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NITROGEN CYCLE DYNAMICS IN A MEDITERRANEAN AGROFORESTRY SYSTEM UNDER DIFFERENT MANAGEMENT REGIMES

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Abstract

The long-term sustainability of agroforestry systems is influenced by the relative balance between the removal of nutrients by harvesting and the addition of nutrients by fertilisation. Potential site nutrient depletion and high water use concerns have led to an interest being developed in linking SRF crop production with the recycling of nutrients from the land treatment of aqueous wastes, such as sludge and effluent arising from animal housing. The application of animal manure to soil can result in increased gaseous emissions such as NH_3, N_2O, CO_2 and CH_4 as well as nitrate leaching, contributing to climate warming and ground and surface water pollution. The application of nitrification inhibitors to soil offers the chance to reduce N losses and to increase fertilizer use efficiency.

Thus, the main goal of this PhD project work was to evaluate and to establish the effectiveness of the nitrification inhibitor DMPP on a Mediterranean agroforestry system combined with a cattle effluent, and to study its effects on soil microbiota involved in N cycling processes. The results of the long-term incubation study markedly show the efficacy of DMPP as nitrification inhibitor under every laboratory conditions investigated. Microbiological results in microcosm studies show high efficacy in the inhibition of the ammonia-oxidizing populations and it was demonstrated the inhibition of both ammonia-oxidizing bacteria and archaea transcripts in soil by DMPP. Given that archaea contribute significantly to nitrification, as their abundance now suggests, estimates of the ecological impact of ammonia oxidation, including greenhouse gas emissions, based on bacterial ammonia-oxidizing activity should be re-assessed.

Key-words: short-rotation forestry; GHG emission; nitrification inhibitors; DMPP; cattle effluent; N use efficiency; ammonia-oxidizing bacteria and archaea; N cycling genes.

Riassunto

L'orientamento di fondo delle principali azioni promosse in sede internazionale dal Protocollo di Kyoto è legato alla riduzione sostanziale dell'uso di fonti d'energia fossile o non rinnovabile, in favore delle risorse rinnovabili. Tra queste l'impiego di energia da biomasse è indubbiamente strategico sia per la disponibilità e la varietà di forme, sia per l'efficienza della trasformazione dell'energia solare in energia fissata. Per promuovere uno sviluppo ecocompatibile delle colture da bioenergia è di primaria importanza l'ottimizzazione della nutrizione azotata e dell'apporto idrico, nonché l'impiego di strategie volte a limitare le perdite di azoto per lisciviazione e volatilizzazione, incrementando l'efficienza di assorbimento dell'elemento da parte della pianta.

La presente attività di ricerca è stata finalizzata alla comprensione delle dinamiche del ciclo dell'azoto in un sistema agroforestale sottoposto a fertilizzazione con un effluente zootecnico ed un inibitore della nitrificazione (3,4-dimetilpirazol fosfato, DMPP). Prove di incubazione a lungo termine in microcosmo hanno dimostrato l'efficacia del DMPP nell'inibire il processo di nitrificazione nel suolo in diverse condizioni sperimentali. L'approccio microbiologico utilizzato nella ricerca ha confermato l'efficacia della molecola nei confronti dei batteri e archaea ammonio-ossidanti attraverso l'inibizione dell'espressione genica di tali popolazioni. Alla luce delle recenti scoperte che attribuiscono agli archaea un ruolo centrale nel processo di nitrificazione nel suolo, le stime dell'impatto ecologico della nitrificazione, comprese le emissioni dei gas serra, dovrebbero essere rivisitate.

Parole chiave: short-rotation forestry; gas serra; inibitori della nitrificazione; DMPP; effluent zootecnici; efficienza dell'assorbimento di azoto; batteri e archaea ammonio-ossidanti.

Chapter 1 – Introduction

1.1. Generals on Short-Rotation Forestry (SRF)

The plantation-based silvicultural model has had many different names: short-rotation forestry, short-rotation woody crops, short rotation coppice, intensive culture of forest crops, biomass and/or bioenergy plantation culture, biofuels feedstock production system, etc. It is commonly defined as "*a silvicultural system based upon short clear-felling cycles, generally between one and 15 years, employing intensive cultural techniques such as fertilization, irrigation and weed control, utilizing genetically superior planting material* (Drew *et al.*, 1987) *and often relying on coppice regeneration*" (Dickmann, 2006). Agroforestry systems could be also defined as "*any form of permanent land use which combines the production of agricultural and/or animal products and tree crops and/or forest plants simultaneously or sequentially on the same unit of land, which aims at optimal sustained, multiple purpose production under the beneficial effect of improved edaphic and micro-climate conditions provided by simulated forest conditions, and management practices which are compatible with the cultural practices of the local population*" (Wiersum, 1981).

Although wood has been a resource from time immemorial for human societies, the scientific-technological basis of short rotation forestry for energy purposes were built starting from the beginning of 20th century. In Italy, in the early 1960s, the *Istituto Sperimentale per la Pioppicoltura* (situated at Casale Monferrato) first carried out experimental trials on purpose-grown poplars and willows for biomass production. During the last decades short rotation forestry has become the subject of renewed interest and focused research (Andersson *et al*., 2002), due to a remarkable amount of public incentives allowed.

The main principle behind SRF is to grow a plantation at such a spacing that it quickly captures the site and then fall it when the trees reach a size that is easily harvested and handled. When growing herbaceous and woody crops for energy purposes, the usual practice is to harvest all the above ground biomass in one operation and transport it to the processing plant for conversion. This is the case for SRF crops such as *Salix*, *Populus*, and *Eucalyptus*, already grown extensively in Sweden, United States and

Australasia (Sims and Riddell-Black, 1998). Most utilized fast growing species for SRF in Central and Northern Europe are willows selected varieties, whereas *Populus* and *Eucalyptus* plantations seem to have a greater potential in Southern Europe (Ortiz *et al.*, 2010).

Eucalyptus is an ideal energy crop with certain species and hybrids having excellent biomass productivity, relatively low lignin content and a short rotation time (Hinchee *et al.*, 2009). The genus *Eucalyptus* contains over 600 species; less than 20 of these have been planted exstensively around the world. *E. globules* and *E. grandis* are used widely in the tropical and sub-tropical regions of the globe, while *E. camaldulensis* and *E. tereticornis* can be commonly found in semi-arid zones (LTS International, 2006).

The planting of short-rotation forestry can lead to several economic, territorial, social and environmental advantages, in terms of biodiversity conservation (Muys *et al.*, 1992), improvement of carbon storage (Montagnini and Nair, 2004; Lagomarsino *et al.*, 2009), enhancing the fertility of soils (Manna *et al.*, 2003), yields of goods and services to society, and providing social and economic well-being to people (Pandey, 2007).

On a global basis, soils and, to a lesser extent, the oceans are the major sources of N emissions to the atmosphere (Jenkinson, 1982). Soil processes contribute about 30% of NO_x, 70% of N_2O, 20% of NH_3 and 30% of annual global CH_4 emissions to the atmosphere (Mosier, 1998). Microbial and chemical processes that occur in the soil affect global change through their impact upon the concentrations of greenhouse gases (e.g., CO_2, CH_4, N_2O, O_3) in the atmosphere. In 1992, the United Nations Framework Convention on Climate Change (UNFCCC) wrote down climate change and global warming firmly on the international policy agenda. Most OECD countries agreed to reduce their greenhouse gas (GHG) emissions by at least 5% below the 1990 levels in the period 2008-2012. Evidence is now emerging that agroforestry systems can act as C sinks and increase the aboveground and soil C stocks through the enhancement of the uptake of CO_2 and the reduction of its emissions.

Crop systems that increase in soil organic C yield positive changes in structure, water-holding capacity, and the storage and availability of nutrients; this in turn leads to increased abundance and diversity of soil biota, as well as increased resistance to compaction (Mann and Tolbert, 2000). In contrast to other arable crops, SRF species can be colonized by ectomycorrhizal fungi, and consequently positive changes in soil

microbial diversity and activity can be achieved in the soils beneath (Baum *et al.*, 2009). Ectomycorrhizal communities have also been shown to be important in *Eucalyptus* trees (Pampolina *et al.*, 2002).

Agroforestry systems have the potential for improving water use efficiency by reducing the unproductive components of the water balance, as run-off, soil evaporation and drainage (Kort *et al.*, 1998; Turner and Ward, 2002). Trees and woodland can also play an important role in the rehabilitation of derelict land, including landfill sites (French *et al.*, 2006); from a water perspective, a key benefit would be reducing the mobilisation and leakage of contaminants, such as adsorbed phosphate, that have the potential to pollute both surface and ground waters (Hutchings, 2002).

1.2. Sustainability of agroforestry crops

Growing and harvesting SRF as a supply of energy on the same site under a continuous rotation over a long term, implies the removal of large volumes of material at a regular intervals and this will lead to a non-sustainable depletion of nutrient from the site. For a non-deciduous tree such as *Eucalyptus*, increased levels of nutrients will be removed off-site, since the leaf component is also harvested. Hence, more nutrients may need to maintain the balance.

Agroforestry poses a number of potential threats to water use; trees and forests are well known to generally use more water than shorter types of vegetation (Nisbet, 2005). This is mainly due to the interception of rainwater by their aerodynamically rougher canopies, but also to higher transpiration rates sustained by deeper rooting on drier sites. The potential high water use of SRF crops could have an adverse impact on local water resources. As in many agricultural production systems, where nutrients are made available at a steady high level with sufficient water, growth is maximized. The long-term sustainability of agroforestry systems is influenced by the relative balance between the removal of nutrients by harvesting and the addition of nutrients by fertilisation. Macronutrients have been studied extensively in biomass crops (Börjesson, 1999).

Potential site nutrient depletion and high water use concerns have led to an interest being developed in linking SRF crop production with the recycling of nutrients from the land treatment of aqueous wastes, such as sludge and effluent arising from animal

housing (cow slurry, pig slurry, poultry manure and liquid sewage sludge), municipal sewage and food and fibre processing industries. The use of waste materials for maintaining and improving soil productivity presents a desirable and economically attractive alternative as well as assisting in the disposal of otherwise troublesome wastes. The benefits of using waste materials as soil amendments justify the extensive investigation required to determine reasonable limits for cumulative adventitious addition of potential toxicants and the close scrutiny required to assure that these limits are not exceeded. The more variable the amendment material, the more complex is the task of determining suitable application rates and maximum cumulative applications. Applications of sewage sludge and nutrient-rich wastewater have been shown to enhance biomass yields of purpose-grown willow plantations, while the nutrient demand for growth helped to reduce leaching (Britt *et al.*, 2002; Christensson and Verma, 2006).

1.3. Zootechnical wastes

The term “livestock wastes” may have many meanings, such as fresh excrement including both the solid and the liquid portions, total excrement but with bedding added to adsorb the liquid portion, the material after liquid drainage, evaporation of water or leaching of soluble nutrients, only the liquid which has been allowed to drain from the total excrement, or material following aerated or anaerobic storage. The characteristics of these items are different. The moisture content of fresh waste is a function of the type of feed and environmental temperature and humidity; evaporation of water may occur under certain conditions. Addition of water occurs from rainwater, wash water, or water added to increase the flow and pumping characteristics of the wastes. Differences in animal waste characteristics can also be a result of changes in the environment and the level of productivity among animals.

Animal manures have long been used as soil amendments and provide valuable macronutrients as well as trace elements. As with many soil amendments, this provides a waste disposal method as well as benefits to agricultural soils. The composition of manures varies widely with species, diet, presence of bedding materials, water intake, management system and sex (MAFF, 1994); this is, perhaps, the reason for the wide

variations in N availability among different types of animal manures (Van Kessel *et al.*, 2000). This is however attributed to be due to the chemical composition such as total N content (Aulakh *et al*., 2000) C/N ratio (Rowell *et al*., 2001; Qiu *et al*., 2008) and lignin/N ratio (Kumar and Goh, 2003). Poultry birds feed mainly on grains with higher protein and fat content compared with forages fed to ruminant; however, microbes in the stomach of the ruminants may also contribute to the nutrient enrichment of their faeces. The feces of livestock consist chiefly of undigested food, mostly cellulose fiber, which has escaped bacterial action; a portion of the other nutrients also could escape digestion. Undigested proteins are excreted in the feces and the excess N from the digested protein is excreted in the urine as uric acid for poultry and urea for animals. Potassium is absorbed during digestion but eventually almost all is excreted. Feces also contain residue from the digestive fluids, waste mineral matter, worn-out cells from the intestinal linings, mucus, bacteria and foreign matter such as dirt consumed along with the food.

Thus, an understanding of some of the predictable differences (e.g., poultry vs. ruminant) is essential to gain maximum benefit with minimal harm, since they may have significant effect on the elemental and nutritional composition of the manures and thus, contributed to the varying magnitudes in net mineralization of different manure N.

The average volume of faeces and urine largely differ from one type of animal to another and mainly depend on their age and lifeweight. For comparison, the general "livestock unit" (LU) is accepted widely. One LU represents a live weight of 500 kg and equals to 1 cow, 6 fattening pigs or 250 laying hens. Up to six varieties of the different types of animal housing are commonly in use, resulting in large variations of total solids (2 - 10 %) and organic dry matter content in manure. Cow slurry is typically collected from feedlots by a scraper system. Straw is often added in the feedlots resulting in slight variations of total solids. In most cases, cows are kept in feedlots with open floors, where the excrements are collected through slots with high amounts of liquid. Chickens are usually kept in large scale units holding up to several hundred thousand animals. Chicken manure is characteristically high in TS contents (~20%) and NH_4-N concentrations (~8g l^{-1}). In most cases, water dissolved ammonia is excreted.

Heavy metals and organics are generally not present near the concentrations found in municipal sludges and ashes, but some unique problems may occur. Feeding and

refeeding of animal manures may cause spiraling trace element accumulation (Capar *et al*., 1978). Although transient in nature, the presence of certain biocides and other medications in animal manures may render them temporarily unsuitable for land disposal unless well-diluted with manures from untreated animals.

Organic fertilizers contain appreciable quantities of nitrogen (N) in soluble forms and should, therefore, only be applied to land when plants can use the N supply. The preferred approach, from a sustainable point of view, is to manage the waste in a carefully designed manner by distributing it at lower rates over a sufficiently large area of land and at acceptably long time intervals such that the nutrients can be effectively taken up by the crops and therefore recycled.

1.4. Nitrogen in the soil

Nitrogen constitutes 78% of earth atmosphere volume. From the total N found in nature, 99,96% is present in the atmosphere, and biosphere contains only 0,005%. The major additions of N to the soil occur through the processes of wet and dry deposition and by the action of microorganisms and, to a lesser extent of lightning that fix atmospheric N_2. The triple bond in molecular nitrogen (N_2) is one of the strongest one between two atoms, therefore, converting N_2 into other compounds requires a large amount of energy, and in nature only specific microorganisms (e.g. *Rhizobium* bacteria) can utilize this compound biologically. Hence, it is only after industrial or microbial conversion of N_2 to NH_3 that this element is available to plant nutrition.

The largest N pool in soil is organic matter, which is mostly unavailable to plants. Nitrogenous organic residues are microbially decomposed with the release of NH_4^+-N (mineralization), which can then be oxidized by microorganisms to NO_3^--N (nitrification). These mineral forms of N are utilized by microorganisms (immobilization), particularly during decomposition of organic residues with a low N content. N can then be returned to the atmosphere through the biological anaerobic reduction of NO_3^- to N_2 (denitrification). Nitrate is readily available for plants and microbe nutrition, but it's highly soluble and mobile in the soil, as it's negatively charged and more subjected to losses; NH_4^+ occurs in the soil as free ions or bounded to mineral or organic colloids, therefore it's more stable than NO_3^-. Decomposition and

mineralization of organic matter mediated by microbial biomass allow N release as available forms to plants. All forms of N, including NH_4^+, NO_3^-, NO_2^-, NO and N_2O, can exist simultaneously inside the soil.

