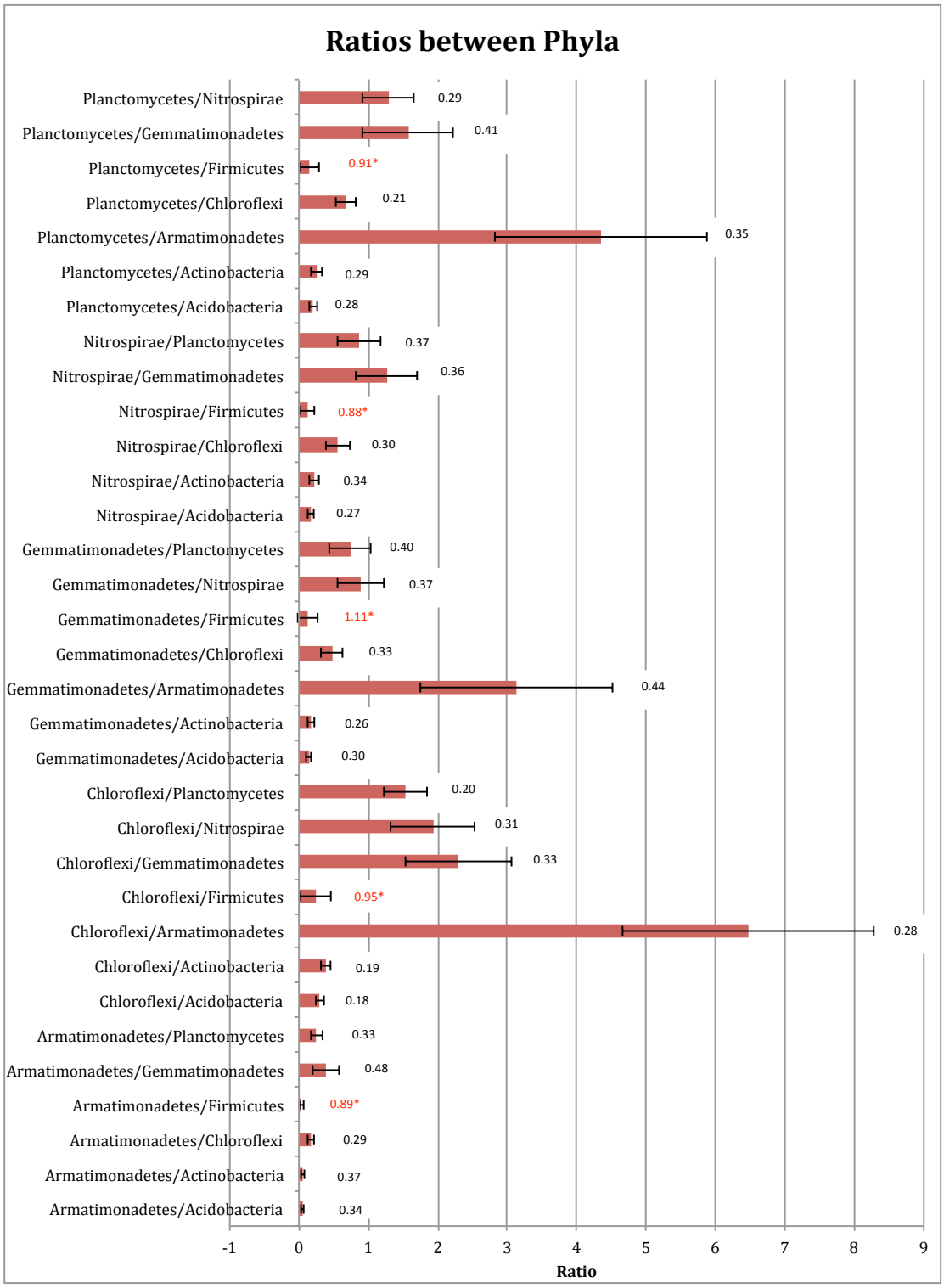

Figure 2.3.8.5

Mean values of the ratios between the presence percentage of a determined phylum in each single soil sampled at day 9 and the presence percentage of the correlated phylum in the same soil sample. The error bars represent the standard deviation of the mean. Over each column, the value of the coefficient of variation for each ratio is reported. *The CV corresponding to negative correlations is highlighted in red.

Considering the proportions of the phylum Armatimonadetes with its correlated phyla (Figure 2.3.8.5), it is evident that the variability in the different soil samples is slightly more pronounced than with the more abundant phyla, such as Acidobacteria and Actinobacteria. In fact, the coefficients of variation present values higher of 0.3 in the majority of the cases. This result is probably due to the fact that this phylum represents a less abundant group of bacteria.

The phylum Chloroflexi (Figure 2.3.8.5), on the other hand, seems to be present in soil samples in a quite constant proportion, in respect to the other phyla with which, considering the correlation results, it has strict interaction. Only with Gemmatimonadetes and with Nitrospirae, this relationship appears to be slightly less stable.

Gemmatimonadetes and Nitrospirae (Figure 2.3.8.5), being less abundant phyla, also present coefficients of variation with values higher than 0.3 in most of the cases, with the exception of the ratio Gemmatimonadetes/Actinobacteria and Nitrospirae/Acidobacteria. In these two cases, the relationship between the bacterial groups is more constant.

The phylum Planctomycetes (Figure 2.3.8.5) presents a stable proportion with the phyla Acidobacteria, Actinobacteria, Chloroflexi and Nitrospirae, while it shows slightly more variation in the ratios calculated with the other phyla.

Figure 2.3.8.6 shows a completely different situation from the graphs reported in the figures 2.3.8.4 and 2.3.8.5. Here, the coefficient of variation is very high in all the cases, as the Pearson's correlation coefficient was always a negative value.

This kind of analysis, in particular considering the strongly positively correlated phyla, is a second validation of the results obtained with the correlation coefficients. Indeed, these observations strengthen the assumption that, in these soils, bacterial groups respond to external stimuli following some rules that are determined by the interactions between each other, and that relative proportions among some specific groups (positively correlated) are maintained, while the decreasing or increasing in abundance of determined groups could be caused by the presence of an antagonistic phylum (negatively correlated).

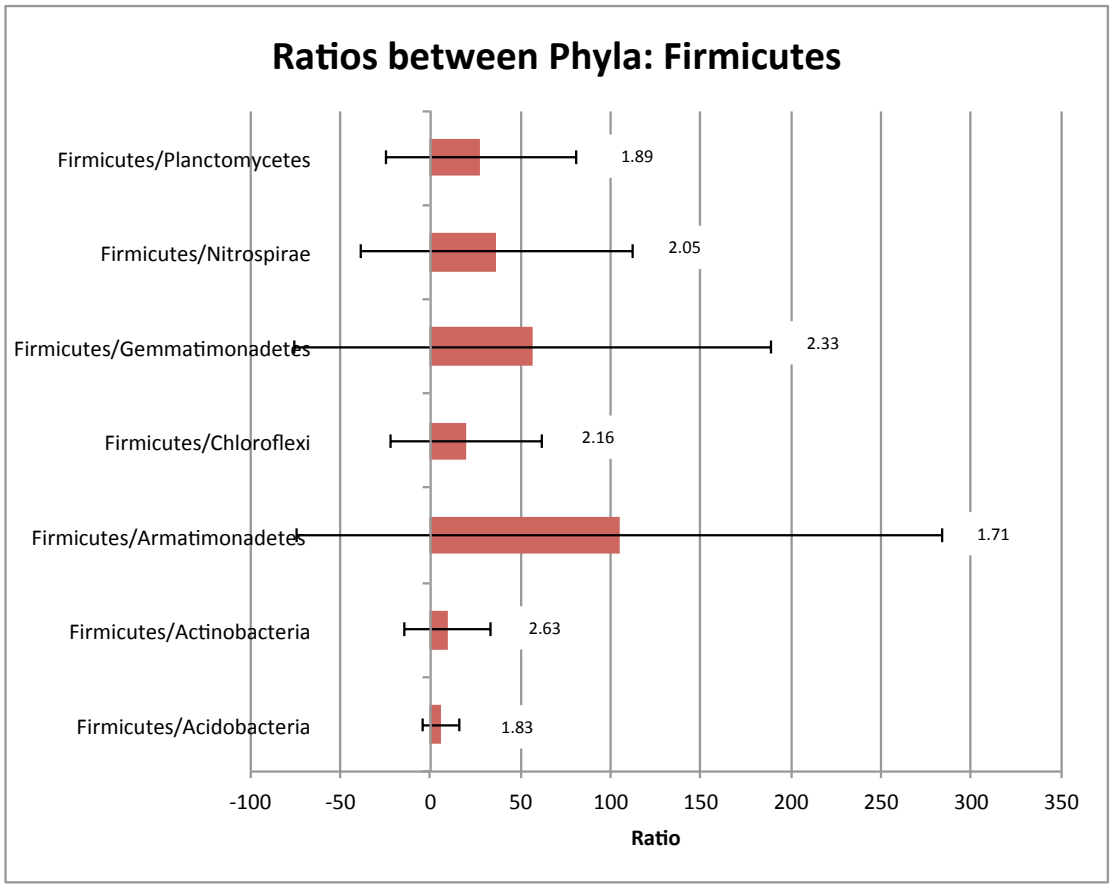

Figure 2.3.8.6

Mean values of the ratios between the presence percentage of the phylum Firmicutes in each single soil sampled at day 9 and the presence percentage of a (negatively) correlated phylum in the same soil sample. The error bars represent the standard deviation of the mean. Over each column, the value of the coefficient of variation for each ratio is reported.