1.5. Nitrification

Nitrification is the oxidation of NH_4^+ or NH_3 to NO_3^- via NO_2^-. These reactions are carried out by two groups of microorganisms: the first step of the process up to NO_2^- production is conducted by the ammonia-oxidizers, whereas in the second step nitrite-oxidizers are involved (Bock *et al*., 1986). These two groups are typically together addressed as *Nitrobacteriaceae* (Buchanan, 1917). *Nitrosomonas europaea* is the best studied autotrophic ammonia-oxidizer, although it's not the most common specie found in soils (Macdonald, 1986). Ammonia oxidation is typically thought to be carried out by a few groups in β- and γ- Proteobacteria, referred to as ammonia-oxidizing bacteria (AOB). However, recent environmental metagenomic analysis reveals that ammonia monooxygenase α-subunit (*amoA*) genes are also present in archaea (AOA) (Schleper *et al*., 2005), belonging to different lineages within the *Crenarchaeota*. The *Nitrobacteriaceae* are aerobes and many are obligate autotrophs. The energy for the CO_2 fixation originates from nitrification, although NH_3 and NO_2^- are not very effective energy sources. This explains the slow growth of these organisms and their relatively high substrate requirements make them difficult to cultivate.

Comparative 16S rRNA sequence analysis demonstrated that all recognized AOB are either members of the β- or γ-subclass of Proteobacteria and seven clusters are currently recognized in this group (Fig. 1.1) (Kowalchuk and Stephen, 2001). The genera *Nitrosomonas, Nitrosospira, Nitrosolobus* and *Nitrosovibrio* form a closely related monophyletic assemblage within the β-subclass of Proteobacteria (Head *et al*., 1993), whereas the genus *Nitrosococcus* constitutes a separate branch within the γ-subclass of Proteobacteria (Woese *et al*., 1995).

The first step of nitrification occur with the following net reaction:

$$NH_3 + O_2 + 2H^+ \rightarrow NH_2OH + H_2O \text{ (Hooper, 1984) (Fig. 1.2).}$$

The reaction is catalized by ammonia monooxygenase (AMO) (Wood, 1986), a

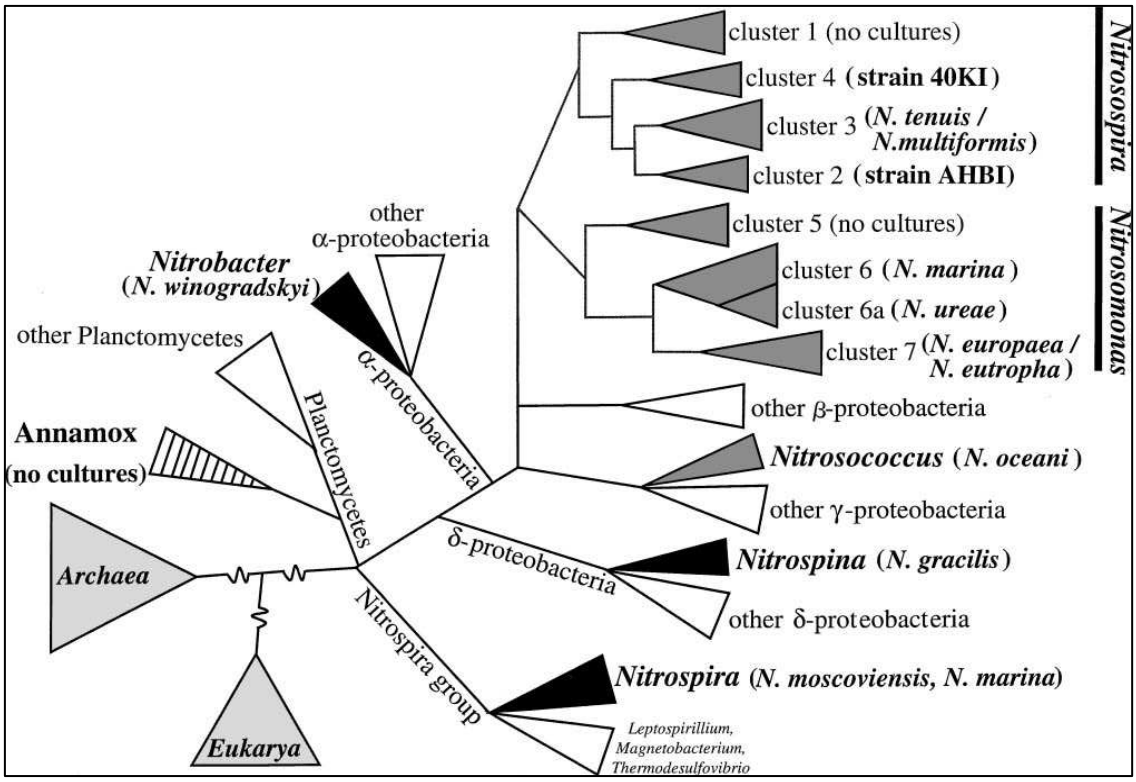

Figure 1.1. Phylogeny of autotrophic nitrifiers. Schematic representation (not to scale) of the known autotrophic nitrifiers based on *16S rRNA* gene sequences. Aerobic ammonia oxidizers are shown in dark gray, nitrite oxidizers in black, and anaerobic ammonia oxidizers are striped. From Kowalchuk and Stephen, 2001.

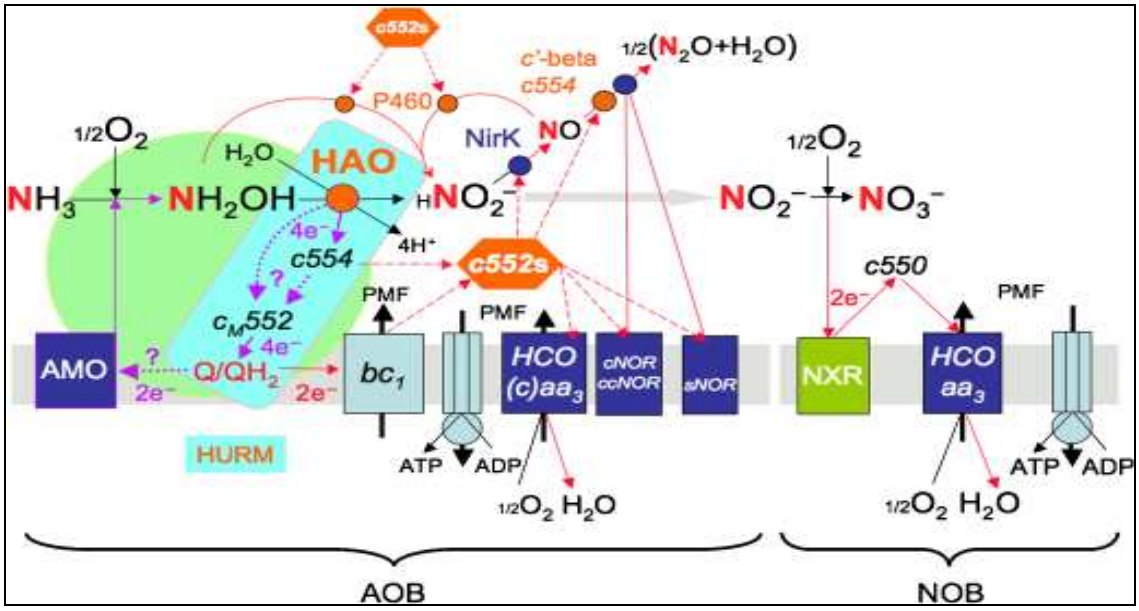

Figure 1.2. Flow of energy and reductant in the nitrification process through pertinent catabolic modules in ammonia-oxidizing (AOB) and nitrite-oxidizing (NOB) bacteria. From Koltz *et al*., 2007.

membrane-bound, multisubunit, copper-containing enzyme which has not been functionally isolated yet (Kowalchuk and Stephen, 2001). AMO genes and the accompanying cytochromes have been most intensively studied in *N. europaea*, which has multiple copies of these primary nitrification genes. The AmoA protein is a 31.8 kDa (McTavish *et al*., 1993), probably containing the active site of AMO (Hyman and Arp, 1992), and consists of five transmembrane sequences and one periplasmic loop. In the same *amoCAB* operon, a second gene *amoB* is located adjacent to *amoA*. whose protein has a molecular weight of 43 kDa (Bergmann and Hooper, 1994), and is characterized by two transmembrane domains and two periplasmic loops (Vanelli *et al*., 1996). Upstream of the genes *amoA* and *amoB*, a third open reading frame *amoC* is located which might encode a chaperone helping the AmoA and AmoB protein subunits to integrate into the membrane properly (Klotz *et al*., 1997). *N. europaea* has a duplicated *amo* operon containing a continuous arrangement of the genes *amoC*, *amoA* and *amoB*, which are cotranscribed as a 3.5-kb mRNA (McTavish *et al*., 1993).

In this reaction, two electrons are needed for the reduction of one of the atoms of O_2 to H_2O, whose source is the further oxidation of the hydroxylamine by the following reaction, catalized by hydroxylamine oxidoreductase (HAO), a periplasm-associated enzyme:

$$NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^- \text{ (Fig. 1.2).}$$

The other two electrons produced are passed via an electron transport chain to the terminal oxidase, thereby generating a proton motive force.

The NO_2^- produced is further used by nitrite-oxidizers in a one-step reaction to NO_3^-, catalized by nitrite oxidoreductase (Bock *et al*., 1986).

Ammonia-oxidizing bacteria thrive in environments where ammonia is often present in very low concentrations. In these habitats, the capability to efficiently make use of temporal flushes of ammonia might represent an important selective advantage for an ammonia oxidizer.

1.5.1. Ammonia-oxidizing archaea (AOA)

Until recently, ammonia oxidation was considered to be performed largely by autotrophic AOB; metagenomic studies have now revealed that members of the kingdom *Crenarchaeota* within the domain of the *Archaea* contain and express genes related to those of bacterial ammonia monooxygenases (Schleper *et al.,* 2005). Up to now, AOA appear not to form a monophyletic clade but rather to belong to different lineages within the *Crenarchaeota. Crenarchaeota* are distributed globally and constitute a considerable proportion of prokaryotic biomass in mesophilic marine and terrestrial environments. Phylogenetic analyses revealed distinct lineages within the non-thermophilic *Crenarchaeota* that reflect some level of ecological differentiation (Fig. 1.3). Most of the planktonic marine sequences are placed within the group 1.1a lineage. This group was estimated to represent about 20% of all planktonic prokaryotes in different oceans and to dominate specifically in deeper regions (Karner *et al.,* 2001; Herndl *et al.,* 2005). In most soil samples analysed, group 1.1b dominate (Ochsenreiter *et al.,* 2003) and represent about 1–5% of prokaryotic 16S rRNA genes, hybridised cells (Sandaa *et al.,* 1999) or extracted rRNA (Buckley *et al.,* 1998). The two abundant groups of soil and marine *Crenarchaeota* share 80% 16S rRNA gene identity. Within these two groups, genes that potentially encode ammonia monooxygenase, a key enzyme in nitrification, have recently been discovered (Schleper *et al.,* 2005).

Phylogenetic analysis of both bacterial and archaeal *amoA–pmoA* shows that crenarchaeal genes are comparatively distant to their bacterial homologues. No significant homology is apparent at the DNA level between AOA and AOB *amo*-like sequences, whereas a substantial amount of sequence identity exists between all *amo–pmo* sequences from Proteobacteria. However, about 25% sequence identity and 40% sequence similarity can be found at the protein level between archaeal and bacterial variants with conserved amino acid residues that coordinate potential metal centres. This indicates that these enzymes share an evolutionary history and belong to the same protein family (Treusch *et al*., 2004).

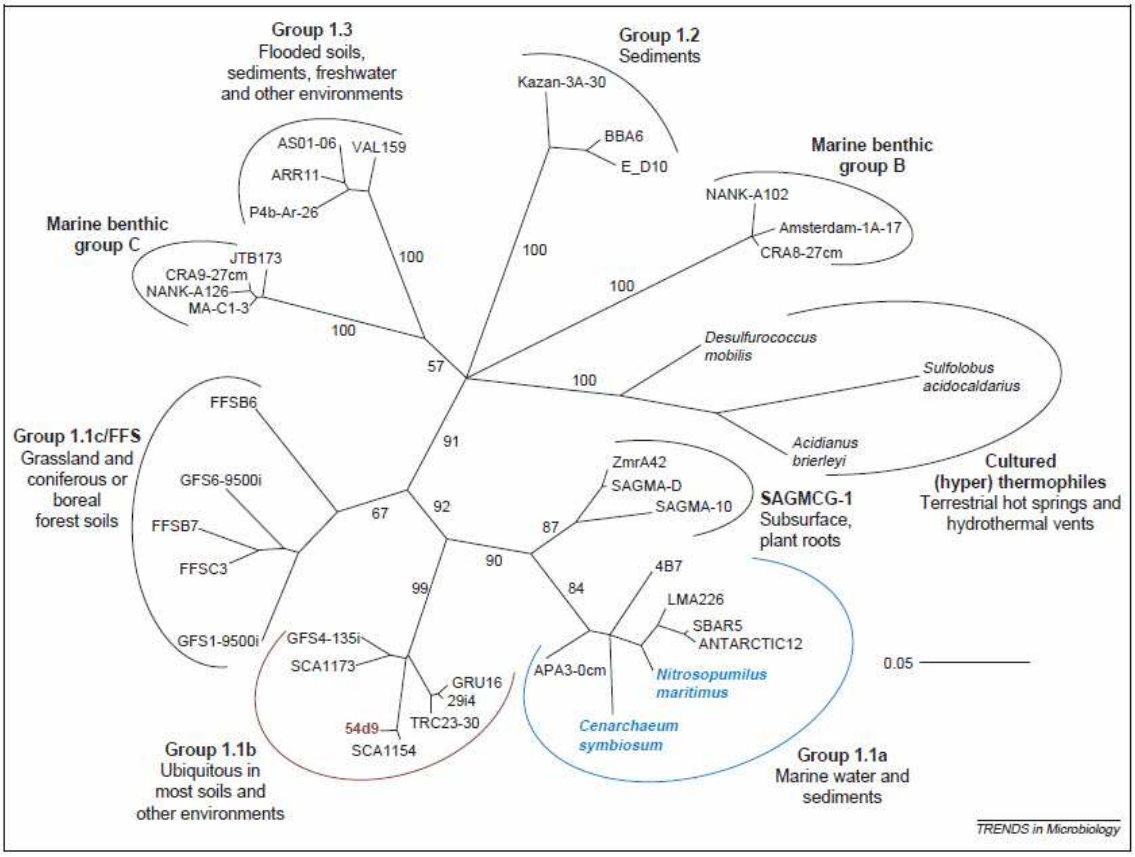

Fig. 1.3. Phylogenetic tree describing major non-thermophilic and cultivated (hyper) thermophilic lineages within the kingdom *Crenarchaeota*. (Nicol and Schleper, 2006).

1.6. Denitrification

Denitrification is the main biological process by which the N cycle is completed and fixed N is returned to the atmosphere through the microbial anaerobic reduction of NO_3^- to N_2 in optimal conditions. However, other intermediates such as N_2O and NO can be released to the atmosphere, contributing to the greenhouse effect (Lashof and Ahuja, 1990) and the destruction of the ozone layer (Waibel *et al*., 1999).

The reactions are carried out by denitrifiers, which are widely distributed across the bacterial taxa, including *Pseudomonas, Bacillus, Thiobacillus, Propionibacterium* and others (Firestone, 1982). These predominantly heterotrophic microorganisms are facultative anaerobes that are able to use NO_3^- in place of oxygen as an electron acceptor in respiration to cope with low-oxygen or anaerobic conditions. Bacteria capable of denitrification are frequently isolated from soil, sediment and aquatic

environments (Gamble *et al.*, 1977). These studies demonstrated that this alternative respiratory process is widespread among microrganisms belonging to phylogenetically distinct groups of bacteria and archaea (Cheneby *et al*., 2000).

Metalloenzymes catalysing the reactions are nitrate reductase, nitrite reductase, nitric oxide reductase and nitous oxide reductase (Fig. 1.4) (Hochstein and Tomlinson, 1988) endoded by *nar*, *nir*, *nor* and *nos* genes, respectively. These enzymes are usually induced sequentially under anaerobic conditions. In contrast to nitrification, N_2O is a regular intermediate of denitrification.

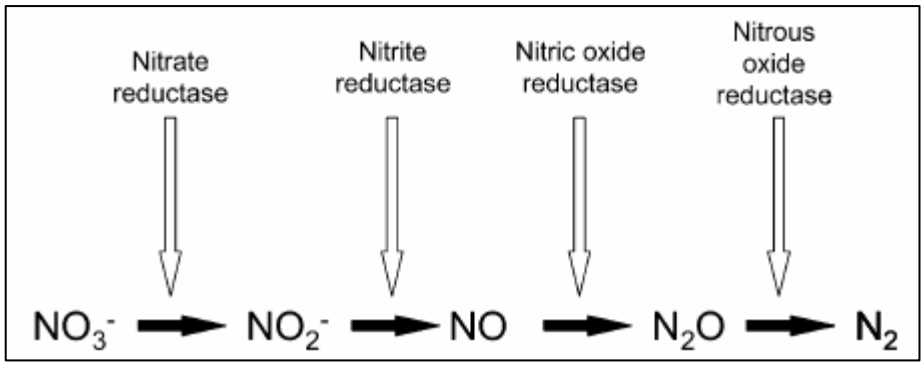

Figure 1.4. Denitrification: outline of the pathway and enzymes involved. From Hochstein and Tomlinson, 1988.

The portion of intermediate N_2O that is released is higher if the pH is low, as N_2O reductase is inhibited at low pH (Knowles, 1982). The ratio N_2O/N_2 also rises if NO_3^- is abundant in the soil because the latter is preferred to N_2O as electron acceptor (Schlegel, 1992). At low O_2 concentrations N_2O reductase is inhibited more strongly than other reductases involved in the pathway, so this ratio increases as well (Knowles, 1982). At high O_2 concentrations the aerobic metabolism of denitrifiers is promoted so that NO_3^- doesn't take place.

Many groups of heterotrophic soil bacteria are known to contain genes required for denitrification although one third of sequenced denitrifier genomes lack the gene for nitrous oxide reductase (*nosZ*) responsible for the final step that results in the release of N_2 rather than N_2O (Jones *et al.*, 2008) and the relative abundance of the gene is reported to be a predictor of the ratio of the two gasses emitted (Philippot *et al.*, 2009).

Indeed denitrifiers are more numerous than any other functional groups involved in the N cycle, reported to comprise up to 5% of all soil bacteria (Philippot *et al*., 2007). The two alternative genes for nitrite reductase, *nirK* and *nirS*, may be present in different but closely-related bacterial species, although they appear incompatible in the same individual (Zumft, 1997).

1.7. Nitrogen uptake by plants

In both fertilized and unfertilized soils NH_4^+ and NO_3^- are the only major ionic forms of N actively absorbed by plant roots (Haynes and Goh, 1978). In soil, plant roots compete with different types of microbes for available N, particularly in a N-limited ecosystem (Neumann G, and Römheld, 2000), but this competition is a short-term phenomenon (Jackson *et al*., 1989). Soil microbes uptake substantially more NH_4^+ and NO_3^- than plants, and the rate of uptake for NH_4^+ is much higher than NO_3^- (Herrmann *et al*., 2005). Microbes are better competitors for NH_4^+ and NO_3^- uptake than plants, so microbial uptake is a major factor controlling NO_3^- availability to plants; plants however compete better for NO_3^- than for NH_4^+ (Jackson *et al*., 1989). Plant uptake of NH_4^+ and NO_3^- is a function of their concentration in soil solution, root distribution, soil water content and plant growth rate. High NH_4^+ concentration is toxic to plants (Schjoerring *et al*. 2002), therefore, after its uptake it is assimilated into amino acids, or translocated elsewhere. Many investigations indicate that NH_4^+ as a sole N form, or as the main form in combination with NO_3^- has inhibitory effects on plant growth (Gerendas *et al*., 1997; Zhang and Rengel, 1999); NO_3^- can return this inhibitory effect of NH_4^+ on leaf growth (Walch-Liu *et al*., 2000). Nitrate on the other hand, after uptake can be reduced and assimilated in root or shoot by nitrate reductase enzyme which is synthesised in response to NO_3^- uptake. Assimilation of NO_3^- by plants involves the reduction of NO_3^- to NO_2^- , and reduction of NO_2^- to NH_4^+ by nitrite reductase and it's therefore an energy-consuming process.

1.8. Nitrogen balance in soil-plant ecosystem

Repeated cycles of fast growing SRF would eventually require regular N additions to maintain productivity; however, such treatments have potential for contamination of water by NO_3^- leaching or by surface run-off of N fertilizer, and air by NH_3 volatilization or by the release of N oxides through denitrification, and this risk is higher in soils with high nitrification rates (Börjesson, 1999). Under ideal cultivation systems the utilization rate of applied N fertilizer is only 50-70%, and in most of cases more than 50% of N applied enters into air or ground water (Velthof *et al.*, 1998). Optimal use of an organic N fertiliser for soil should be based on the slow release of the available N so as to significantly reduce N loss and guarantee the crop with an adequate N supply throughout the entire vegetative cycle (Benedetti, 1983).

Most of the applied N-fertilizer to soil in the form of NH_4^+ is usually oxidized quite rapidly to NO_3^- through nitrification process by ammonia-oxidizing microorganisms (Subbarao *et al.*, 2006). The application of inorganic and organic fertilizer to land is a key factor controlling the amount of N leached: there is no significant adsorption of NO_3^- onto soil surface, since nitrate is negatively charged and generally not held by the soil colloids, and there are no common insoluble nitrates (Wild, 1988); thus, if sufficient water is added, NO_3^- can reach streams and rivers. Nutrients enrichment of surface waters can lead of eutrophication, causing decreased biodiversity, changes in species composition and dominance, and having implications for drinking-water supplies affected by toxic algal growth (Collingwood, 1977). Many factors, such as fertilization, soil texture, land use, crop rotation and cultivations, can have a strong effect on the quantity of NO_3^- leached from a soil.

Nitrate plays important role in both nitrification and denitrification processes. Nitrous oxide, generated from incomplete reduction of NO_3^-, is one of the most contributors to the greenhouse effect and is also considered to be a partial cause of the depletion of the Earth's stratospheric ozone layer.

Agricultural land is a significant source of emissions of N_2O. Within the European Union, agriculture has been estimated to contribute 63% of N_2O emissions (Duchateau and Vidal, 2003), which derives directly or indirectly from the turnover of mineral fertilizers and field-applied animal manure, and from the decomposition of crop

residues (Kroeze *et al*., 1999).. Soil structure and water content are the most important factors affecting the balance between escape of N_2O and its further reduction to N_2. Denitrification rates are correlated to some extent with concentrations of NO_3^- in the soil: where fertilizers cointaining NO_3^- are applied, much of the loss through denitrification occurs in the period immediately after the application.

Nitrogen can be significantly lost from agricultural soils by the release of NH_3 into the atmosphere, whose predominant source is the urea in the faeces and urine and effluents of livestock, both voided directly onto the land by grazing animals or spread as slurry or effluent. The ammonia lost to the atmosphere is a major contributor to acid deposition (Melillo *et al*., 1989). When NH_3 is applied to the soil, it is rapidly hydrolized by the urease enzyme to ammonium and bicarbonate ions, which tend to raise the soil pH near the surface and promote the NH_3 volatilization. The amounts of the loss are influenced by the amount of urea or organic N applied and its rate of hydrolysis, the temperature, the initial pH, the buffer capacity of the soil, the soil moisture level and the depth of application.
Since both denitrification and leaching losses are of major concern when N is present as NO_3^- and when soils are excessively wet, N management programs should be designed to minimize the amount of NO_3^--N present during those period of the year when the probability of excess soil water is high. Factors to consider in designing a N-management program include timing of N application, source of N, soil temperatures, soil type, soil moisture, and time of N need for particular crop.

During the last decades attention has focused on N-prevention measures as a result of the EC Nitrate Directive (91/676), which forms part of a comprehensive framework of EU legislation to protect the environment. The Directive recognizes that excessive use of both inorganic and organic fertilizers could constitute an environmental risk, and it aims to design areas of land sensitive to water pollution from N compounds. The key changes to farming practices include an accurate determination of crop N requirements, the application of N fertilizers when crop uptake of the supplied N is maximised, splitting applications of fertilizers rather than one or two major dressings and encouraging the use of slow-release N fertilizers.

Consequently to the Nitrate Directive, a *Code of Good Agricultural Practice* was published, which is a practical guide to help farmers, growers and land managers protect the environment in which they operate, and it is primarily concerned with instigating voluntary controls on the timing, amount and application method associated with the utilization of organic wastes on agricultural land. Five stages are identified in this Code which should be followed prior to the application of organic fertilizer to land: identification to areas of land on which organic fertilizer should not be spread at any time; undertaking measures to match the land area available for spreading to the nitrogen load of the waste; estimating the risk of pollution from spreading; choosing the size of storage facilities to contain organic wastes at times when spreading is not feasible, and provisions for the design and building of storage facilities for organic waste.

More recently, the " IPPC Directive" (2008/1/EC) was issued, whose purpose is to achieve integrated prevention and control of pollution arising from new or existing industrial and agricultural activities with a high pollution potential, including waste management, livestock farming, energy and chemical industry.

The main N losses in soil through nitrification, leaching and denitrification involve NO_3^- as central point, so limiting NO_3^- in the soil is a potential tool to restrict N leaching, NO_x emissions and even NH_3 volatilization from soils in one hand, and increasing N use efficiency on the other hand, obtaining both environmental and economic benefits.

1.9. Nitrification inhibitors

Nitrification inhibitors (NI) are natural or synthetic compounds that delay the microbial oxidation of NH_4^+ to NO_2^-, the first step of nitrification process, for a certain period of time. Despite huge works and great interest in NI only a few compounds are allowed in agriculture, as their development, production and usage are quite expensive. High efficiency at lowest possible concentration and minimum side effect is very important in selection of a NI. It needs to persist in soil for longer time, and resist against being washed out from the soil profile, and be environmental friendly (McCarty and Bremner 1989).

By far Nitrapyrin, Dicyandiamide (DCD) and 3,4-Dimethyl pyrazole phosphate (DMPP) are three major commercial NIs (Subbarao *et al*., 2006). The first two molecules have some disadvantages: DCD is very soluble in water, resulting in spatial separation of NI and NH_4^+, and for large scale is much expensive, as well as its efficiency is not high. In addition, it may cause phytotoxic problems to plants under particular conditions (Reeves and Touchton, 1986). Nitrapyrin is adsorbed to soil minerals more strongly than NH_4^+ and this causes spatial separation to NH_4^+, too. Moreover, this compound is explosive, corrosive and can cause phytotoxicity (Sahrawat *et al*., 1987).

The effects of NIs, depend on soil conditions, are too complicated and are most likely to be greater on soils rich in N with high risk of leaching and denitrification. Inhibitory effect of NIs and related plant growth is largely depends on soil N status. Meanwhile, efficiency of these compounds largely depends on soil N status, soil physiochemical factors (texture, temperature, moisture, organic matter, pH) and climatic factors (temperature, rainfall intensity and frequency) which on one side, determine the size of these losses, and on the other side, influence dynamics of NIs in the soil (Adair and Schwartz 2008).

NIs were first introduced into the commercial market in 1976. At that time, the major emphasis was aimed at the market for fall-applied N on corn (Zea mays L.) since that time, both research and farmer experience have shown that the products could be effectively used on other crops, and that they could be effectively used with spring and to some extent side-dress applications for corn.

Their introduction to the market place raised several questions:

1) Do the compounds reduce the rate of nitrification?
2) How long will they be effective in soil?
3) What N fertilizers can be used with the inhibitors?
4) Will the inhibitors maintain N in the NH_4^+ form too long?
5) Will the use of the inhibitors produce economical benefit?

The nearer N is applied to the time of crop need, the lower the potential for N loss. Therefore, the nearer N is applied to time of crop need, the lower the potential for benefit from NIs.

1.10. Modes of action of nitrification inhibitors

Considerable effort has been devoted towards understanding the biochemistry of autotrophic oxidation of NH_3 and the modes of action for specific inhibitors of this process. The ammonia monooxygenase is a nonspecific enzyme; ammonia oxidizers are capable of co-oxidizing a range of hydrocarbons (includine methane and even xenobiotics). The broad substrate range of AMO also is responsible for inhibition of ammonia oxidizers by a variety of substances. Over 40 compounds have been shown to be substrates of AMO which can competitively inhibit NH_3 oxidation. Alternative substrates can influence AMO activity by three distinct mechanisms:

1) direct binding and interaction with AMO;
2) interference with the supply of reductant needed for monooxygenase activity;
3) the oxidation of substrates to give products that are highly reactive and inactivate AMO and/or other enzymes (Keener and Arp 1993).

Mechanism-based inhibitors of an enzyme can be defined as compounds that inhibit as a result of the normal catalytic cycle of the enzyme, producing an inhibitory product from the compound. Usually this mode of inhibition involves irreversible inactivation of the enzyme through its covalent modification by the product of catalysis. With mechanism-based inhibition causing covalent modification of protein(s) in the organism, the re-establishment of AMO activity in a population of nitrifying microorganisms after the loss of an inhibitor requires de novo synthesis of protein. The rate of this recovery depends on the extent of damage to the metabolic machinery of the organism. The degree to which cellular components other than AMO become covalently modified is a function of the stability of the intermediate reactive compound formed during oxidation.

1.10.1. S compounds

A broad range of S-containing compounds inhibit nitrification, including thiosulfates, thiocarbamates, xanthates, S-containing amino acids, and several pesticides and fungicides. Specific compounds include: carbon disulfide (CS_2), thiourea, allylthiourea, guanylthiourea, 2-mercaptobenzothiazole, 3-mercapto-1,2,4-triazole, thioacetamide, sodium diethyldithiocarbamate, sodium thiocarbanate, thiosemicarbazide,

diphenylthiocarbazone, dithiocarbamate, *s*-ethylidipropylthiocarbamate, ethylene-*bis*-dithiocarbamate, and *N*-methyldithiocarbamate (Hauck 1980). The general ability of C=S compounds to complex Cu would inhibit the catalytic cycle of AMO required for mechanism-based inhibition.

1.10.2. Acetylenic compounds

C_2H_2 was first found to inhibit NH_3 oxidation in pure cultures of *N. europaea* (Hynes and Knowles 1978) and was then established as a potent inhibitor of nitrification in soil (Bremner and Blackmer 1979). Studies of the inhibitory effects of acetylene on AMO activity involving the use of ^{14}C-labeled acetilene demonstrated the covalent modification of a single polypeptide with an apparent molecular weight of 28 kDa (Hyman and Wood 1985), therefore provided strong evidence that the modified protein was directly involved in the catalysis of NH_3 oxidation with the formation of a highly reactive intermediate by the cooxidation of C_2H_2.