2.4 Conclusions

Soil is one of the most heterogeneous systems on earth, and in this environment thousands of species of microorganisms find a favourable habitat for their lives and metabolisms. Bacteria, archaea and fungi adapted to live in soil are often part of complex mechanisms regulating both plant growth and health and atmospheric and groundwater conditions, such as organic matter decomposition, biogeochemical cycles, plant growth promotion, etc. The structure of soil microbial communities, and in particular the presence of specific microorganisms taking part in fundamental processes, such as nitrogen fixation, nitrification, denitrification, deserves attention and importance from the scientific community. Indeed, a better understanding of the mechanisms regulating soil life and functionality is fundamental to improve soil management, and in particular nitrogen management in agriculture, and to reduce the threats to environment, and in turn human, health.
In the last years, classic microbiological techniques have been substituted, in the study of soil microorganisms, by culture-independent methods, which involve direct DNA extraction from soil and subsequent analyses through molecular biology techniques. This change was necessary, as it was demonstrated that only about 0.1-1% of soil microorganisms are culturable in laboratory.

The first part of this thesis work aimed at comparing and optimizing DNA extraction techniques and at determining how this first step of the analysis influences subsequent processing, and how different kinds of soils could respond differently to the same extraction method. It was observed, in effect, that the purification from humic acids of DNA samples using the commercial DNA extraction kit by MoBio is dependent from the soil type, and for this reason PCR amplification does not present the same efficiency in all the cases.
A new automatic DNA extraction method using the workstation BioSprint 96 (Qiagen) was tested, and permitted the purification of relevant quantities of soil DNA, even if the fragmentation of nucleic acids was significant. This kind of automatic DNA extraction method could be an important improvement for soil analysis, because it would permit the simultaneous extraction of DNA from 96 samples with limited manipulation and working time. However, this method has still to be optimized in order to obtain samples with a better quality.

Real Time PCR technique is widely used in the study of nitrogen cycle, in particular for the detection of functional genes involved in this process in soil samples. In this work,

SybrGreen-based quantitative Real Time PCR was optimized in a reaction volume of 10 µl using the QuantStudio 12K Flex Real Time PCR System, in 384 wells plates. The optimization of this technique permitted to perform Real Time PCR experiments of 384 reactions, and to significantly decrease time and costs for this kind of analysis.

Thanks to quantitative Real Time PCR experiments targeting functional genes of the nitrogen cycle, and in specific bacterial and archaeal *amoA* genes (ammonia monooxygenase A) for nitrification, *nirK*, *nirS* (nitrite reductase) and *nosZ* (nitrous oxide reductase) genes for denitrification and *nifH* gene (nitrogenase) for nitrogen fixation, it was possible to answer to some important questions.
A first analysis of the nitrogen cycle in soils coming from neighbouring areas but subject to different environmental conditions permitted to confirm the hypothesis that remarkable differences are detectable in the abundance of microorganisms involved in the N-cycle in soils from different environments. Moreover, it was observed that in natural environments the balance between nitrification and denitrification processes is near the equilibrium, while in soils that are in some way manipulated by humans, one of the processes dominates on the other, likely causing pollution.

In the majority of the soils analysed in this work, archaeal *amoA* gene was detected at significantly higher levels in respect to all the other N-cycle genes, with a gene copy number exceeding the others of an order of magnitude in most of the cases. Moreover, it was observed that the level of these microorganisms did not change significantly in response to different treatments, and that they were present at high levels also in low-nutrient soils. These observations indicate a possible adoption of the K-selection strategy by archaeal ammonia-oxidizers. K-strategy organisms, indeed, are usually very abundant in their habitats, being, by definition, close to the carrying capacity K of that given environment, and tend to grow slowly but continuously, with no rapid responses to external stimuli.
A different behaviour was instead detected for bacterial nitrifiers and denitrifiers, which seem to have adopted a R-selection strategy, with lower numbers and rapid responses to stimuli such as soil fertilization, and with the capability of increasing exponentially their reproduction in the presence of favourable conditions.

The capacity of N-cycle bacteria to respond to environmental changes and to external inputs makes these organisms good indicators of the functionality and equilibrium of this biogeochemical cycle in soils. Both nitrifying and denitrifying bacteria showed to be influenced by fertilization practices, and in particular by the presence of organic matter. Major effects of fertilization were detected after a long period from the addition of the fertilizer. However, rapid responses were also observed.

Through the analysis of different soils subject to different conditions (environmental conditions, water table level, fertilization), two genes were observed to respond significantly to changes: bacterial *amoA* gene for nitrification and *nosZ* gene for denitrification. Moreover, these two genes showed a significant positive correlation with plant growth in the fertilization trial. In specific, bacterial *amoA* gene resulted to be positively correlated with the plant growth parameters after about 10 days from fertilization. The denitrification gene *nosZ*, on the other hand, presented significant positive correlation after 58 days from fertilization.
Bacterial *amoA* gene, therefore, could be used as a precocious indicator of soil quality, while *nosZ* gene could be a tardive one, giving evidences for a more settled situation. For these reasons, bacterial *amoA* and *nosZ* genes are good indicators of the functionality of nitrification and denitrification processes, and of their equilibrium in soils, and could be useful proxies to predict crop productivity.

Results obtained in different soil samples pointed out a certain similarity in the behaviour of nitrifying and denitrifying microorganisms in the soil environment. Indeed, significant positive correlations were detected between bacterial nitrifiers and denitrifiers in all the soil samples analysed in this work. This result is important as it could indicate a strong topological and biochemical connection between these two groups of microorganisms, which could be explained by the fact that denitrifiers, when using complex organic matter, may release ammonia that could be then used by nitrifiers and, on the other hand, the products of nitrification, nitrates and nitrites, are the substrates used by denitrifying organisms. Nitrifying and denitrifying bacteria, therefore, could be part of the same environmental niche, and appear to live in the same microenvironments in soils. Nitrifying archea on the contrary show to be substantially independent from the other groups and are likely inhabiting different niches, presumably less nutrient-endowed such as the bulk mineral portion of soils.

An analysis through T-RFLP experiments and next generation sequencing techniques (454 sequencing, Roche) of the structure of bacterial communities in soils subject to different fertilization practices was performed. This kind of investigation permitted the observation of strong changes at the community level in response to the addition of the fertilizers, and the variation of the community structure over time. In specific, treatments caused a rapid change in the first days, followed by a gradual stabilization of an equilibrium and the raising of evenness. This rapid change short after the addition of the treatments, apart from being due to the responses of bacteria already present in the substrate soil, could be also caused by a significant arrival of new microorganisms present in the fertilizers. Indeed, results from T-RFLP experiments indicated that an increase in soil microorganisms was present after one day from the addition of the

fertilizers, in all the cases, and this fact was further demonstrated by the possibility to extract DNA and to amplify 16S rRNA gene from 5 out of 7 of the considered fertilizers.

The variations in the abundance of the detected phyla over time were investigated by comparing the presence percentage of each phylum after 9 and 58 days from the addition of the fertilizer. It was observed that some major groups of bacteria, Firmicutes and Bacteroidetes, were present at high levels at day 9 and were subject to a decrease in abundance over time. This diminishment of some abundant groups permitted the raising in number of some minor groups of bacteria, which were then present at a more relevant percentage at the end of the trial, after two months. Proteobacteria, which was the most abundant phylum in all soil samples, on the other hand, did not change in number over time in almost all the cases, and its abundance was also not influenced significantly by different fertilization practices. Acidobacteria and Actinobacteria, which are also two very important groups of microorganisms found in soils, showed a similar trend in the presence of the fertilizers and tended to change similarly over time.