1.10.3. Heterocyclic compounds

Several strong inhibitors of NH_3 oxidation in soil can be classified by their heterocyclic ring structures. This classification includes some of the most potent inhibitors of nitrification in soil, namely nitrapyrin, etridiazole, 2-ethynylpyridine, 4-amino-1,2,4-triazole, and 3-methylpyrazole-1-carboxamide. Current understanding of the mode of action of these compounds is generally limited, and study of their activity has largely been performed in soil systems (Mc Carthy, 1999). Compounds containing two or three adjacent ring N atoms (pyrazole, 1,2,4-triazole, pyridazine, benzotriazole, and indazole) significantly inhibit nitrification in soil, whereas those containing two or three non-adjacent ring N atoms (pyrimidine, imidazole, *s*-triazine, benzimidazole) or only one ring N atom (pyrrole, pyridine, indole) have little or no effect on NH_3 oxidation (McCarty and Bremner 1989). Also several substituted pyrazoles, 1,2,4 triazoles, pyridines, and thiadiazoles inhibit nitrification in soil (McCarty and Bremner 1989). Several of the heterocyclic N compounds found to inhibit NH_3 oxidation are structurally similar in that they contain Cl and/or trichloromethyl (CCl_3) groups substituted on C

atoms adjacent to a ring N (e.g., nitrapyrin, etridiazole, 2-chloropyridine, 2,6-dichloropyridine, 6-chloro-2-picoline, and 3,4-dichloro-1,3,4-thiadiazole). Nitrapyrin has been found to be a substrate of AMO and apparently a weak mechanism-based inhibitor of the enzyme, with largely indiscriminate binding of the product to membrane proteins. The product of nitrapyrin oxidation by AMO is 6-chloropicolinic acid (Vannelli and Hooper 1992).

1.11. 3,4-Dimethylpyrazole phosphate (DMPP)

3,4-Dimethylpyrazole phosphate (DMPP) belongs to the heterocyclic NI class, highly specific in inhibiting nitrification at low concentrations of 0.5–1.0 kg active compound ha^{-1} over a period of 4–10 weeks and typically applied at 1% of total N (Zerulla *et al*., 2001). Compared to other NI such as DCD, and N-Serve, it has no toxicity or other side effects, but instead, it offer potential advantages for cropping systems (Zerulla *et al*., 2001; Barth *et al*., 2001).

The duration of action depends on climatic conditions (Pasda *et al*. 2001), site characteristics (Barth *et al.* 2001; Pasda *et al*. 2001) and probably the cultivated crop. The period of time over which nitrification inhibitors are effective strongly depends on soil temperature; the effect of temperature on the efficacy of DMPP has been investigated in an incubation study (Irigoyen *et al.,* 2003). At 10 °C addition of DMPP stabilized the NH_4^+ content in soil over a period of more than 100 days. At 20 °C and even more at 30 ◦C NH_4^+ degradation markedly accelerated with half-lives of NH_4^+-N of 18 and 8 days, respectively.

In short-term incubation experiments, decreasing sand content and pH value and increasing catalase activity reduced the efficacy of DMPP to retard the NH_4^+ oxidation (Barth *et al.,* 2001). It was shown that the adsorption of DMPP to inorganic soil constituents was a crucial factor under these conditions.

The inhibitor concentration plays an important role particularly when DMPP and NH_4^+ fertilizers are applied to the soil as a liquid, *i.e.*, as fertigation or in combination with liquid organic fertilizers or in solid form as fertilizer granules. The application as a liquid may lead to a more uniform distribution of NH_4^+ and DMPP within the soil

compared to an application as fertilizer granules, where it can be supposed that fertilizer and inhibitor only react within a smaller area in the vicinity of the granule.

1.12. Objectives of the research

Organic and inorganic fertilizers have had a significant impact on food production in the recent past and today are an indispensable part of modern agriculture. They guarantee the production of food for a steadily growing population. However, the external costs of environmental degradation and human health pose a major limitation to their excessive use and urge for careful designing of its application.

Input efficiency of N fertilizer is one of the lowest and, in turn, contributes substantially to environmental pollution. Nitrogen equivalent to 30-50% of the fertilizer N applied may be lost to the atmosphere through denitrification, while up to 30% may be lost through leaching (Frissel and Van Veen, 1982; Jenkinson, 1982). Nitrate in ground and surface water and the threat to the stability of ozone layer from gaseous oxides of nitrogen are major health and environmental concerns. These environmental considerations dictate that biological alternatives, which can augment and in some cases replace, N fertilizers, must be exploited. Long-term sustainability of agricultural systems relies on effective management of internal resources. Great benefits can be gained through sustainable agroforestry and waste recycling. Application of nitrification inhibitors could inhibit this rapid nitrification and thus potential wastes N losses via NO_3^- leaching and N_2O emissions.

Thus, the main goal of this PhD project work was to evaluate and to establish the effectiveness of the nitrification inhibitor DMPP on a Mediterranean agroforestry system combined with a cattle effluent, and to study its effects on soil microbiota involved in N cycling processes. Firstly, a long-term incubation experiment was conducted to evaluate the influence of fertilizer (mineral or organic), temperature (30°C and 20°C), soil microbiological properties (using two soils that differ mainly in C and N content and in microbial activity and biomass) and timing of application of the inhibitor (if concomitant or prior to fertilizer addition) on the effectiveness of DMPP as nitrification inhibitor. Furthermore, experimental microcosms were set up to evaluate the effect of the addition of the nitrification inhibitor DMPP combined with a cattle

effluent as organic fertilizer on culturable heterotrophic microbial metabolism, as well as on the abundance and expression both of target (ammonia oxidizers and denitrifiers populations), and non-target microbial populations. Finally, nitrogen cycle dynamics were approached in a Mediterranean agroforestry system managed with zootechnical wastes and the nitrification inhibitor DMPP.

1.13. References

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Chapter 2 – Effectiveness of 3,4-dimethylpyrazole phosphate (DMPP) as nitrification inhibitor in soil as influenced by fertilizer, temperature, soil microbiological properties and timing of application

2.1. Abstract

The element nitrogen (N) is an essential nutrient for plant development and its intake is a key factor for crop production; however, its real availability in soil, as well as its chemical form, are crucial for the N uptake by crops. The main N losses in soil driven by microbial processes like nitrification, leaching and denitrification involve NO_3^- as key point, so limiting NO_3^- in the soil is a potential tool to restrict N leaching, NO_x emissions and even NH_3 volatilization from soils in one hand, and increasing N use efficiency on the other hand, obtaining both environmental and economic benefits (Jenkinson, 1982). The application of nitrification inhibitors to soil offers the chance to reduce N losses and to increase fertilizer use efficiency. The present study therefore aimed to evaluate the influence of fertilizer, temperature and soil biological properties on the effectiveness of DMPP in a long-term incubation experiment.

The application of DMPP combined with ammonium sulphate and a cattle effluent (AS, AS+D, E, E+D) as inorganic and organic N fertilizer, respectively, to a low biological fertility soil (Casalotti soil) resulted in a reduction of the nitrification rate starting from the second week of incubation until at least the fourth week, while the inhibition occurred immediately in the higher biological fertility soil (Celimontano soil) but after four weeks no difference were detected. Temperature had little or no effects on N mineralization trends during the first 28 days of incubation. The inhibitory effect started immediately after 7 days and ended by the fourth week of incubation in Celimontano soil, indicating a strong influence of soil biological properties on N mineralization. The simultaneous application of DMPP and fertilizer resulted to be the more efficient option when compared to the application of DMPP prior to mineral N addition, since in the latter case more N was mineralized in both soils at the end of the incubation period.

Keywords: nitrification inhibitor; DMPP; cattle effluent; N mineralization; N use efficiency

2.2. Introduction

Nitrification is the process in which a relatively immobile cationic N form (NH_4^+) is converted into a more mobile anionic one (NO_3^-). This conversion has important implications for physical, chemical and biological soil functioning; hence, it may also affect ecosystem productivity.

Ammonium fertilizers after application to the field are oxidized to nitrate within few days (Abbasi and Adams, 2000; Azam *et al*., 2002). Under ideal cultivation systems the utilization rate of applied N fertilizer is only 50–70%, and in most of cases more than 50% of those fertilizers enters into air or ground water (Velthof *et al*., 1998; Ishikawa *et al*., 2003).

Delaying this oxidation in order to have ammonium rather than nitrate in soil is achieved through application of synthetic nitrification inhibitors such as N-Serve, Dicyandiamide (DCD) and 3,4-Dimethylpyrazole phosphate (DMPP). To large extent, the efficiency of these compounds depends on their chemical structure, mode of action, dynamics inside the soil, and their resistance to biological and chemical degradation. Because of simultaneous absorption of ammonium and DMPP to clay minerals (Pasda *et al*., 2001; Azam *et al*., 2002), the difference between kinetics of adsorption for NH_4^+ and DMPP regulates the effectiveness of inhibition. Therefore, it has the highest efficiency in light soils (Barth *et al*., 2001; Pasda *et al*., 2001).

DMPP is one of the most effective and widely used nitrification inhibitor with high efficiency and no side effect (Weiske *et al*., 2001; Pasda *et al*., 2001; Irigoyen *et al*., 2003). It is not as liable to loss through leaching (Fettweis *et al*. 2001) and is effective at much smaller doses (Weiske *et al*. 2001) and, therefore unlikely to have damaging effects on the crop plant. DMPP has been evaluated under field (Merino *et al*., 2005; Li *et al*., 2008; Chen *et al*., 2010) and laboratory (Hatch *et al*., 2005; Barth *et al*., 2008; Pereira *et al*., 2010) conditions, mixed with both inorganic (Weiske *et al*. 2001; Linzmeier *et al*., 2001) or organic (Dittert *et al*. 2001; Macadam *et al*., 2003) fertilizer. It has the potential to reduce losses of N_2O (Weiske *et al*., 2001; Zerulla *et al*., 2001; Hatch *et al*., 2005) even if added to slurry (Dittert *et al*. 2001), and to increase yield (Zerulla *et al*., 2001; Pasda *et al*., 2001; Linzmeier *et al*., 2001). However, only recently attention has been paid to the possibility of using inhibitors with slurry (Dittert *et al*. 2001; Macadam *et al*. 2003), especially in Mediterranean ecosystems, where

temperature more than 25-30°C normally occurs and nitrification can proceed fastly. Furthermore, to our knowledge, the application of nitrification inhibitors combined to zootechnical effluents for short rotation forestry purposes and its effects on soil N dynamics has never been studied.

Laboratory experimentation offers the possibility of better evaluation of treatment effects through closer control of environmental variables and the capability of taking measurements with high temporal resolution.

The present study therefore aimed to evaluate the effectiveness of DMPP as influenced by (i) fertilizer (mineral or organic), (ii) temperature (30°C and 20°C), (iii) soil microbiological properties (using two soils that differ mainly in C and N content and in microbial activity and biomass) and (iv) timing of application of the inhibitor (if concomitant or prior to fertilizer addition) in a long-term incubation experiment.

2.3. Materials and Methods

2.3.1 Soils and cattle effluent

The soil used (Casalotti soil) was collected from a *Eucalyptus*, short rotation, high-density plantations field managed by the research unit for intensive wood production (CRA-PLF) located in Rome (Italy). The 20 year-old experimental field with initial low-density plant spacing (1100 plants/ha) was converted in 2007 into a high-density plant spacing (5000 plants/ha). Six samples of soils from the top 30 cm were collected in June 2009 and kept in sterile plastic bags. The soils were air-dried, homogenized by sieving (2-mm mesh size), pooled and stored at room temperature. Main physico-chemical and some microbiological properties of the soil are reported in Table 2.1.

A natural top soil (Celimontano soil) located in the experimental field of the CRA-RPS research centre, Rome, (41°53'N, 12°29'E) was also used which has similar texture but differs mainly in organic C and N content and in microbiological parameters from Casalotti soil (Table 2.1).

The cattle effluent used was obtained from a dairy farm situated in Rome. Sampling was performed in June 2009 and sample was stored in PVC barrels at 4 °C until required. On day 0, it was sampled after thorough stirring and blending and analyzed by standard laboratory methods to assess the physico-chemical properties shown in Table 2.2.

Table 2.1. Main physico-chemical and microbiological characteristics of the soils used.

Parameters	Casalotti soil	Celimontano soil
Sand (%)	63	67
Silt (%)	16	22
Clay (%)	21	11
N_{tot}(%)[a]	0.06	0.15
TOC (%)[b]	1.06	2.49
pH[a]	7.5	7.9
qCO_2[c]	0.17	1.56
Cumulative C-CO_2 evolved over 28 days (mg kg^{-1} soil)[d]	282,11	856,49
Respiration measured at 28 days (mg C-CO_2 kg^{-1} soil)[d]	0,4	11,3
C_{mic} (mg-C kg^{-1} soil)[e]	95,53	307,27

[a]Analysis was performed as described in Violante (2000)

[b]TOC was determined as reported by Springer and Klee (1954)

[c]Metabolic quotient (specific respiration of the microbial biomass of the soil) calculated from basal respiration values with the formula (mg-C/kg soil)

[d]Soil respiration was determined as reported by Isermayer (1952)

[e]Microbial-C biomass was calculated as reported by Vance *et al.*, (1987).

Table 2.2. Physico-chemical properties of the cattle effluent used.

Parameters	Cattle effluent
Moisture (%)	88.9
Dry matter (%)	11.1
N_{tot} (%)	0.32
N-NH_4^+ (%)	0.17
N-NO_3^- (%)	0.0025
Density (g/cm^3)	1
Cu (ppm)	0.3
Zn (ppm)	0.2

2.3.2. Potentially mineralized N

Two kinds of fertilizer were applied: mineral as ammonium sulphate (AS) and organic as cattle effluent (E). Liquid formulation of DMPP (25%, provided by K+S Nitrogen, Italy) containing 1,86% mineral N (1,1% N-NO_3^-, 0,76% NH_4^+-N) was used with mineral (AS+D) and organic (E+D) fertilizer at a final concentration of 1% according to the NH_4^+-N content, as recommended by the providers (Zerulla *et al.*, 2001). A

treatment with no fertilizer was included as a control (C). Nitrogen (250 mg N kg soil^{-1}) from mineral or organic fertilizer and nitrification inhibitor were added as mixed solution to 50 g air-dried soil mixed with quartz sand in a 1:1 ratio. The mixture was incubated at 60% water holding capacity (pF 2.5) in the dark at 30°C or 20°C for different periods of time (Benedetti *et al*. 1994). The buchners funnel used were covered by cling film which prevented water loss but allowed gas exchange. Every 3-4 days buchners were aerated and moisture was replenished throughout the experiment duration. Nitrate-N and NH_4^+-N produced during the incubation were monitored at 1, 2, 4, 8 and 12 weeks. The soils were eluted with 900 ml 0.01 M $CaSO_4$ solution, and then with 100 ml N-free solution [0.002 M $CaSO_4$, 0.005 M $Ca(H_2PO_4)_2$, 0.0025 M K_2SO_4, 0.002 M $MgSO_4$] in order to reintegrate nutrient elements. Nitrogen forms were determined colorimetrically in the eluate by continuous flux analyser (Autoanalyser Technicon II), according to Wall *et al.* (1975) for N-NH_4^+ and Kamshake *et al*. (1967) for N-NO_3^-. Net N mineralization (the sum of NH_4^+-N, NO_2^--N, and NO_3^--N) and net nitrification (the sum of NO_2^--N and NO_3^--N) were calculated as the percentage of total mineral N or total nitrates leached, respectively, with respect to the added N. Each treatment was replicated three times.

2.3.3. Data analysis

Excel and SPSS 11 softwares were used for analysis of data, and comparisons of means were conducted using one way ANOVA and Duncan test at level of $p<0.05$. Results in the tables, text and figures are given as means ± SD.

2.4. Results and discussion

2.4.1. Effects of fertilizer

Values net mineralised N and net nitrification eluted after addition of mineral and organic fertilizer to the soil are reported in Figure 2.1 and 2.2 together with the cumulative percentages of N mineralization and nitrification.

Influence of different N sources applied as mineral or organic fertilizer to Casalotti soil on ammonium oxidation dynamics during the 12 weeks of incubation was similar from

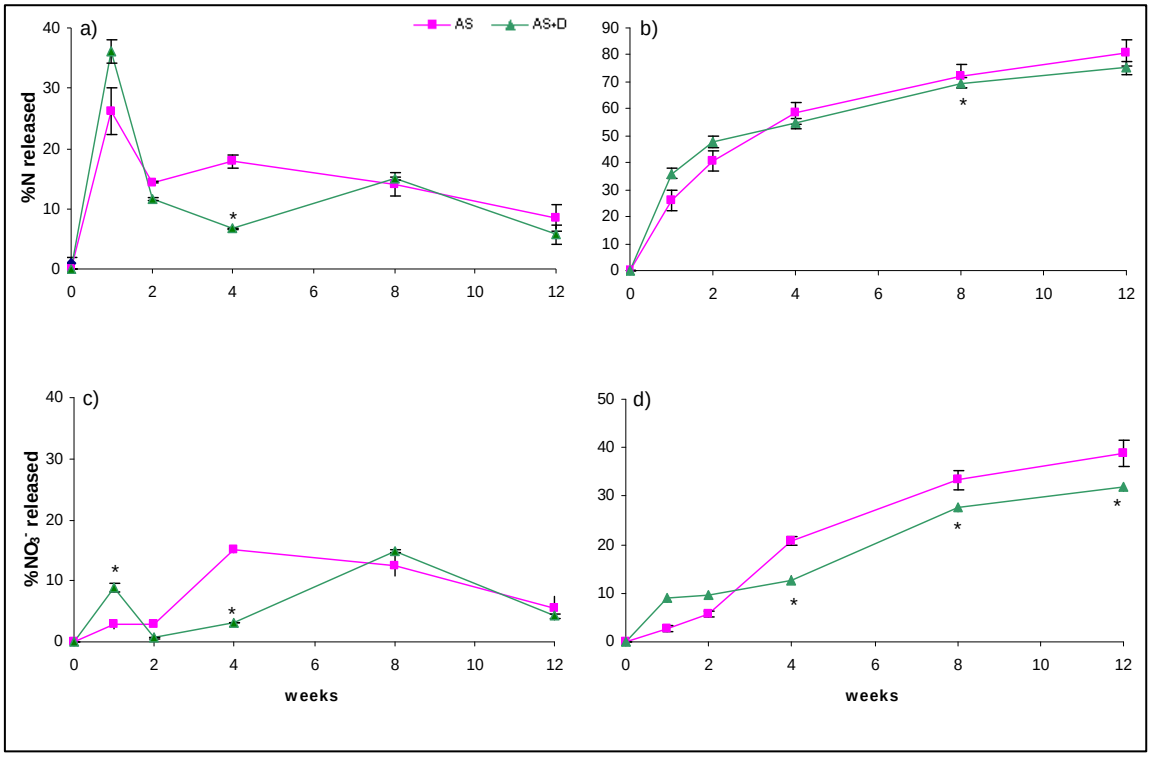

Fig. 2.1. Net N mineralization (2.1a, weekly; 2.1b, cumulative), and net nitrification (2.1c, weekly; 2.1d, cumulative) as the percentage of total N leached or nitrates leached with respect to the added N during the incubation period. Significant effects of the inhibitor are indicated by asterisks, Duncan's test, $P<0.05$. Treatments: $(NH_4^+)_2SO_4^-$ as mineral fertilizer (AS) combined or not with DMPP (AS+D).

the beginning to the end of the incubation period. At the first week of incubation more N was nitrified from both mineral and organic fertilizer combined with DMPP (AS+D, E+D) (Fig. 2.1c and 2.2c). This difference was significant ($p<0,05$) for net nitrification rate.

Unexpectedly, low N mineralized content was recorded at 7 days; in a low-biological fertility soil (Casalotti soil) the addition of mineral or organic nutrients could have stimulated an initial proliferation of microbes in the soil and thus the immobilization of N compounds in soil microbial biomass, since the nutrients provided could be insufficient to fulfil the N needs for formation of cell organic N constituents during growth of soil microbial populations (Jarvis *et al.*, 1996). With a certain content of nutrients added to the soil, the reproductive rates of the microbes were expected to increase and thus high competition for the nutrients and the subsequent immobilization occurred. For AS+D treatment, a little more N was added compared to AS treatment,

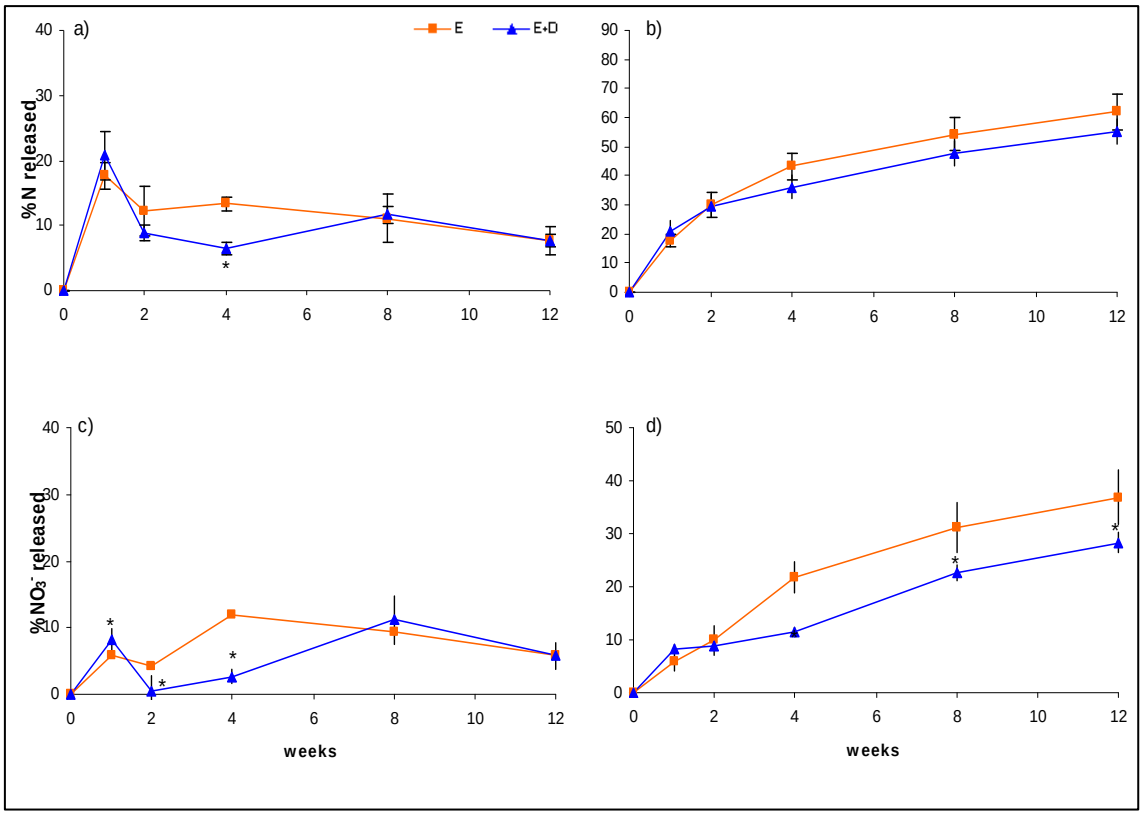

Fig. 2.2. Net N mineralization (2.2a, weekly; 2.2b, cumulative), and net nitrification (2.2c, weekly; 2.2d, cumulative) as the percentage of total N leached or nitrates leached with respect to the added N during the incubation period. Significant effects of the inhibitor are indicated by asterisks, Duncan's test, $P<0.05$. Treatments: cattle effluent as organic fertilizer (E) combined or not with DMPP (E+D).

due to a small mineral N content contained in the liquid formulation of DMPP; this was perhaps the reason for the higher amount of NO_3^--N leached observed at 7 days.

From the second week of incubation on the N mineralization and nitrification rate were lower in the treatments where DMPP was applied. At 14 days DMPP reduced net nitrification by 77,5% (AS+D) and 86,4% (E+D), and at 28 days by 79,1% (AS+D) and 77,4% (E+D). The smaller values and percentages in the "plus DMPP" treatments indicate the effectiveness of DMPP during this period.

After 8 and 12 weeks, the percentages of net mineralised N and net nitrification from each treatment were not different when compared to the control and the inhibition effect declined, suggesting that DMPP has undergone biological decomposition since it is non-volatile (Zerulla *et al.*, 2001).

In treatments where DMPP was applied, net nitrification rate was significantly lower with respect to the control starting from the fourth week until the end of the incubation period (Fig. 2.1d and 2.2d). After 12 weeks of incubation, 80,73±4,90% and 75,26±2,52% of the total N added as mineral fertilizer was mineralized (AS and AS+D, respectively), whereas 62,02±6,14 and 55,27±4,31 of N from organic fertilizer was mineralized (E and E+D, respectively). It is possible that ammonia volatilization were occurring during the incubation.

2.4.2. Effects of temperature

Net N mineralization and net nitrification dynamics after the first week of incubation at 20°C (Fig. 2.3) were similar to those at 30°C incubation, with gradually increasing N mineralization throughout the incubation period. At the fourth week of incubation nitrification rate in DMPP-contained treatment was significantly different from the control, confirming the trend occurred at 30°C incubation experiment. Nitrogen mineralization and nitrification were not measured at 20 °C after the fourth week of incubation, but it is reasonable to hypothesise that the trends were the same of those occurred at 30 °C at 8 and 12 weeks.

The effect of temperature on the efficacy of DMPP has been investigated previously. Incubation studies at constant soil temperatures have shown that at 5 °C there was practically no nitrification of the NH_4^+ from ammonium sulphate nitrate to which DMPP had been added, whereas for the control fertilizer without DMPP nitrification was completed within approximately 140 days (Zerulla *et al.*, 2001). At 20 °C, and under otherwise unchanged conditions, nitrification for ammonium sulphate nitrate was completed within 7–21 days, compared to 40 days with DMPP (Zerulla *et al.*, 2001).

Irigoyen *et al.* (2003) reported that at 10 °C addition of DMPP stabilized the NH_4^+ content in soil over a period of more than 100 days. At 20 °C and even more at 30 °C NH_4^+ degradation markedly accelerated with half-lives of NH_4^+-N of 18 and 8 days, respectively. Chen *et al.* stated that DMPP seems to be a viable nitrification inhibitor for areas experiencing soil temperatures less than 25 °C at the time of fertilizer application.

In our region, where temperatures can be slightly higher than those registered in Central

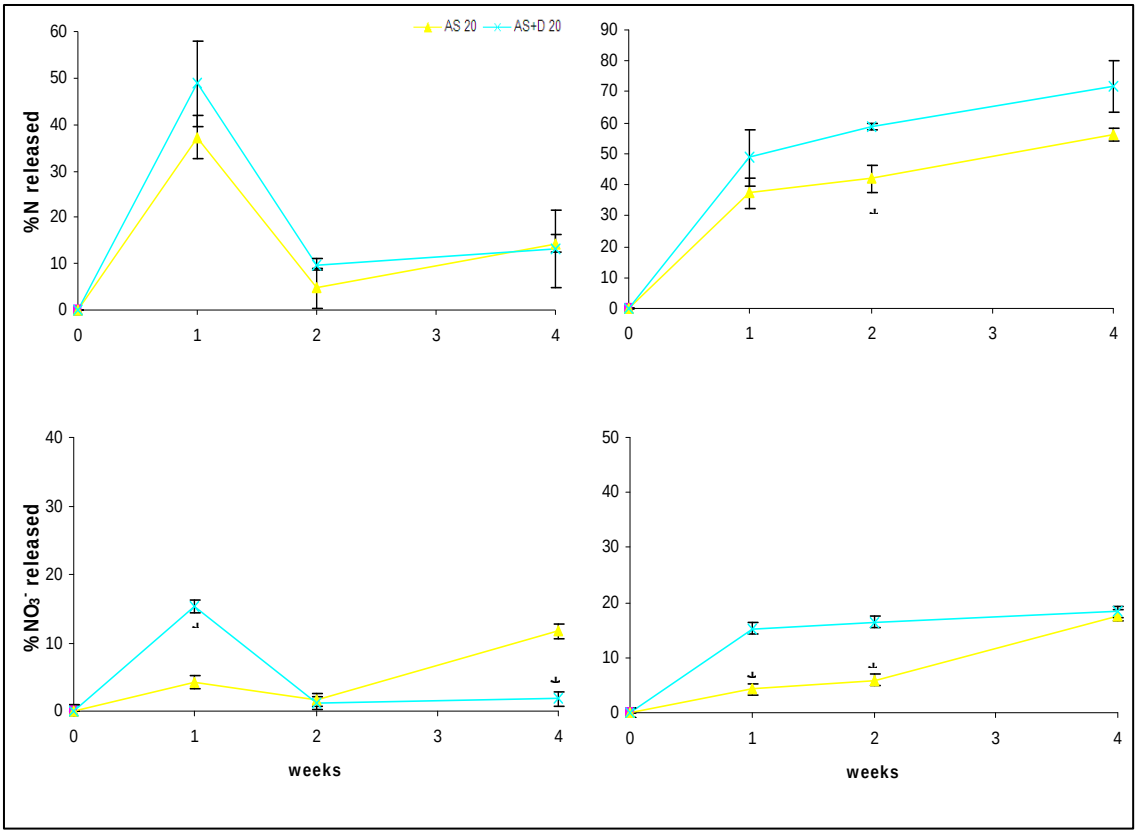

Fig. 2.3. Net N mineralization (Xa, weekly; Xb, cumulative), and net nitrification (Xc, weekly; Xd, cumulative) as the percentage of total N leached or nitrates leached with respect to the added N during the incubation period. Significant effects of the inhibitor are indicated by asterisks, Duncan's test, $P<0.05$. Treatments: 20°C incubation with $(NH_4^+)_2SO_4$ (AS 20) combined or not with DMPP (AS+D 20)

and Northern Europe, it is crucial to assess whether and to what extent warm temperatures may affect DMPP performance as a nitrification inhibitor, in order to guarantee the plant with an adequate N supply throughout the entire vegetative cycle.

2.4.3. Effects of soil microbiological properties

Values of total mineralised N and net nitrification rate of Celimontano soil after mineral N addition combined or not with DMPP are reported in Figure 2.4. After the first two weeks of incubation weekly and cumulative percentage of both net N mineralization (Fig. 2.4a, 2.4b) and nitrification (Fig 2.4c, 2.4d) were significantly reduced where DMPP was applied. After 7 and 14 days 57,5% and and 62,7% inhibition of nitrates

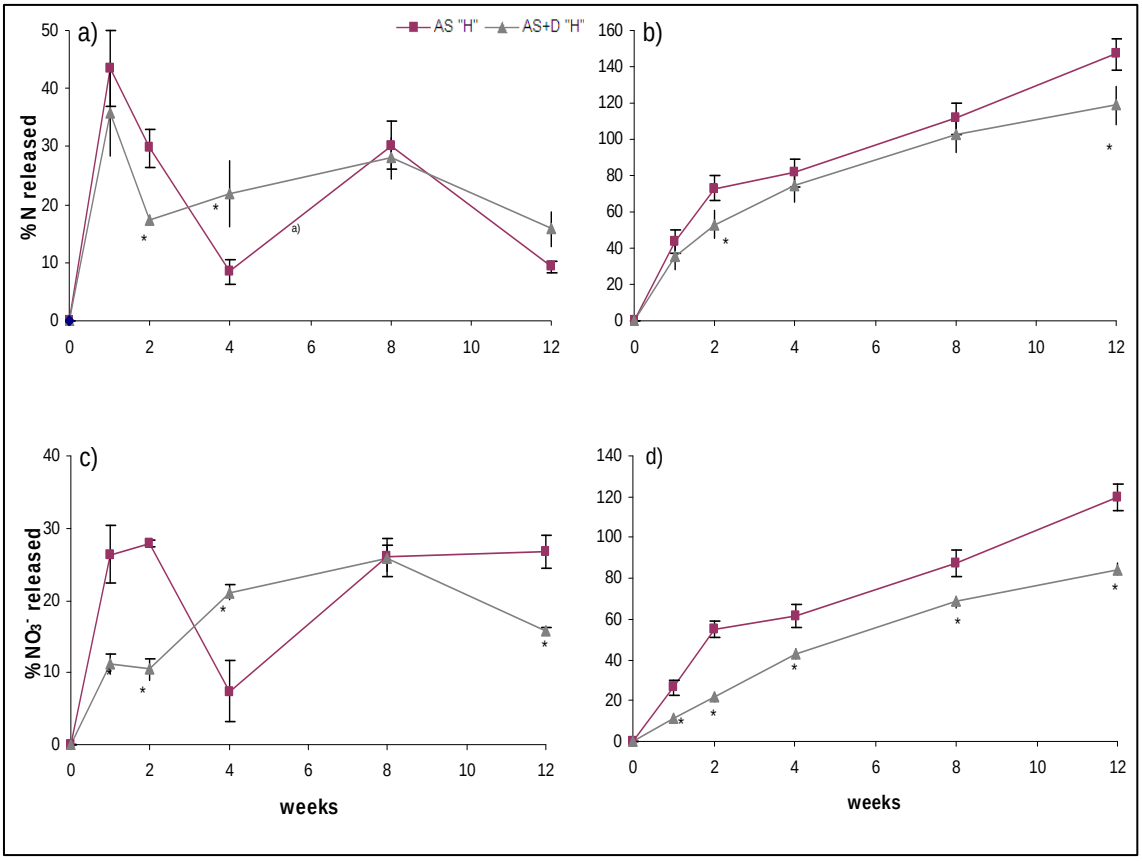

Fig. 2.4. Net N mineralization (2.4a, weekly; 2.4b, cumulative), and net nitrification (2.4c, weekly; 2.4d, cumulative) as the percentage of total N leached or nitrates leached with respect to the added N during the incubation period. Significant effects of the inhibitor are indicated by asterisks, Duncan's test, $P<0.05$. Treatments: $(NH_4^+)_2SO_4^-$ as mineral fertilizer (AS "H") combined or not with DMPP (AS+D "H") using a high biological fertility soil (Soil Celimontano).

leached was found. After the fourth week more N was released in DMPP-cointaining treatments, whereas at the eighth week of incubation no differences were observed (Fig 2.4a, 2.4c). The efficacy of DMPP as nitrification inhibitor in a laboratory study (Pereira *et al.*, 2010) was observed for 30 days, whereas Zerulla *et al.* (2001) stated that nitrification for ammonium sulphate nitrate was completed within 7-21 days, compared to 40 days with DMPP.