Major differences were detected in the structure of soil microbial communities in the presence of fertilizers of different kinds and origin. Particularly evident was the effect of the fertilizer Yeast on soil community structure. This treatment caused a rapid and strong increase in the abundance of Firmicutes and Bacteroidetes, which in turn negatively influenced the impact of less abundant phyla. Another treatment having a peculiar effect on bacteria was OATr, which instead was the only one causing a decrease in the number of Firmicutes and an increase in abundance of the low-number phyla. The two composite fertilizers UTY and UTN often caused similar changes at phylum level, which were often different from the effects caused by single component organic treatments.

Interesting observations could be done evaluating the correlations among the bacterial phyla detected in the fertilized soil samples. It was indeed evident that the major bacterial phyla changed in abundance in parallel, or in opposition, to other, either abundant or less, bacterial species. Acidobacteria and Actinobacteria, for example, presented a strong positive correlation, with a very low p-value, indicating a possible relationship between these two main soil bacterial groups, or in any case that these bacteria respond likely to the same stimuli.
The bacterial group Firmicutes, instead, presented strong and negative correlations with the majority of the other abundant phyla. Specifically, the most marked negative correlations were detected with Acidobacteria, Actinobacteria, Cyanobacteria, Gemmatimonadetes, Planctomycetes, Verrucomicrobia. Firmicutes, differently from other bacterial groups such as for example Proteobacteria, Acidobacteria and

Actinobacteria, seem to respond with a rapid increase to the addition of organic treatments, and to cause in this way the decrease in abundance of other low-number or abundant bacterial groups.
The structure of soil bacterial communities was investigated also at genus level. About 900 genera were detected in the agricultural soil used for this fertilization trial.

Concluding, in this part of this thesis work molecular biology techniques were used to identify the structure and the function of soil microbial communities, and in particular to detect the responses to external stimuli of major groups of microorganisms, important for both their function, such as N-cycle microorganisms, or for their quantity. This kind of analysis is important as the role of soil microorganisms in crop production and in atmosphere and groundwater pollution has been widely recognized, and raises the necessity of new methods for monitoring the changes in the structure and in the functionality of soil microbial communities to improve soil management.

3 Intercellular communication among bacteria: Quorum Sensing

3.1 Introduction

3.1.1 The discovery of Quorum Sensing

Historically, it was thought that bacteria were solitary organisms, and that each bacterial cell was growing independently from the surrounding population.
In 1970 Nealson and his collegues discovered that bacteria can sense and respond to the rest of the population. Nowadays, this phenomenon is called Quorum Sensing (QS) and is defined as the cell density-dependent regulation of gene expression (Gonzàlez and Marketon, 2003).
This surprising biological phenomenon came to light for the first time with the observation that certain marine bacteria, such as *Vibrio fischeri* and *V. harveyi*, could produce luminescence. These bacteria, though being non-luminescent at low cell density, for example when free-living in sea water, generate a blue-green light when grown to high cell densities, like in laboratory conditions.
Interestingly, *V. fischeri* commonly forms symbiotic relationships with different fish (such as the Japanese pinecone fish *Monocentris japonica*) and squid species such as *Euprymna scolopes* (Visick and McFall-Ngai, 2000), and lives in a specialized cavity, called the light organ, in these marine animals. In dark environments, squids as *E. scolopes*, can appear bioluminescent thanks to the presence of a *V. fischeri* population at high density (10^{10}-10^{11} cells ml^{-1}) in its light organ.
This bioluminescent phenotype is exploited by the squid in order to perform a behavioural phenomenon called counter-illumination. At night, the squid camouflages itself from predators transiting below by controlling the intensity of light that it projects downwards, thus eliminating a visible shadow created by moonlight. This is a case of perfect symbiosis, as in return *E. scolopes* provides the *V. fischeri* population with nutrients.
Further studies on *V. fischeri* revealed that the bacterium grows very fast, directly entering the exponential phase, but the luminescence increases only at about mid-log phase of its growth (Hastings and Greenberg, 1999).
The increase in luminescence is due to the transcriptional activation of the enzyme luciferase, which responds to a threshold density of cells.
This whole circuit is based on the bacterial assessment of its population density by means of release of chemical signalling molecules, also called autoinducers. Thus, the autoinducer is responsible for the establishment of the communication between the

cells, and could cause the change of the expression profile of single bacteria; in this case, the luciferase gene (*lux*) is activated.
For a long time, bioluminescence expressed by *V. fischeri* remained a model system to study density dependent expression of a gene function.

The bioluminescence gene cluster of *V. fischeri* consists of eight *lux* genes (*luxA–E*, *luxG*, *luxI* and *luxR*), which are arranged in two bi-directionally transcribed operons.
One of the two units contains *luxR*, and the other, which is activated by the LuxR protein along with the autoinducer, contains the *luxICDABEG* operon (Engerbrecht and Silverman, 1987).

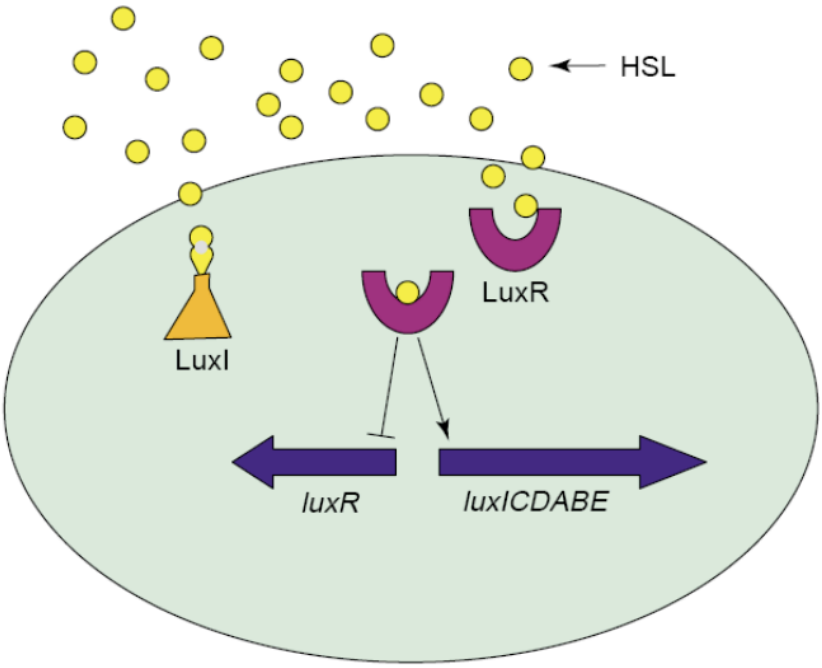

Figure 3.1.1
Quorum sensing in *V. fischeri*. The *luxI* gene encodes for an autoinducer synthase (LuxI), which produces the autoinducer N-(3-oxohexanoyl)- homoserine lactone (HSL). HSL exits the cell and re-enters freely against a gradient when the external concentration reaches a threshold value. Upon re-entry into the cell HSL binds to the gene product of *luxR* (LuxR), a transcription factor. The HSL–LuxR complex binds upstream of the *luxICDABE* operon, facilitating the transcription of all the necessary components of the luciferase system in addition to an exponential increase in *luxI* transcription. LuxR also binds to the *luxR* promoter in a positive feedback loop (the presence of LuxR inhibits its synthesis) (March and Bentley, 2004).

In *V. fischeri*, the only gene required for the synthesis of the autoinducer (3-oxo-hexanoylhomoserine lactone, 3-oxo-C6-HSL) is *luxI*.
The initial stage of the induction of bioluminescence involves the interaction between the autoinducer and the transcriptional regulator protein, LuxR. The gene *luxI* is always

expressed at a basal level in *V. fischeri* cells, even when the population density is low, and therefore the concentration of the molecule in the mean remains low. When the population density increases, also the concentration of the autoinducer increases, until a critical threshold is achieved. This critical density, at which the autoinducer diffuses back into the cells and binds to LuxR activating the transcription from *luxICDABEG* operon, corresponds to the so-called 'quorum' of bacteria. This regulatory process is referred to as autoinduction, as it causes the increase of cellular levels of mRNA for both bioluminescence and autoinducer synthesis.
Thus, autoinduction ensures that bioluminescence and signalling molecule production continues (Gera and Srivastava, 2006).