Net nitrification rate was significantly reduced even after 12 weeks of incubation (41,1%) (Fig. 2.4c). In long-term incubation experiments the adsorption of the active substance on soil constituents and its degradation could represent crucial factors for the efficacy of DMPP as a nitrification inhibitor. If adsorbed DMPP, as opposed to DMPP

in soil solution, is better protected against microbial degradation and is then remobilised at a sufficiently high equilibrium concentration, this may ultimately result in an extended inhibitory effect in soils with higher adsorption capacities (Barth *et al.*, 2001). This could be perhaps the reason for the inhibition of nitrification observed after 12 weeks of incubation under these conditions.

The overall nitrification process was constantly inhibited during the entire incubation period (Fig. 2.4d). Globally, higher N release occurred in Celimontano soil when compared to Casalotti soil (Fig. 2.1b) at the end of the 12 weeks.

2.4.4. Effects of timing of application of DMPP

In order to test the best technical way of application of liquid formulation of DMPP to the soil, mineral N fertilizer was added one week after the application of the DMPP both to Casalotti and Celimontano soil (Fig. 2.5-2.6) soil.

The conversion of the N in the soil to nitrate was identical after one week of incubation of Casalotti soil (corresponding to two weeks of incubation of Casalotti soil after the addition of the inhibitor) (Fig. 2.5); at 14 days a significant decrease in nitrates leached in AS+D treatment occurred. After seven weeks of incubation however no difference in net nitrification could be detected.

The pattern of total mineral N (NH_4^+-N, NO_2^--N, + NO_3^--N) mineralized is similar to that of nitrification when DMPP was applied one week before the addition of mineral N fertilizer to Celimontano soil (Fig. 2.6).

As in the concomitant application of DMPP and $(NH_4^+)_2SO_4^-$ (Fig. 4), mineral N leached at 7 days of incubation was reduced in AS+D “H” treatment (Fig. 6a) but at 21 days it increased significantly when compared to the control. At the end of the incubation there was no difference between the two treatments.

At the end of the incubation, more total mineral N was released in AS and AS+D treatment both in Casalotti (114,83±1,29 and 96,64±1,20, respectively) and Celimontano soil (149,12±0,97 and 174,71±1,13, respectively) when compared to the concomitant application of the inhibitor and the N source (80,73±4,90%/75,26±2,52%

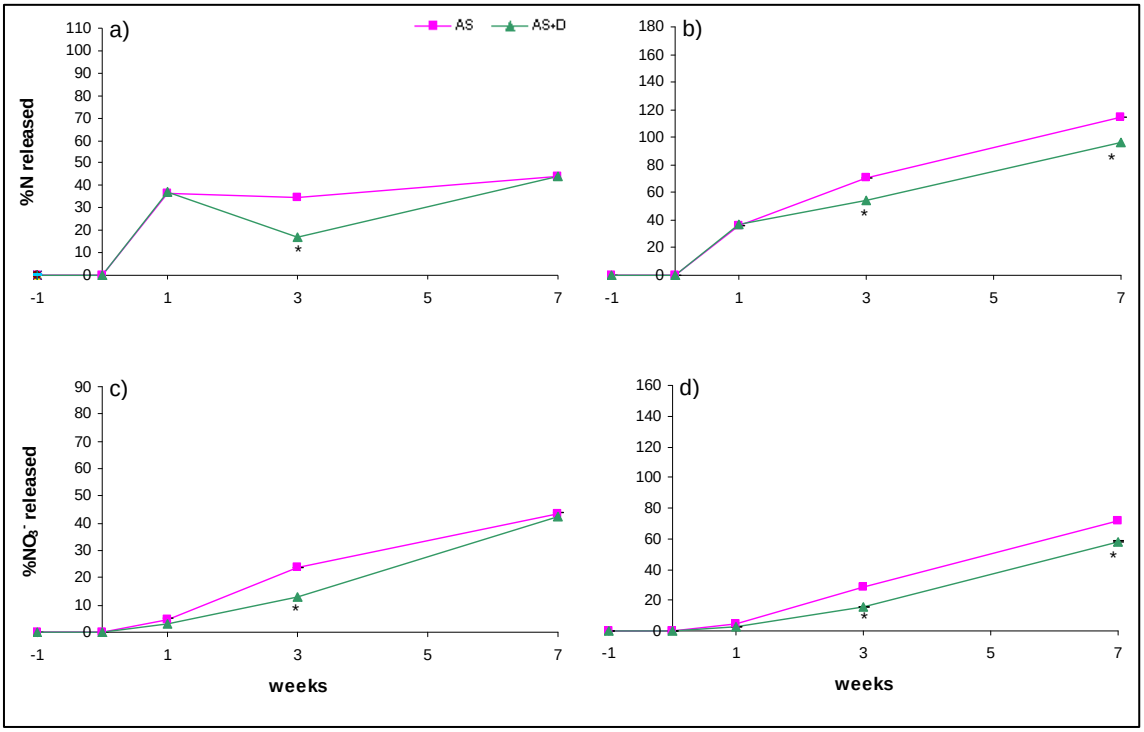

Fig. 2.5. Net N mineralization (2.5a, weekly; 2.5b, cumulative), and net nitrification (2.5c, weekly; 2.5d, cumulative) as the percentage of total N leached or nitrates leached with respect to the added N during the incubation period. Significant effects of the inhibitor are indicated by asterisks, Duncan's test, $P<0.05$. Treatments: $(NH_4^+)_2SO_4^-$ as mineral fertilizer (AS) combined or not with DMPP (AS+D) using a low-biological fertility soil (Casalotti soil). Mineral N fertilizer was added one week before the application of DMPP.

in Casalotti soil and 147,04±8,68%/118,76±10,45%, in Celimontano soil).

2.5. Conclusions

In conclusion, the results of the present study markedly show the efficacy of DMPP as nitrification inhibitor under every laboratory conditions investigated. However, distinct differences in the extent and the duration of this inhibition among treatments were observed. The inhibitory effect of DMPP with respect to nitrification resulted in reducing nitrates leached in Casalotti soil starting from the second week of incubation and it lasted over the fourth week of incubation, when fertilizing with both mineral or organic fertilizer. Temperature had little or no effects on N mineralization trends during

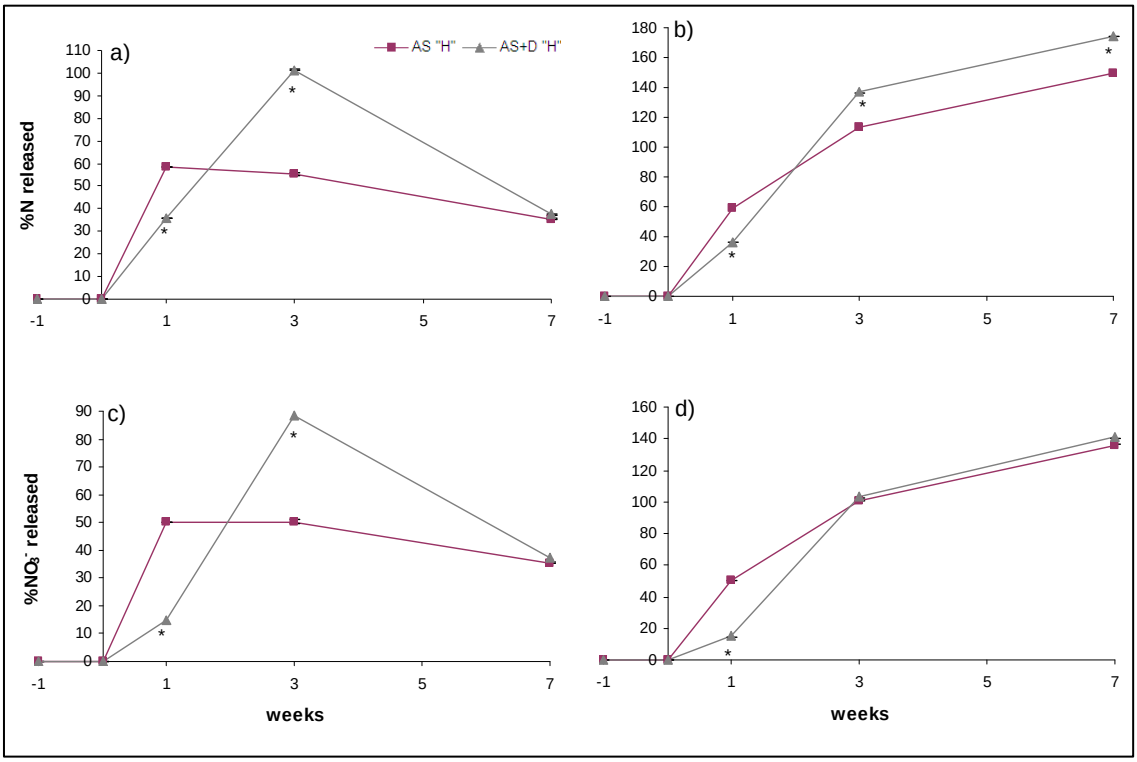

Fig. 2.6. Net N mineralization (2.6a, weekly; 2.6b, cumulative), and net nitrification (2.6c, weekly; 2.6d, cumulative) as the percentage of total N leached or nitrates leached with respect to the added N during the incubation period. Significant effects of the inhibitor are indicated by asterisks, Duncan's test, $P<0.05$. Treatments: $(NH_4^+)_2SO_4^-$ as mineral fertilizer (AS "H") combined or not with DMPP (AS+D "H") using a high-biological fertility soil (Soil Celimontano). Mineral N fertilizer was added one week before the application of DMPP.

the first 28 days of incubation. The inhibitory effect started immediately after 7 days and ended by the fourth week of incubation when using a soil (Celimontano soil) with higher C and N content and higher microbial activity and biomass, indicating a strong influence of soil biological properties on N mineralization. The simultaneous application of DMPP and fertilizer resulted to be the more efficient option with respect to the application of the nitrification inhibitor prior to mineral N addition, since in the latter case more N was mineralized in both Casalotti and Celimontano soil at the end of the incubation period.

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Chapter 3 – Effects of the nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP) on soil microbial communities after cattle effluent application in experimental microcosms

3.1. Abstract

The application of animal manure to soil can result in increased gaseous emissions such as NH_3, N_2O, CO_2 and CH_4 as well as nitrate leaching, contributing to climate warming and ground and surface water pollution.

The application of nitrification inhibitors to soil offers the chance to reduce N losses and to increase fertilizer use efficiency. In this study, we assess the effects of the nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP) on microbial community-level physiological profiling (CLPP) and on the abundance of ammonia-oxidizing bacteria (AOB) and archaea (AOA) using RT-qPCR in soil microcosms amended with cattle effluent. DMPP reduced the nitrification rate starting from the second week of incubation until at least the fourth week. DMPP treatments induced rapid changes in microbial heterotrophic metabolism showing significant differences after 24h of incubation: DMPP seemed to decrease metabolic activity when applied without a N source but AWCD values were increased in DMPP + cattle effluent treatment, suggesting that a few effluent strains were the main responsible of substrates consumption. As expected, total bacteria population was higher where cattle effluent was applied, as it cointains its own microbial population, but no massive differences were found between treatments in qPCR. On the contrary, rapid changes in gene expression occurred in DMPP + effluent treatment: after 24h both bacterial and archaeal *amoA* gene copy numbers were lower with respect to the effluent treatment, due to a high efficacy in the inhibition of the ammonia-oxidizing populations, but even bacteria 16S gene expression was reduced, suggesting a possible effect on non-target populations by DMPP.

Keywords: Nitrification inhibitor, DMPP, ammonia-oxidizers, RT-qPCR, T-RFLP

3.2. Introduction

According to the Soil Science Society of America nitrification can be defined as a "*biological oxidation of ammonium to nitrite and nitrate, or a biologically induced increase in the oxidation state of nitrogen*" and it is usually assumed that autotrophic bacteria are responsible for it in most soils, even if some studies suggested heterotrophic nitrifier microorganisms may contribute to nitrification and N_2O emissions related with, more than is commonly believed (Schimel *et al*., 1984; Tortoso and Huchinson, 1990; Williams *et al*., 1992).

Ammonia oxidizing bacteria (AOB) all have in common the ability to utilize ammonia as a sole source of energy and carbon dioxide as the chief source of carbon (Hooper *et al*., 1997). It is generally accepted that ammonia (NH_3) rather than ammonium (NH_4^+) is used as substrate, and the ammonia/ammonium ratio may therefore affect growth (Suzuki *et al*., 1974; Wood, 1986). Ammonia oxidation is thought to be the rate-limiting step for nitrification in most systems, as nitrite is rarely found to accumulate in the environment (De Boer *et al*., 1990; De Boer *et al*., 1992; El-Demerdash and Ottow, 1983; Prosser, 1989). Nitrification can positively or negatively affect nitrogen retention in a system, depending on the environmental conditions. Ammonia is highly volatile and therefore readily lost from some systems. In such cases, nitrification may facilitate nitrogen retention by oxidizing ammonia to less volatile nitrogen forms. Nitrification can lead to a net loss of nitrogen from environmental systems via at least three different mechanisms. First, the eventual production of nitrate can fuel denitrification, which is the main pathway for the elimination of fixed nitrogen from environmental systems. Second, the product of ammonia oxidation, nitrite, can undergo chemodenitrification, and it is not known to what extent this process affects nitrogen losses. A third important route to nitrogen loss is through nitrate leaching from soil. Whereas anionic NH_4^+ molecules bind well to cationic soil particles, NO_3^- is far more mobile and therefore readily lost to adjacent watercourses by leaching (Kowalchuk and Stephen, 2001).

One of the greatest problems associated with nitrification is nitrate contamination of ground- and freshwater supplies due to nitrate leaching from agricultural lands. Human and animal consumption of nitrate can lead to health risks, and government regulations strictly limit the amount of nitrate permitted in drinking water sources. Nitrate infiltration in freshwater systems may also lead to the eutrophication of such

environments, stimulating the growth of some phototrophic and/or heterotrophic organisms. This can lead to decreased biodiversity and the creation of anoxic conditions, respectively. The high productivity of modern agricultural practices is dependent upon the use of nitrogen fertilizers, which are generated either chemically or via the recycling of organic wastes, typically animal excreta. Nitrogen loss from such fertilizers, either before or after application, compromises their effectiveness. Nitrification can lead to nitrogen loss from such systems via two major mechanisms: increasing nitrogen leaching owing to conversion of ammonia to nitrate or linking nitrification with denitrification activities (Abbassi and Adams, 1998). Incomplete denitrification can also lead to the production of the ozone-depleting gas NO or the greenhouse gas N_2O. AOB can also produce these deleterious gases either at low levels as by-products of normal nitrification (Zart *et al*., 2000) or at high levels (up to 20 mol%) (Bremner and Blackmer, 1978; Goreau *et al*., 1980; Lipschultz *et al*., 1981; Poth *et al*., 1985) via partial-denitrification processes under reduced oxygen conditions (Colliver and Stephenson, 2000). Several groups of microorganisms contribute significantly to the production of these trace gases (Bouwmann, 1990), but the relative contributions of different microbial groups is not yet fully understood. It is unknown to what extent AOB contribute to the global production of these gases. However, it is now known that production of N_2O under suboptimal aeration varies drastically between AOB species (Jiang and Bakken, 2000), providing further urgency to the understanding of the factors regulating population size and structure of environmental AOB. The process of ammonia oxidation leads to a net acidification of the environment. Where ammonia deposition and nitrification levels are high, this may contribute to a lowering of the environmental pH (Beiderbeck *et al*., 1996). The acidification of forest soils can have a detrimental effect on tree health, and high levels of nitrification may intensify problems involving the effects of acid rain. Furthermore, N transformations that lead to an increased proton load can lead to the release of metals such as aluminum, which can contribute to root damage and forest decline. Such problems can be exacerbated by the role of nitrification in changing the relative pools of NH_4^+ and NO_3^-, the most common nitrogen forms taken up by plants. Some tree species show a strong preference for the uptake of NH_4^+ over NO_3^-, and thus nitrification may affect seed-dormancy breaking, germination, seedling establishment, and growth (Kronzuker *et al*., 1997). In contrast,

many weed species show a preference for NO_3^- and may thrive under conditions of elevated nitrification. AOB may therefore affect vegetational succession by the relative abundance of inorganic N sources (Kronzuker *et al*., 1997).

In agricultural systems, control of nitrification for a limited time may help to apply the exact amounts of N-fertilizer which plants actually need (Bronson and Mosier, 1994; Patra *et al*., 2001; Subbarao *et al*., 2006). Consequently, this leads to an increase in plant N use efficiency through reduced N application rates and numbers. Depending on soil conditions and more importantly plant species, these beneficial effects could be increasing N use efficiency, reduction in N losses and consequently environmental and health risk, a better and more balanced nutrition of micronutrients which specially are not present in available forms in soil (calcareous or high pH conditions), better utilization of unavailable phosphorus forms such as Ca-P or rock phosphate (Hinsinger *et al*., 2003), and more effective bioremediation of contaminated soils.

Analysis of natural populations of autotrophic ammonia-oxidizing microorganisms using cultivation-based techniques presents particular problems, due to their slow growth, difficulties in separating ammonia oxidizers from heterotrophic contaminants, and the limited number of characteristics for identification and classification. This has hampered meaningful study of the population ecology of the ammonia oxidizers, limiting our understanding of their important role in carrying out oxidation of ammonia to nitrite, the first step in nitrification. Increased understanding of the relationship between taxonomic and physiological diversity is important for our understanding of nitrogen cycling in natural environments. With the exception of a small number of cultured marine strains belonging to the γ-proteobacteria, analysis of 16S rRNA gene sequences places autotrophic ammonia oxidizers in a monophyletic group within the β-Proteobacteria, containing two genera, *Nitrosomonas* and *Nitrosospira* (Head *et al*., 1993). Analysis of 16S rRNA gene sequences recovered from natural populations of ammonia oxidizers indicates further subdivisions, with the *Nitrosospira* and *Nitrosomonas* genera containing at least four clusters and three clusters, respectively, of related sequences (Stephen *et al*., 1996).

Quantification of ammonia-oxidizing bacteria (AOB) and archaea (AOA) has been attempted using several different methods, including the most probable number technique (Kowalchuk *et al*., 1999), in situ hybridization (Schramm *et al*., 1999), a

competitive enzyme-linked immunosorbent assay using monoclonal antibodies (Sanden *et al*, 1994) and competitive PCR (Kowalchuck *et al*., 1997) based on traditional methods of amplification. However, all of these methods have significant disadvantages. Thus, a reliable and reproducible method for quantifying AOB and AOA would be valuable for evaluating correlations between microbial activities and cell numbers, the effect of different treatments on cell density, and populations changes in time and space. Determination of DNA concentrations with real-time PCR, which is based on measurements obtained during the early exponential phase, overcomes some of the problems associated with traditional PCR.

Blocking ammonium oxidation in order to have stabilized ammonium in bulk soil, is achieved through application of synthetic nitrification inhibitors such as N-Serve, Dicyandiamide (DCD) and 3,4-dimethylpyrazole phosphate (DMPP). To large extent, the efficiency of these compounds depends on their chemical structure, mode of action, dynamics inside the soil, and their resistance to biological and chemical degradation. DMPP however is one of the most effective and widely used nitrification inhibitor with high efficiency and no side effect (Weiske *et al*., 2001; Pasda *et al*., 2001; Irigoyen *et al*., 2003). Its typical dosage is 1% $N\text{-}NH_4^+$ which is too much lower than other inhibitors such as Dicyandiamide (DCD) (Zerulla *et al*., 2001). Potential distinct advantages such as significant and more effective inhibition of nitrification and reduction of N_2O emission (Weiske *et al*., 2001; Zerulla *et al*., 2001; Hatch *et al*., 2005), and CO_2 emission (Weiske *et al*., 2001), increasing yield (Zerulla *et al*., 2001; Pasda *et al*., 2001; Linzmeier *et al*., 2001) and consequently increasing N use efficiency are attributed to DMPP, compare to other NIs.

In most environments, autotrophic AOB are considered to be the most important contributors to ammonia oxidation. However, in most mesophilic environments, members of the kingdom *Crenarchaeota* within the domain of *Archaea* are considerably more abundant than characterised AOB populations. For example, soil typically contains 10^4–10^6 β-proteobacterial AOB cells g^{-1} (Mendum and Hirsch, 2002; Okano *et al*., 2004). By contrast, extrapolating from 16S rRNA and whole cell probe data, soil might contain 10^7 *Crenarchaeota* g^{-1} or even more (Buckley *et al*., 1998; Sandaa *et al*., 1999). Furthermore, inhibition experiments have also indicated that AOB are not the major contributors to nitrification processes in certain habitats (Jordan *et al*., 2005). It

will, therefore, be important to determine whether crenarchaeal AMO is also sensitive to AOB inhibitory compounds such as acetylene, and whether nitrification processes can be attributed specifically to AOA populations.

To date there have been few studies on effects of nitrification inhibitors on non-target soil biota. Given the importance of maintaining soil quality and function, it is essential that soil treatments such as nitrification inhibitors do not negatively impact on activity of non-target soil microbes and fauna.

Thus, the aim of this study was to evaluate the effect of the addition of the nitrification inhibitor DMPP combined with a cattle effluent as organic fertilizer on microbial community structure and functioning. Soil microcosms were set up as described in paragraph 3.3.2; microbial heterotrophic metabolism was investigated by means of Biolog's community-level physiological profiling, whereas molecular techniques (qPCR, RT-qPCR, T-RFLP) were used to investigate the abundance and expression both of target (ammonia oxidizers and denitrifiers), and non-target microbial populations. To determine whether *amoA* genes from AOA and AOB populations, bacterial 16S rRNA and *nosZ* genes are actively transcribed in soil, total RNA from soil microcosms was isolated and relative cDNA copies of these genes after reverse transcription.

3.3. Materials and methods

3.3.1. Soil and cattle effluent

The soil used (Casalotti soil) was collected from a *Eucalyptus*, short rotation, high-density plantations field managed by the research unit for intensive wood production (CRA-PLF) located in Rome (Italy). The 20 year-old experimental field with initial low-density plant spacing (1100 plants/ha) was converted in 2007 into a high-density plant spacing (5000 plants/ha). Six samples of soils from the top 30 cm were collected in June 2009 and kept in sterile plastic bags. The soils were air-dried, homogenized by sieving (2-mm mesh size), pooled and stored at room temperature. Main characteristics were: sand 63%, loam 16% and clay 21%; pH 7.5; TOC 1.06%, total N 0.06%.

The cattle effluent used was obtained from a dairy farm located in Rome. Sampling was performed in June 2009 and sample was stored in PVC barrels at 4 °C until required. On

day 0, it was sampled after thorough stirring and blending and analyzed by standard laboratory methods to assess the following physico-chemical properties: moisture 88.9%; dry matter 11.1%; density 1 g/cm^3; total N 0,32%; N-NH_4^+ 0.17%; Cu 0.3 ppm; Zn 0.2 ppm.

3.3.2. Experimental setup

Microcosms containing 1.1 Kg of homogenized soil were set up to examine microbial community structure and functioning in Casalotti soil after cattle effluent as organic fertilizer and DMPP addition. Nitrogen (250 mg N kg soil^{-1}) from organic fertilizer was added (E); liquid formulation of DMPP (25%, provided by K+S Nitrogen, Italy) containing 1,86% mineral N (1,1% N-NO_3^-, 0,76% NH_4^+-N) was used with organic fertilizer (E+D) at a final concentration of 1% according to the NH_4^+-N content, as recommended by the providers (Zerulla *et al.*, 2001). A treatment with no fertilizer (C) and DMPP only (D) were included as controls. Microcosms were incubated at 60% water holding capacity (pF 2.5) in the dark at 30°C for 28 days, and covered by cling film which prevented water loss but allowed gas exchange. Every 3-4 days moisture was replenished throughout the experiment duration. Each treatment was replicated three times. 10g subsamples were taken at days 1, 4, 7, 14 and 28: 2g were used for Biolog analysis, while the remaining 8g were immediately frozen at -80°C and stored for molecular analysis until required. After each sampling soil was homogenized by mixing.

3.3.3. Microbial community-level physiological profiling (CLPP)

The functional diversity of microorganisms, particularly as defined by the substrates used for energy metabolism, is integral to our understanding of biogeochemistry (Hooper *et al.*, 1996); functional aspects related to substrate utilisation can provide important information beyond that afforded by taxonomic level investigations or structural investigations based on rRNA or rRNA analysis (Grayston *et al.*, 1998).

The metabolic profiles of the microbial communities were generated by means of the Biolog® Microstation System 4.2 (Biolog Inc., Hayward, CA, USA) using ECOPlates,

specifically designed for community analysis and microbial ecological studies. The ECOPlate contains 31 of the most useful carbon sources for soil community analysis, repeated 3 times to give more replicates of the data. Oxidation wells contain a redox indicator, tetrazolium violet, which undergoes a colour change (from colourless to dark violet) whose intensity is proportional to the intensity of the microbial metabolism (which in turn may be due to the number of the cells and/or the nature of the species involved).

Two g soil subsamples were mixed with 20 ml sterile physiological solution (NaCl 9 g l^{-1}), stirred with 10g glass beads for 30', and centrifuged at 3000 rpm for 3'. One hundred and twenty µl supernatant were used to inoculate each plate, according to Torsvik (1995). The absorbance values corresponding to colour changes are read by the E-MAX reader at 590 nm wavelength for 10 days.

Each well of Ecoplate contains a redox dye tetrazolium, which is reduced by NADH produced by respiration pathways. The rate and the extent of color formation indicate the rate and extent to which respiration occurs with the substrate present in that well (Garland and Mills 1991; Garland 1996a, b).

Organization of results was performed with Biolog MicroLog System 4.2 software. Raw OD data are corrected by blanking each response well against its own first reading (immediately after inoculation). This blanking not only avoids the intrinsic absorbance of the carbon sources but also the negative values when compared to subtracting the control well from the response well. The absorbance profile obtained for each trial at each reading time was transformed into the average well colour development (AWCD) index (Garland 1997) with the formula AWCD = $\sum ab_t/96$ (ab_t being the absorbance value at a certain reading time, calculated as previously reported, and 96 the total number of the wells) and their temporal evolution plotted as AWCD curves. Data analysis have been further elaborated by calculating the area under the curve for each well OD for the entire period of incubation and by estimation of kinetic parameters (K, r, s) by fitting the curve of OD versus time to a density dependent logistic growth equation $Y = OD_{592} = K/(1 + e^{-r\,(t-s)})$, where K is the asymptote (or carrying capacity), r determines the exponential rate of OD change, t is the time following inoculation of the microplates, and s is the time when the mid point of the exponential portion of the curve (i.e., when y = K/2) is reached (Insam and Goberna, 2004).

Analysis of variance (ANOVA) and Post-Hoc (Duncan) test was applied on profiles for statistical testing with SPSS 13.0 (SPSS Inc., Chicago, IL).

3.3.4. Acid nucleic extraction

RNA and DNA were extracted from 2 g of each soil sample using a RNA PowerSoil® Total RNA Isolation Kit and RNA PowerSoil® DNA Elution Accessory Kit, respectively (Mo Bio, Carlsbad, CA, USA) following slight modification to the manufacturer's instructions, whereby the 15 min shaking on a flat bed vortex was replaced by a 30 s bead beading step (5.5 m s^{-1}, Fastprep). DNA and RNA concentrations were determined using the Qubit quantification platform with Quant-iT^{TM} dsDNA high sensitivity (HS) Assay Kit and Quant-iT^{TM} RNA Assay Kit (Invitrogen NZ). DNA and RNA were diluted to 5 ng/µL and stored in -80°C freezer for the following molecular applications.

3.3.5. Real-time PCR (qPCR)

The real-time PCR technique is based on continuously monitoring fluorescence throughout the reaction. Quantification of DNA by real-time PCR is based on measurements obtained during the early exponential phase, when amplification of the PCR product is first detected and the amount of the amplified product is proportional to the concentration of the template DNA (Heid *et al*., 1996). The abundances of bacterial 16S rRNA, bacterial *amoA*, archaeal *amoA*, *nirK*, *nirS* and *nosZ* genes in all the soil samples were quantified using real-time PCR. Quantitative real-time PCR was performed as follows: bacterial 16S rRNA (as a measure of total bacterial populations) used primers Muyzer For (5' - CCT ACG GGA GGC AGC AG – 3') (Nadkarni *et al.*, 2002) and Muyzer Rev (5' – ATT ACC GCG GCT GCT GG – 3') (Muyzer *et al*., 1993); bacterial *amoA* (as a measure of ammonia-oxidizing bacteria) used primers amoA-1F (5' – GGA CTA CVS GGG TAT CTA AT – 3') and amoA-2R (5' – CCC CTC KGS AAA GCC TTC TTC – 3') (Rotthawue *et al.*, 1997); archaeal *amoA* (as a measure of ammonia-oxidizing archaea) used primers arch-amoAF (5' – STA ATG GTC TGG CTT AGA CG – 3') and arch-amoAR (5' – GCG GCC ATC CAT CTG

TAT GT – 3') (Francis *et al.*, 2005); *nirK* (as a measure of denitrification bacteria) used primers nirK876 (5' – ATY GGC GGV CAY GGC GA – 3') and nirK1040 (5' - GCC TCG ATC AGR TTR TGG TT – 3') (Henry *et al*., 2004); *nirS* (as a measure of denitrification bacteria) used primers nirS_cd3a-F (5' – GTS AAC GTS AAG GAR ACS GG – 3') (Michotey *et al*., 2000) and nirS_R3cd_R (5' – GAS TTC GGR TGS GTC TTG A – 3') (Throback *et al*., 2004); *nosZ* (as a measure of denitrification bacteria) used primers nosZ2RHenry (5' – CAK RTG CAK SGC RTG GCA GAA – 3') and nosZ2FHenry (5' – CGC RAC GGC AAS AAG GTS MSS GT – 3') (Henry *et al*., 2006).