3.1.2 Quorum Sensing in other microorganisms

After the discovery of this phenomenon in *V. fischeri*, Quorum Sensing mechanisms were searched and characterized also in other organisms.
Examples of other genes regulated by Quorum Sensing include the *tra* (Ti plasmid transfer) genes in *Agrobacterium tumefaciens*, exoenzymes and virulence factors in *Pseudomonas aeruginosa* and *Erwinia carotovora*, swarming motility in *Serratia liquefaciens*, antibiotics and violacein pigment in *Chromobacterium violaceum*, exopolysaccharide production in *Pantoea stewartii* and nodulation in *Rhizobium* (Fuqua *et al.*, 1994; Salmond *et al.*, 1995; Swift *et al.*, 1996). In all these organisms, one or more LuxR and LuxI homologues were detected.
It is not surprising that symbiosis, pathogenesis, and Quorum Sensing are intertwined in a complex story of gene regulation, as most of these organisms are able to establish symbiotic or pathogenic relationships with eukaryotic hosts.
Furthermore, it has been shown that also in natural habitats, different bacterial species communicate with one another to coordinate their behavior (Bassler, 1999; Bassler, 2002). An example of this is the bacterial community that naturally colonizes the roots of tomato plants (Steidle *et al.*, 2001). In specific, it has been suggested that the AHLs act as signals for coordination of the functions of the different populations within this rhizosphere community.

3.1.3 AHL molecules

AHLs consist of an HSL head group attached to a variable acyl side chain (R2 in figure 3.1.2). The amphipathy of the AHL molecule seems to be a balance between the hydrophobic side chain and the hydrophilic HSL ring. It is probable that these characteristics allow the AHLs to pass through the phospholipid bilayer of the cell membrane and to move in the aqueous intracellular and extracellular environments (Fuqua *et al.*, 2001). The acyl chain can vary in length, from 4 to 18 carbons in those AHLs identified so far. Variability also exists in the third carbon position (R1 in Figure 3.1.2) of the acyl chain, where there can be a hydrogen, hydroxyl, or oxo substitution.
These QS signals are specific thanks to the overall length of the side chain and the chemical modification at the third carbon position. Moreover, most QS organisms can produce more than one type of AHL and many organisms can produce the same AHL. Therefore, there is some overlap in the production and recognition of AHLs by different organisms (González and Marketon, 2003).

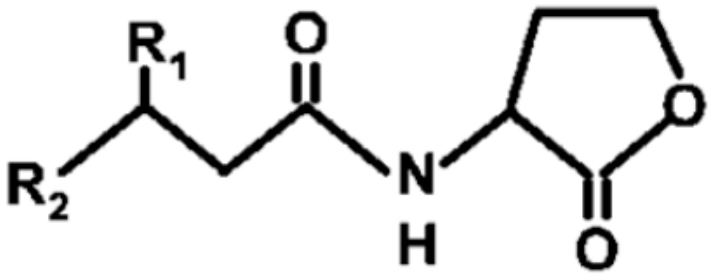

Figure 3.1.2
The general structure of AHLs is shown in bold. R1: –H, –OH or =O; R2: – CH3, –(CH2)2–14CH3 or –(CH2)5CH=CH(CH2)5CH3 (Soto *et al.*, 2006).

3.1.4 Methods for the detection of AHL signals

Screening for AHL production from bacterial strains has typically relied on bacteriological monitor systems (Swift *et al.*, 1993; Cha *et al.*, 1998; Gram *et al.*, 1999). Many bioassays and sensor systems have been developed to allow the detection, characterization and quantification of microbial acyl HSLs34–40.
These systems base on the detection of a phenotypic response, such as bioluminescence, violacein production, Beta-galactosidase activity, growth inhibition, which are activated through an AHL-receptor protein. Such strains contain an easily assayable reporter gene

and lack all AHL synthases, such that reporter activity requires exogenous AHLs. Each receptor protein responds to a different range of AHLs (McClean *et al.*, 1997; Cha *et al.*, 1998; Winson *et al.*, 1998).
One reporter is for example *Agrobacterium tumefaciens* NTL4 pZRL4 (Shaw *et al.*, 1997). This strain permits to perform an assay for these signals that couples separation by thin-layer chromatography with detection. Using *A. tumefaciens* harboring *lacZ* fused to a gene that is regulated by autoinduction, it is possible to detect acyl-homoserine lactones with 3-oxo-, 3-hydroxy-, and 3-unsubstituted side chains of all lengths tested. Moreover, the intensity of the response is proportional to the amount of the signal molecule chromatographed.
Other two strains used as such biosensors are *Chromobacterium violaceum* CV026 for its production of violacein in response of AHL from exogenous sources, and *Rhizobium leguminosarum* bv. *viciae* A34 for its sensitivity to C14 AHL.

3.1.5 Project outline

The major aim of this part of the work was to define experimental procedures to analyse the physical diffusion of Quorum Sensing signal molecules in artificial systems approximating real-life microbial contexts, as the biofilm.
In particular, combining physics and microbiology approaches, the consequences of boundary conditions (signal-reflecting vs. signal-adsorbing) on bacterial intercellular communication were investigated.
In this chapter, a paper reporting the results obtained with this kind of analysis, which is now in press in FEMS Microbiology Letters, is reported.

3.2 FEMS Microbiology Letters 2014, in press.

Relevance of absorbing/reflecting boundary conditions in Quorum vs. Diffusion Sensing: a quantitative analysis.

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Abstract:

The consequences of the boundary conditions (signal-reflecting vs. signal-adsorbing) on bacterial intercellular communication were addressed by a combined physics and microbiology approach. A predictive biophysical model was devised considering system size, diffusion from given points, signal molecules decay, and the behaviour of boundaries. The theoretical predictions were tested by two experimental setup concepts respectively providing reflecting or absorbing boundaries within a gel matrix, whereby diffusing AHL concentration profiles were measured using the *A.tumefaciens* NTL4 bioassay. While for the reflecting system borders the polystyrene petri dishes or microtiter plates were found to perform with such behaviour, the absorbing borders were achieved by providing a constant water flow over and exposed side of given surface of the matrix. The properties of the boundaries, in terms of absorbing vs. reflecting nature, were recognized to play a primary critical role on the AHL concentration profiles. A non-reflecting boundary side could practically equate the effect of a hundred-fold lower cell concentration. Results allow to extrapolate that the kinetics of signal accumulation vs. signal removal and its consequent threshold-mediated phenotypes within actual natural biofilms are directly linked to the boundary properties, stressing the relevance of the Diffusion Sensing component in bacterial communication.

Keywords: quorum sensing; diffusion kinetics: boundary conditions; AHL

INTRODUCTION

The bacterial capability of sensing their own emitted signal molecules and turning the information into action is nowadays regarded as a central aspect in microbiology studies. Originally referred to as Quorum Sensing (Fuqua *et al.*, 1994), the phenomenon has shown many facets and led to different perspective interpretations, including that of Diffusion Sensing (Redfield, 2002) where the standpoint is shifted from the population density to the single cell environment topology. Further work integrated these two basic views leading to concept of Local Efficiency Sensing (Hense *et al.*, 2007). In parallel, mathematical models have been proposed to analyze molecular signalling between cells (Ward *et al.*, 2001; Dockery & Keener, 2001; Mueller *et al.*, 2006). Our prior work (Alberghini *et al.*, 2009) introduced novel conceptual views and terms (Positional Sensing, Cluster Sensing, Cumulative Gradient Sensing) emphasizing the issue of the diffusion of signal molecules in a tridimensional matrix and providing mathematical formulas to predict concentrations in different point of spatial systems approximating real-life microbial contexts as the biofilm. The work has been commented in reviews on the abridged Quorum Sensing terminology (Platt & Fuqua; 2010) and cited by papers devoted to the implications of spatial signal deployment; among these, upon manipulating the diffusive properties of the growth medium, it was shown that for *Streptococcus peneumoniae* there is no fixed quorum for competence, and induction cell density scales with diffusivity (Yang *et al.*, 2010); other authors, capitalizing on the above proposed concepts, hypothesized that community stability in an open environment relies on a predominantly local steady state of intercellular communication between bacterial colonies (Venturi *et al.*, 2010). The issue on how dynamic chemistry of natural environments affects cell-to-cell chemical signalling was accordingly reviewed (Decho *et al.*, 2011) . The importance of confinement-induced quorum sensing has been stressed and it is now acknowledged that, in addition to cell density, other factors such as the dimensions and diffusional characteristics of the environment could influence quorum sensing and induction of genetic reprogramming (Carnes *et al.*, 2010). Dulla and Lindow (2008) demonstrated that QS confined on dry leaves could occur with rather small numbers of cells. Other studies addressed the optimal density over which QS is predicted to become advantageous (Pai and You., 2009). In microfluidic devices confinement was shown to induce QS response even for single cells (Boedicker et al. 2009). The kinetics of spatiotemporal activation of cells has been investigated and modelled by Dilanji et al. (2012). Studying the transition to QS in Agrobacterium Goryachev et al., (2005) showed that in biofilms the event required a much lower cell density than in liquid cultures and deduced that QS could be used by bacteria as a detector of biofilm formation.

Understanding the dynamic ranges of AHL signals produced in natural biofilms, in which quorum sensing is a primary aspect, calls for an accurate definition of the effects of the underlying boundaries on molecule diffusion, retention, withdrawal or backwards reflection. Biofilms laying on submerged solid surfaces face different effects exerted from the basal side when compared to those from the flowing fluid side. The reach of an active signal threshold depends from supposedly cell-density dependent AHL production, and from a balance between its outflow from the system versus its local accumulation due to the molecules bouncing back from reflecting surfaces. In the work presented hereby we address the properties of a system that turned out to faithfully represent totally reflecting boundaries. A subsequent test is instead devoted to the opposite situation upon setting up absorbing boundary conditions apt to draw out completely AHL molecules from the system.

In the present account we moreover characterized the issue of diffusion of the gram negative QS signals N-Acyl homoserine lactones by devising a biophysical model to predict their space- and timewise-dependent concentration in an agarose matrix, comparing systems of different size. Being gels of this organic type characterized by irregular mesh size permeated by a water-based solution, they were also deemed to approximate and represent a biofilm-like structure suitable to mimic a natural setting and allowing to analyze thereby the bacterial signal diffusion kinetics. The theoretical model was verified by the actual tridimensional setups which showed a robust coherence with the predictive computing.

MATERIALS AND METHODS

Diffusion test setup and predictive model definition

The AHL of choice was OHL (N-Octanoyl-L-Homoserine lactone, Fluka Chemie Gmbh Buchs, Switzerland, molecular weight $w_{OHL} = 227.3)$ and we considered its diffusion in agar cylindrical disks with radius R and height h (the volume is $V = \pi R^2 h)$. Experimental data were obtained using two different disk sizes; a larger disk (*R=4.2* cm, *V=20* ml, $h \approx 3.61$ mm) obtained by pouring 20 ml of a 0.7% water solution of molten agarose in plastic petri dishes with a diameter of 8.4 cm, and a smaller disk (*R=1.7* cm, *V=5* ml, $h \approx 5.51$ mm) for which 5 ml of the same agarose solution were poured in mini-petri dishes with a diameter of 3,4 cm.

$m_0 = 100$ ng of OHL were initially dispensed at the center of the disks (10 µl of a 10 ng/ µl solution). OHL molecules diffuse in the disk and their radial concentration

profile is detected after either 22 hours or 94 hours, by using an overlay of reporter cells, that are activated if OHL concentrations are greater than its quorum threshold.

The reporter of choice was *Agrobacterium tumefaciens* NTL4 pZRL4 (Shaw *et al.*, 1997). The strain was grown in liquid AB medium (Chilton *et al.*, 1974) supplemented with 30 µg/ml gentamycin at 30°C. 10 ml of an overnight preculture, were inoculated in 100 ml of AB and grown for 18 h under the same conditions. 67 ml of this culture were subsequently mixed with 133 ml of molten AB agarose (10 g/l) containing 30 µg/ml gentamycin and 60 µg/ml X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) which was previously cooled to 42 °C. 10 ml of overlay were poured in the larger petri dishes and 2.5 ml in the smaller ones.

In the mathematical modeling of AHL molecules diffusion in agarose disks, we compute our AHL molar concentration $c_{OHL}(\rho,z,t)$ as a function of the distance ρ from the cylinder axis ($0 \le \rho \le R$), of the distance *z* from the bottom face of the disk ($0 \le z \le h$), and of the time *t* from the moment when AHL is poured in the disk. AHL concentration can be derived by solving the diffusion equation in the presence of AHL degradation (k is the AHL degradation rate) :

$$\frac{\partial c_{OHL}(\rho,z,t)}{\partial t} = \frac{D}{\rho}\frac{\partial}{\partial \rho}\left[\rho \frac{\partial c_{OHL}(\rho,z,t)}{\partial \rho}\right] + D\frac{\partial^2 c_{OHL}(\rho,z,t)}{\partial z^2} - k\, c_{OHL}(\rho,\mathrm{z},\mathrm{t}) \qquad (1)$$

We set the initial condition $c_{OHL}(\rho,z,t=0) = \frac{n_0}{\pi\rho}\delta(\rho)\delta\left(z - h/2\right)$ for all values of ρ and *z*, where $\delta(x)$ is the Dirac delta, assuming that the AHL is initially poured right at the disk center, $n_0 = \frac{m_0}{w_{OHL}}$ is the corresponding number of AHL moles that start to diffuse. D is the diffusion coefficient of AHL in our system that we expect to be smaller than the diffusion coefficient (D_{H_2O}) of AHL in a water-based enviroment that has been estimated to be $D_{H_2O} = 4.9\times10^{-10}\ \mathrm{m^2/s}$ m (Stewart, 1998; Stewart, 2003). Furthermore, we consider reflecting boundary condition both at the agarose/plastic interface (i.e. at $\rho = R$ and at $z = 0$), so that $\left.\frac{\partial c_{OHL}(\rho,z,t)}{\partial \rho}\right|_{\rho=R} = 0$ for all *z* and *t*, and $\left.\frac{\partial c_{OHL}(\rho,z,t)}{\partial z}\right|_{z=0} = 0$ for all ρ and *t*, and at the agar/air interface (at $z = h$), so that $\left.\frac{\partial c_{OHL}(\rho,z,t)}{\partial z}\right|_{z=h} = 0$, for all ρ and *t*.

The solution of the diffusion equation (1) with the above specified boundary conditions can be worked out (Pikulin & Pohozaev, 2001) as a sum over all possible relaxation modes of the system, labeled by *n,m*:

$$c_{OHL}(\rho,z,t) = 2\frac{n_0}{V}\sum_{n=0}^{\infty}\sum_{m=0}^{\infty} B_{nm} e^{(-\omega_{nm}t)} J_0\left(\mu_n \frac{\rho}{R}\right)\cos\left(\tau_m \frac{z}{h}\right); \qquad (2)$$

$$B_{nm} = \frac{1}{\left[J_0(\mu_n)\right]^2}\frac{\cos\left(\tau_m/2\right)}{1+\delta_{m,0}}; \qquad (3)$$

$$\omega_{nm} = k + D\left(\frac{\mu_n^2}{R^2} + \frac{\tau_m^{\,2}}{h^2}\right); \quad J_1(\mu_n) = 0; \quad \tau_m = m\pi \qquad (4)$$

where $J_0(r)$ and $J_1(r)$ are Bessel functions of the first kind (Arfken &Weber, 2005) and μ_n are the roots (zeroes) of $J_1\left(\mu_0 = 0 < \mu_1 < \mu_2 < \ldots\right)$; $\delta_{a,b}$ is the Kronecker delta ($\delta_{a,b} = 1$ if *a=b*; $\delta_{a,b} = 0$ otherwise).

The different relaxation modes describe in detail how the system relaxes to the equilibrium ($t = \infty$) solution $c_{OHL}(\rho,z) = 0$ corresponding to the achieved degradation of all AHL molecules initially poured into the disk. Each mode is characterized by its own relaxation frequency ω_{nm}, or half-life time $\tau_{nm} = 1/\omega_{nm}$, and by an intrinsic amplitude B_{nm}. The low frequency modes are the more relevant ones in describing system behaviour at long times. Mode frequency increases with n,m. The slowest mode n=m=0 (with frequency $\omega_{00} = k$), corresponds to the asymptotic long time solution $c_{OHL}(\rho,z,t) = \frac{n_0}{V}e^{-kt}$ describing the degradation with rate k of n_o AHL moles uniformly distributed within the whole disk. Other modes with n,m≠0 take into account the spatial dependence of AHL concentration profile within the disk. In practice, only a few modes (around 10) need to be considered in the two sums in eq. (2), in order to get a reliable estimate of the c_{OHL} concentration profiles shown in Figure 1.

In the absence of bacterial cells, under the present neutral pH conditions AHL molecules are sufficiently stable. Their half-life at pH7 is reported to be around 7 days (Englmann et. al., 2007) although earlier estimation (Schaefer et al. 2000) were suggesting lower values (1 day). Eq. (2) can be used to predict the concentration of AHL whose effects can be measured through a bacterial report as described below.

Our theory has three free parameters: the diffusion constant D, the degradation rate k and the quorum sensing concentration threshold Q_s to induce a given bacterium. These three parameters can be optimized to better fit the experimental results.

We do not report here for simplicity the solution of the diffusion equation (1) in the presence of absorbing boundary conditions. It consists of a similar sum over different relaxation modes having different profiles, frequencies and intrinsic amplitudes with respect to the reflective boundary case.

AHL dilution bioassay in microtiter plates.

In order to quantify the AHL concentration attained at different distances from the center after the two incubation times, a variation of the same reporter technique was devised. Such modification overcomes a common limitation in the use of chromogenic AHL-reporting overlays when assessing diffusion gradient profiles, which is the fact that once the signal amount is above the QS threshold, no quantitative information on its actual concentration can be deduced. Instead of pouring an overlay of the reporter cells over the diffusion plates, cored samples of agarose (100 µl aliquots) were withdrawn with a plastic borer and transferred into 0.5 ml eppendorf tubes which were placed in a thermal block set at 95°C for 15 minutes to melt the samples. As already discussed, and as confirmed (see below) by our analysis, the AHL half-life time, at room temperature and pH=7, is rather long (7 days). We do not expect that raising the temperature for a much shorter time may induce a significant degradation of C8-OHL signal molecules (Yates et al. 2000), but we want to stress that a potential high temperature degradation would not at any rate affect the relative concentrations between different samples because they were all treated in the same way. High temperature degradation would possibly lower the absolute concentration in each sample by the same factor, so that the only effect in our parameter tuning would be a potential overestimation of the quorum concentration threshold.

A series of twofold dilutions were performed by transferring 20 µl of molten agarose into an equal volume of water kept at the same temperature in the thermal block, and proceeding accordingly up to a 1:8 dilution.

To reveal active concentrations within the serially diluted portions of the system, 10 µl of molten material were transferred into wells of a microtiter plate containing the *Agrobacterum tumefaciens* NTL4 reporter. The revealing microtiter plate had been prepared as follows. After the same above described procedure for the overlay preparation aliquots of 200 µl of *A. tumefaciens* suspension in molten AB agar with gentamycin and X-gal were dispensed in each of the wells of a sterile 96-wells microtiter

plate (Cellstar, Greiner bio-one, Kremsmuenster, Austria). Upon solidification, 10 µl molten aliquots of the supernatants from the diffusion plates and of their serial 1:1 stepwise dilutions were layered in the wells, and the plates were incubated for 24 h at 30°C. Digital images of the results were acquired directly on an Epson Perfection 1240U flatbed digital scanner. The whole experiment was repeated three times providing constantly coherent and fully reproducible data.

Absorbing boundary conditions assembly.

In order to provide a set up that could ensure boundary conditions of the absorbing kind for the diffusing AHL molecules, we modified a 96-wells microtiter plate by piercing holes on the bottom of some of its wells by means of a rotating microblade. Wells' opened bottoms were temporarily sealed with sellotape to allow pouring in molten AB medium agarose (0.7%) with the suspended AHL-reporter A.tumefaciens NTL4 with X-Gal prepared as above but without Gentamycin, and supplemented with a suspension of an AHL-producing bacterial strain (Rhizobium leguminosarum bv. viciae A34). The latter was pre-grown in TY medium for 24 h, centrifuged at 5000 rpm for 15 min, resuspended in 10 ml of sterile physiological solution, counted in a Petroff-Hauser chamber in order to calculate aliquots to be mixed along with the reporter strain in the molten agarose suitable to deliver concentrations of 104 and of 106 cells/ml. The cylinders that resulted had 8 mm height and, upon removing the tape after gel solidification, offered an open circular side which enabled to achieve the signal-absorbing condition by placing the microtiter plate in a leaning reservoir whose floor was constantly flooded by a flow of running water drawn out from a hole at the downside of the reservoir. A direct comparison with reflecting conditions was achieved by using non-pierced control wells. The incubation was carried out for over five days and monitored in continuous by inspecting the onset of the blue colour brought about by X-Gal formation.

RESULTS AND DISCUSSION

The mathematical modelling of the diffusion profile of N-Octanoyl-L-Homoserine lactone, elaborated upon the equations described in the experimental section, predicted curves as the ones shown in Fig.1. The plots represent the expected distribution of the concentrations that would be achieved upon placing a 10 µl drop of solution containing 100 ng of the pure molecule over the agarose gel matrix in the center of plates of small (radius of 17 mm) or large size (radius of 42 mm), and allowing it to diffuse for 22 hours or for 94 hours, subject to degradation with rate k. The behaviour of serially halved concentrations (1:2, 1:4 etc.) is also shown. One main critical parameter in such systems

consists in the properties of the boundaries. Once the diffusing molecules reach the borders, these could be either absorbed or reflected. These two alternative possibilities would profoundly affect the resulting distribution profiles. All AHL molecules would anyway be degraded after a time much longer than 1/k (typically days), but boundaries of indefinitely absorbing nature would correspond to AHL concentrations fading to zero in each point much faster, namely within the typical time $h^2/4D$ needed by a AHL molecule to diffuse from the disk center to the top or bottom adsorbing side at a distance $h/2$ (a couple of hours for our experimental setup). Conversely, in the presence of reflecting walls, the long term behaviour would stabilize the concentration at spatially uniform values, slowly decaying in time with rate k. If this were the case, such situation would be reached earlier in smaller-sized systems than in larger sized ones, as molecules would start bouncing back earlier against the reflecting borders. It needs to be remarked that, in terms of absorption or reflection, the behaviour of a solid surface with respect to diffusing organic solutes is not predictable a priori as it depends on the chemical affinity properties of the specific material and, in case of absorbing behaviour, on the limits of its capacity to accommodate and hold the bound compound.

The plots presented in Fig. 1 are modelled assuming the case of reflecting boundaries. For this reason the small disk (plate with 17 mm radius) at the longer diffusion time (94h, bottom left panel) is predicted to reach flat concentration profiles earlier than the larger disk (bottom right panel) and to attain higher values, being the bounced molecules confined within a smaller space. The critical concentration threshold Q_s (orange broken line) required to induce a given bacterium allows to place that value as a reference cutoff line in the graph, to assess at which distance, time, and system size, one would expect the effective quorum to be reached. The model predictions in the case of absorbing boundaries are simply that for all disk sizes, diffusion times and dilutions considered in this work the AHL concentration curves are well below the quorum activation threshold.

The behaviour of boundaries in their absorbing vs. reflecting performance, is a crucial issue that applies to all contexts in which quorum sensing is taking place, and especially in the biofilm matrix, which represents its most significant occurrence in environmental microbiology (Costerton *et al.*, 2003). It becomes of primary importance to evaluate whether the surface on which the microbial biofilm layer is developing, acts as a 'mirror' for molecules or rather titrates them out. In the human mouth, a bacterial biofilm as the dental plaque would encounter inducing concentrations in a manner that depends on how reflecting is the tooth enamel on which the biofilm rests. In water-covered biofilms, the underlying rock type, its porosity and properties can equally condition the kinetics of signal perception by bacteria. The same applies to biofilms colonizing artificial

materials as metal pipelines tubing, implanted prostheses, and craftsmanship in general wherever wet condition allow microbial development. Under this view the whole issue of diffusion sensing (self-perception of signals in narrow spaces) as opposed to quorum sensing (cell density assessment) acquires new depth, calling for proper focus.

In order to verify the reflective boundary model predictions in a real setup, we performed the test using plastic petri dishes of two sizes and a diffusive agarose gel matrix on whose center the OHL molecules were dispensed (Fig. 2). The molecule distribution profiles were evaluated by means of the reporter *A. tumefaciens* NTL4 in two ways; (a) to inspect the overall diffusion we overlayed the plates after the two diffusion times and observed the blue halo appearance, whose front sets up the cutoff around the Q_s concentration, below which the reporter strain stays colorless. (b) on a parallel identical set of plates, instead of pouring the revealing reporter over the diffusion plates, we cored small agarose cylindrical blocks from the plates at increasing distances from the center, melted those, and diluted them in hot sterile water to lower AHL concentration down to its bioassay-detectable limits. To read all such dilutions we exposed to each of them the *A. tumefaciens* reporter in microtiter wells and observed where the transition between blue to colorless phenotype would occur. As regards the whole plates, Fig. 2 shows how the short term diffusion of 22 h (top panels) in the large plates leads to a situation in which the phenotypic-cutoff front has not yet reached the disk circumference as a colorless circular crown is still visible around the blue halo. In the small plate instead the path is complete and a more saturated tint uniformly covers the plate. In the longer diffusion stage (94h, bottom panels) the active detectable concentration has reached the plate border also for the large disk.

The bioassay in itself would not provide a strict quantitative information but a detailed 'exploded view' of the actual concentrations that are concealed within the blue phenotype can be easily obtained by stepwise dilutions of each desired zone, as the threshold of transition to colourless phenotype is fixed, 5nM being the lowest detection limit value determined by the same assay (data not shown). The agarose matrix allows to perform molecule dilution without extraction, by simple melting and mixing with corresponding hot water volumes. For this purpose we sampled aliquots of the plates at increasing distances from the origin to verify the shape of the diffusion gradient by using the same bioassay in microtiter wells, as shown on the sides of Fig. 2 . The phenotype extinction is observed as function of the variables of position in space, diffusion time, and size of the plate. The results allowed to inspect the validity of the theoretical predictions provided by the model (Fig.1). As explained in the Materials and Methods section we have the flexibility of choosing three parameters to better reproduce the experimental profiles: Fig1 was obtained with $D = 3.0\times10^{-10}\ \mathrm{m^2/s}$, $k = 1/(7\ \mathrm{days})$ and

$Q_s = 5\,\mathrm{nM}$ a set of values selected after a fine-tuned scanning of the possible values. The theoretical prediction fits the expected profiles with remarkable accuracy: to check out the correspondence one can follow the predicted curves in Fig. 1 to observe at which times and distances were they foretelling the drop below the broken orange line of the quorum threshold; e.g. in the large disks at 22 h only the first and the second distance (plate center and area encompassing 10-15 mm) were predicted to have concentrations still above the threshold line for all the 3 dilutions; as Fig 2 confirms the first two columns (A,B) are active, while those coming from cores taken at 25 mm or 40 mm (plate border) are colourless. All the other situations comply as well to the mathematically assessed profiles. It is important to stress that the very accurate correspondence between theoretical predictions and experimental evidence is crucially related to the choice of the free parameters. For instance, a value of $k = 1/(1\,\mathrm{day})$ as estimated in (Schaefer et al., 2000), would have implied much lower values of the AHL concentration profile after 94 hours: in such situation, for both disk sizes and all dilutions, all microtiter wells would have not been activated. Therefore our approach allows for an indirect measure of k which is fully consistent with prior literature (Englmann et al. 2007). In the same way, the estimation of D (smaller than the coefficient diffusion of AHL in water) and Q_s are congruent with expectation. In particular $Q_s = 5\,\mathrm{nM}$ is close to the quorum threshold displayed by the reporter strain A. tumefaciens, resulting from the bioassay when using serial dilutions of AHL (data not shown).

The most prominent issue of the present work is the confirmation that boundaries of such kind behave in a reflecting manner. If the borders were indefinitely absorbing the outcome would have been radically different as no molecules would have bounced back and the long term concentrations would not have led to higher concentration in the small plates compared to the large ones. In a more realistic scenario, AHL molecules may be sequestered on plate walls only up to a certain saturation surface concentration, determined by the chemical properties of the absorption process. After saturation has been reached, the walls become reflecting; the present study shows that in the given experimental conditions with this kind of material a possible adsorption regime is negligible as experimental data are consistently reproduced by theoretical predictions that take into account only the reflecting behaviour.

From this part of the experimentation we thus conclude that the effective behaviour of boundaries in this system is that of reflecting surfaces, although it can not be excluded that in the first stages of the OHL contact with the plate walls a behaviour of absorbing nature could also operate.

The model, consistently supported by the data, will be utilized to devise an accurate predictive tool to study quorum sensing, as well as other of its practical, conceptual or semantic distinctions, in actual microbiological biofilm systems of different permeability, depth, and underlying substrate.

The sensor system applied, the *Agrobacterium tumefaciens* NTL4 reporter, by introducing simple dilution steps to the basic protocol, has shown to possess the adequate sensitivity to study these phenomena at the desired resolution. In this respect, being the bioassay able to detect molecule-specific responses around the 5 nM range, it also proves to be ten times more sensitive than analytical techniques as fluorescence or mass spectrometry, whose limits are instead in the 100 nM range, and moreover require expensive instrumentation and do not provide a straight specific identification of molecule structures. The biological assay qualifies therefore as the most sensible sensor in studies of these phenomena, in which, as the results show, the diffusion component of bacterial signalling and perception (Redfield, 2002) calls for an adequate consideration when modelling biofilm-based cell-to-cell communications and interactions with their environment.

Coming to the alternative system experiment, we devised a set-up apt to enable a signal-absorbing behaviour in a continuous fashion from the open bottom side of the microtiter wells. In these wells, the gel cylinders holding the embedded reporter Agrobacterium along with two different concentrations of the AHL-producing Rhizobium leguminosarum, were exposed from one side to uninterrupted contact with flowing water in order for the signal molecules to be irreversibly drawn away from the system (Fig.3). As controls, wells with intact bottoms filled with the same bacterial suspensions agarose represented the reflecting conditions. The presence of X-Gal in the gel enabled a direct monitoring of the effects in real time. At 96 hours the plate was withdrawn and the image was acquired by means of a flat bed scanner. The result is shown in Fig. 4. It appears that the absorbing conditions have a significant impact on the signal concentration that can build-up in such system. In those open-sided wells, in spite of the fact that the embedded producer cells had the same density of their reflecting conditions control wells, the reporter response indicated a much lower AHL concentration. It is interesting to observe that the threshold response colour of the assay (close to 5 nM AHL as separately verified) is similar in the wells with 106 cells/ml under absorbing conditions and the closed control reflective wells with 104 cells/ml. That translates into the fact that a non-reflecting boundary side equates the effect of a hundred-fold lower cell concentration. It is also worth observing that in the absorbing boundary wells containing 104 cells/ml, the signal withdrawal action consequent to the open side flow is such that the threshold of quorum sensing is never achieved. These data support the view of QS as a dynamic source of information to cells about the diffusion properties of a

biofilm boundary. In other terms, the depth of a cell-containing layer is a variable that can be probed and sensed by the bacteria themselves via quorum sensing system.

In conclusion we have here presented a sophisticated biophysical sensor system based on an Agrobacterium tumefaciens NTL4 bioassay which allows for a quantitative tracking of the presence of AHL molecules at very low concentrations in the nM range. Even the most efficient analytical methods such those using anti-AHL antibodies (Chen et al. 2010) are only capable of detecting molecules in the lower micromolar to nanomolar range, which is much higher that the concentrations that can be detected with the assay reported here. By using the A.tumefaciens bioassay we studied in details two systems by directly measuring the strong effects that different boundary conditions might have on AHL concentrations and by comparing these experimental measurements with theoretical predictions. As a side result we were able to give estimates for the diffusion constant and the degradation rate of AHL and for the quorum sensing threshold of the reporter which are in close agreement with values obtained using direct approaches. Beside their methodological advance, we believe the results are relevant to highlight the hiterto underestimated role of the surrounding conditions in triggering quorum sensing mechanisms.

ACKNOWLEDGMENTS

Leonard Prins and Maria Arduini are gratefully acknowledged for fruitful discussions and precious advice on technical aspects of the methodology. FS acknowledges support from the PRIN project 2009SKNEWA.

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Figure Legends

Figure 1. Theoretical predictions of the diffusion model. The expected OHL concentration profile is plotted against the distance from the plate center. The curves model the concentrations predicted to result from stepwise twofold dilutions. The segments over the X axis correspond to the positions of the cored aliquots that are then sampled in the actual test to quantify the real OHL concentration with the reporter strain as shown in Fig.2. Broken orange line: threshold of the minimum detectable concentration (yielding blue colour in the bioassay) measured for the reporter *A. tumefaciens* NTL4 in the presence of OHL, and corresponding to 5 nM (="Quorum").

Figure 2. Results of the bioassay validating the predictive modeling. Petri plates small and large are shown, that were supplemented with 100 ng OHL at the center and allowed to diffuse for 22h or 94h and subsequently overlayed with the *A. tumefaciens* reporter. In parallel replicas of the same plates not overlayed with the reporter, were used to sample aliquots at the positions shown by the broken circles. Their stepwise twofold dilutions (corresponding capital or small letters) were transferred into the microtiter wells containing the reporter whose development is shown alongside.

Figure 3. Experimental set up to create signal-absorbing boundary condition. (1) A 96-wells microtiter plate is used, on whose bottom holes are pierced in some of the wells and sealed with tape. (2) molten agarose is poured containing in suspension the AHL-producing strain (R.leguminosarum) and the AHL-sensing reporter (A.tumefaciens) and X-gal indicator. Wells on the left (with intact bottom) are the controls offering the reflecting boundary conditions. Wells on the right (bottomless) constitute the ones where the absorbing conditions are realized. (3) When the agarose with the embedded cells is solidified, sealing tape is removed from the bottomless wells and the plate is placed within a tray where a slow and continuous water flow running under the plate bottom and in constant contact with the exposed agarose side at the open bottoms draws away diffusing signals.

Figure 4. Results of the comparison between signal-relecting and signal-absorbing conditions. Microtiter wells containing 8 mm-deep layers of embedded AHL-producing and AHL-reporting cells after 96 h of incubation under the conditions described above.

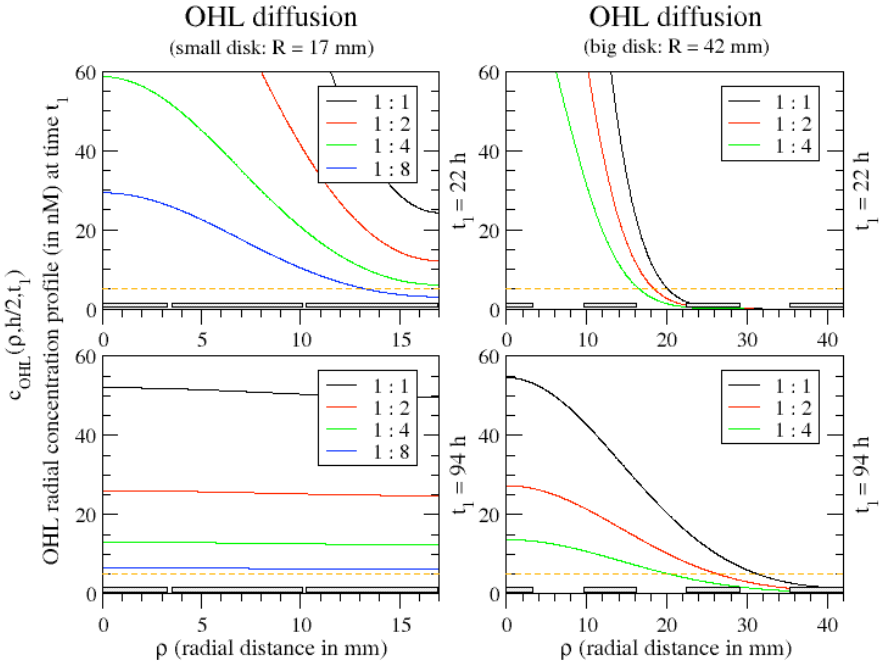
OHL diffusion
(small disk: R = 17 mm)
OHL diffusion
(big disk: R = 42 mm)
OHL radial concentration profile (in nM) at time t1
c_OHL (ρ,h/2,t1)
1 : 1
1 : 2
1 : 4
1 : 8
t1 = 22 h
t1 = 94 h
ρ (radial distance in mm)

Fig.1

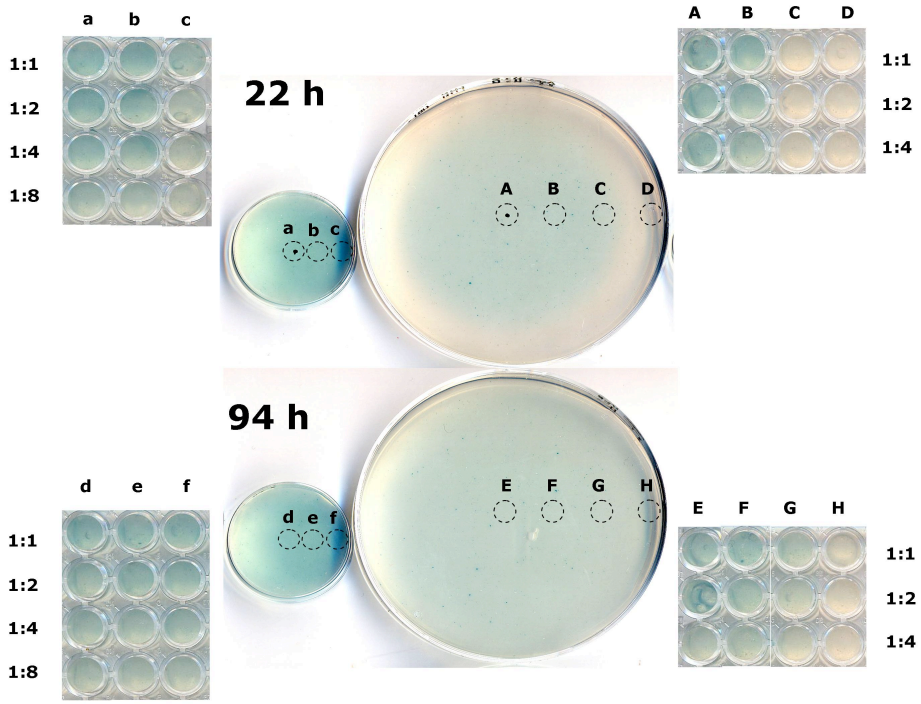
22 h
94 h
a b c
d e f
A B C D
E F G H
1:1
1:2
1:4
1:8

Fig.2

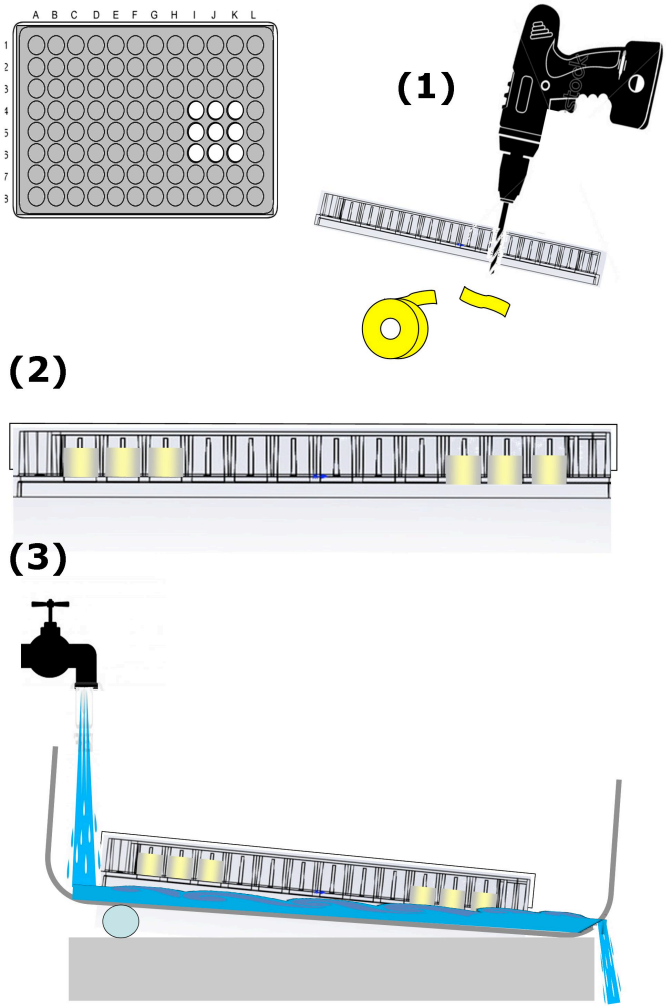
A B C D E F G H I J K L
1
2
3
4
5
6
7
8
(1)
(2)
(3)

Fig.3

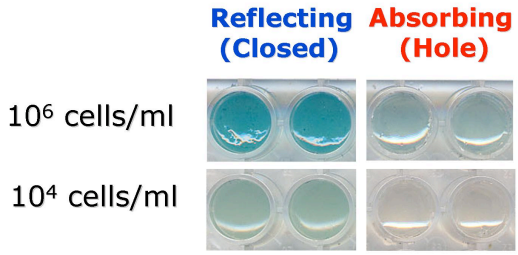
Reflecting (Closed)
Absorbing (Hole)
10⁶ cells/ml
10⁴ cells/ml

Fig. 4

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Acknowledgments

I would like to express my special appreciation and thanks to my advisor Professor Andrea Squartini, for being of great support during my Ph.D., for encouraging my research and for being always available to help and to give me precious advices.

I express my gratitude to Professor Michael Schloter for hosting me in his laboratories at the Helmholtz Zentrum in Munich for six months, and for the collaboration that started in this last year.

I would also like to thank my co-supervisors Prof. Barbara Baldan and Dr. Elisabetta Bergantino for their valuable assistance in these years.

Special thanks goes to all my lab-mates, and to all the people that helped and worked with me during my Ph.D.

Finally, I sincerely thank my family, Francesco and my friends.