Quantitative PCR was performed using a Stratagene Mx3000P QPCR thermal cycler (Agilent Technologies) in a 25 μL reaction mixture consisting of 20 ng DNA template, 12,5 μL QuantiTect SYBR green master mix (Qiagen), and the following primer concentrations: 1μM for bacterial 16S rRNA and archaeal *amoA*, 1.5 μM for bacterial *amoA*, 5 μM for *nirK* and *nosZ*, 7.5 μM for *nirS*. Real-time amplification was performed using the following programs: for bacterial 16S rRNA gene: initial 15 min at 95°C; 30 cycles of 15 s at 95°C for denaturating, 30 s at 58°C for annealing, 30 s at 78°C and 15 s at 84°C (both readout) for extension; for bacterial *amoA* gene: initial 15 min at 95°C; 45 cycles of 15 s at 95°C for denaturating, 30 s at 58°C for annealing, 45 s at 72°C and 15 s at 84°C (both readout) for extension; for archaeal *amoA* gene: : initial 15 min at 95°C; 40 cycles of 15 s at 95°C for denaturating, 30 s at 58°C for annealing, 45 s at 72°C and 15 s at 78°C (both readout) for extension; for *nirK*, *niS* and *nosZ* genes: : initial 15 min at 95°C; 40 cycles of 15 s at 95°C for denaturating, 30 s at 58°C for annealing, 45 s at 72°C and 15 s at 84°C (both readout) for extension.

A melting curve analysis was performed to confirm PCR product specificity after amplification by measuring fluorescence continuously as the temperature increased from 50 to 99 °C. The specificity of the PCR was further evaluated by running randomly selected samples on a 1% (w/v) agarose gel. The corresponding real-time PCR efficiency for each of these genes was estimated based on serial dilutions of the common DNA mixture described above. Triplicate qPCR repetitions were performed for each of the gene for all the samples. The copy numbers of these genes per gram of dry soil was calculated by the copy numbers of the gene per ng of DNA multiplied by the amount of DNA contained in each gram of dry soil.

Standards were generated from PCR products that had been generated from soil DNA extracts, gel purified, and quantified using a Qubit™ fluorometer (Invitrogen NZ) and diluted accordingly. This was to minimise the bias inherent in the usual approach, where one or only a few genes are used to standardize complex communities (Towe *et al.*, 2010). Problems arise because the degenerate primers have only partial homology to the gene variants used to generate the standard curve, and there are differences in the amplification efficiencies of the range of sequences internal to the primers in the environmentally-extracted DNA samples.

The number of copies of each gene was calculated using the following equation: gene copy number = (ng*number/mol)/(base pairs*ng/g*g mol base pairs) (http://www.uri.edu/research/gsc/resources/cndna .html). Standards were diluted accordingly to give a concentration range from 0 to 10^7 (10^9 for bacterial 16S rRNA) gene copies μL^{-1}. All DNA preparations were checked for the absence of inhibitors prior to PCR and all results were analysed using LinRegPCR program version 11.1 (Ramakers *et al.*, 2003; Ruijter *et al*, 2009) to confirm the efficiency of amplification and the absence of inhibition.

3.3.6. Reverse transcription - real-time PCR (RT-qPCR)

Quantitative real-time reverse transcription (RT-PCR) assays were used to specifically measure the bacterial 16S, bacterial *amoA*, archaeal *amoA* and *nosZ* abundance mRNA. The mRNA concentrations of the four genes investigated were quantified with one-step RT-PCR assays. RNA was treated with TURBO DNA-*free*™ Kit (Applied Biosystem, USA). RT-PCR assays were performed using SensiFAST™ SYBR Lo-ROX

One-Step Kit (Bioline, UK) in a 40 µL mix containing 20 µL of 2X SensiFAST SYBR Lo-ROX One-step Mix, 0.4 µL of Reverse transcriptase, 0.8 µL of RiboSafe RNase Inhibitor, 0.2 ng DNA for bacterial 16S rRNA gene, 20ng DNA for *nosZ* gene and 50 ng DNA for bacterial and archaeal *amoA* genes, and the same primers concentrations used in real-time amplification assays described in paragraph 3.3.5.

The RT-PCR reaction programs were the same of those used in real-time amplification assays described in paragraph 3.3.5.

3.3.7. cDNA synthesis

Reverse transcription (RT) was carried out on half of the total RNA extracted using Quantitect Reverse Transcription Kit (Qiagen). Ten μL RNA were added to 2 μL gDNA wipeout Buffer to a final volume of 14 μL, in order to eliminate genomic DNA. The suspension was heated for 2 minutes at 42°C and then placed immediately on ice. An RT mix of 1 μL Reverse transcriptase containing RNase inhibitor, 5X Quantiscript RT buffer containing Mg2+ and dNTPs and 1 μL RT primer mix, to a final volume of 20 μL, was added. Free – genomic DNA obtained previously was added on ice to each tube containing reverse-transcription master mix. The suspension was incubated for 15 minutes at 42°C, and then heated for 3 minutes at 95°C to stop the reaction. The absence of DNA contamination was confirmed by PCR amplification of non reverse transcribed RNA samples.

3.3.8. T-RFLP analysis

The samples collected from soil microcosms described previously were analyzed by terminal restriction fragment length polymorphism (T-RFLP) on metabolic active microbial populations. The bacterial and archaeal 16S rRNA genes were amplified in multiplex with VIC- and NED-labeled (on forward primer) primers, respectively, 63f (5’ - AGGCCTAACACATGCAAGTC)/1087f (5’ - [VIC]-CTC GTT GCG GGA CTT ACC CC) Hauben *et al*., 1997; Marchesi *et al.*, 1998), and Ar3F (5’ – TTC CGG TTG ATC CTG CCG GA – 3’)/Ar927R (5’ - [NED]-CCC GCC AAT TCC TTT AAG TTT C – 3’) Raskin *et al*., 1994; Jurgens *et al*., 1997). with the slight modification that the forward bacterial 16S primer 63F was VIC-labeled and the archaeal 16S primer 344F replaced 3F (Macdonald *et al.*, 2011).

Following PCR amplification (95 °C for 2 min followed by 30 cycles of 30 s denaturing at 94 °C, 30 s annealing at 55 °C and 1 min extension at 72 °C with a final 10 min extension at 72 °C) products were visualised with ethidium bromide staining on a 1% (w/v) agarose gel using UV radiation and purified using Wizard® Genomic DNA Purification Kit (PROMEGA, USA) according to the manufacturer’s instructions. Purified PCR products (about 500 ng) were digested with 20 U of Msp I (Promega, UK) restriction enzyme as previously described (Macdonald *et al.*, 2007). Terminal

restriction fragments (T-RFs) were resolved on an ABI PRISM 3130xl genetic analyzer (Applied Biosystems, UK) and bacterial, archaeal and fungal T-RFLP profiles were generated from the raw data using GeneMarker (version 1.7; SoftGenetics, USA). Fragment analysis was performed between 50 and 500 base pair (bp), whichwas within the line range of the internal size standard (LIZ 500, Applied Biosystems). A baseline threshold of 50 fluorescence units was used to minimize the effect of artifacts.To account for T-RF drift, fragments that differed by less than 0.5 bp were aligned as the same peak i.e. binning (Dunbar *et al*., 2001). The relative abundance of a T-RF in a profile was calculated as a proportion of the total peak height of all the T-RFs in a profile. Any peak that was <0.5% of the total fluorescence unit was removed from the data before statistical analysis.

3.3.9. Data analysis

Excel and SPSS 11 softwares were used for analysis of Biolog and real-time data, and comparisons of means were conducted using one way ANOVA and Duncan test at level of $p<0.05$. Results in the tables, text and figures are given as means ± SD. T-RFLP analisys was conducted with S-PLUS 6 for Windows (Insightful Corp., USA). Eucledian similarity matrices were generated on relative abundance T-RFLP data that had been log transformed prior to PCA (Pricipal Components Analysis) analysis. The key idea of PCA is to represent the variation of a data matrix in a reduced number of dimensions to obtain the general structure of variability. PCA scores were plotted in two dimensions: principal components 1 (PC1) and PC2.

3.4. Results and discussion

3.4.1. Microbial community-level physiological profiling (CLPP)

In Table 3.1 are reported AWCD values, inflection point, area of the curve and catabolic versatility of microbial heterotrophic community during the incubation period. The AWCD measure (Figg. 3.1-3.5) gives a general indication on the metabolic capacity of the community and on the degree of activity with respect to 31 different substrates.
As shown in Table 3.1 (AWCD values), significantly lower potential microbial activity

	AWCD (OD_{590nm})	s (h)	Area	VC
T_1 C	1.408[a]	115.4[a]	216.4[a]	1.43[a,b]
	(0.079)	*(2.9)*	*(7.5)*	*(0.09)*
T_1 D	1.139[b]	134.5[b]	154.2[b]	1.24[a]
	(0.050)	*(5.3)*	*(1.0)*	*(0.12)*
T_1 E	1.152[b]	98.1[c]	196.5[c]	1.48[a,b]
	(0.036)	*(3.3)*	*(3.0)*	*(0.09)*
T_1 ED	1.514[a]	105.2[c]	248.2[d]	1.51[b]
	(0.054)	*(0.5)*	*(8.3)*	*(0.00)*
T_4 C	1.347[a]	139.3[a]	176.2[a]	1.08[a]
	(0.090)	*(2.9)*	*(8.0)*	*(0.26)*
T_4 D	1.128[b]	135.1[a]	152.1[b]	1.22[a]
	(0.015)	*(6.3)*	*(9.1)*	*(0.23)*
T_4 E	1.267[a,b]	96.2[b]	219.1[c]	1.57[a]
	(0.089)	*(5.8)*	*(8.4)*	*(0.22)*
T_4 ED	1.583[c]	113.9[c]	246.8[d]	1.33[a]
	(0.023)	*(5.5)*	*(5.1)*	*(0.10)*
T_7 C	1.414[a]	138.4[a]	186.5[a]	1.45[a]
	(0.056)	*(3.1)*	*(3.6)*	*(0.01)*
T_7 D	1.444[a]	137.6[a]	191.4[a]	1.57[b]
	(0.011)	*(1.6)*	*(3.6)*	*(0.01)*
T_7 E	1.287[b]	102.4[b]	214.4[b]	1.44[a]
	(0.030)	*(7.3)*	*(4.1)*	*(0.04)*
T_7 ED	1.622[c]	88.2[c]	293.9[c]	1.53[b]
	(0.007)	*(0.0)*	*(1.6)*	*(0.01)*
T_{14} C	1.095[a]	105.3[a]	216.4[a]	0.98[a]
	(0.035)	*(2.7)*	*(7.5)*	*(0.01)*
T_{14} D	0.852[b]	88.0[b]	154.2[b]	1.22[b]
	(0.064)	*(3.2)*	*(1.0)*	*(0.04)*
T_{14} E	1.437[c]	105.2[a]	196.5[c]	1.42[b]
	(0.066)	*(0.1)*	*(3.0)*	*(0.05)*
T_{14} ED	1.457[c]	90.6[b]	248.2[d]	1.27[b]
	(0.055)	*(0.3)*	*(8.3)*	*(0.12)*
T_{28} C	0.863[a]	105.9[a]	141.5[a]	1.01[a]
	(0.064)	*(3.0)*	*(13.2)*	*(0.14)*
T_{28} D	0.579[b]	92.7[b]	102.6[b]	0.99[a]
	(0.011)	*(0.2)*	*(2.1)*	*(0.08)*
T_{28} E	0.882[b]	96.3[b]	152.8[b]	1.07[a]
	(0.088)	*(5.3)*	*(10.6)*	*(0.05)*
T_{28} ED	1.055[c]	91.4[b]	188.0[c]	1.14[a]
	(0.051)	*(2.3)*	*(6.9)*	*(0.03)*

Tab. 3.1. AWCD values, inflection point (s), area of the curve and catabolic versatility (VC) after 1, 4, 7, 14 and 28 days of microcosms incubation. Results are given as means ± SD. Comparisons of means were conducted using one way ANOVA and Duncan test at level of $p<0.05$.

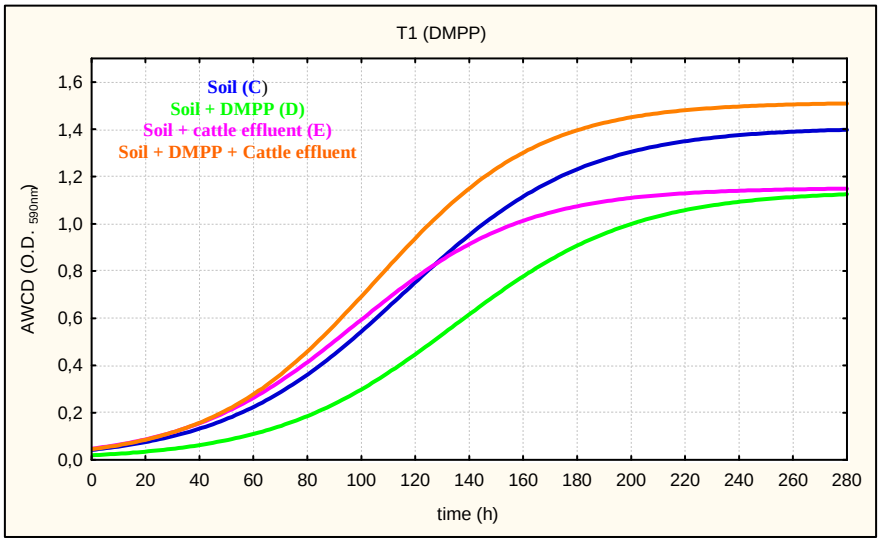

Fig. 3.1. AWCD curves during the plates incubation period after 1 day of microcosms incubation. Treatments: soil only (C), soil + DMPP (D), soil + cattle effluent (E), soil + cattle effluent + DMPP (ED).

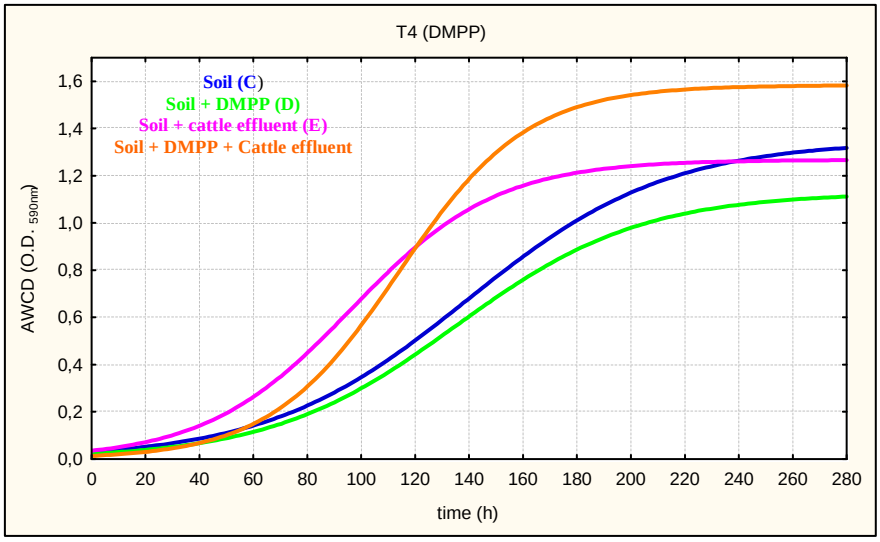

Fig. 3.2. AWCD curves during the plates incubation period after 4 days of microcosms incubation. Treatments: soil only (C), soil + DMPP (D), soil + cattle effluent (E), soil + cattle effluent + DMPP (ED).

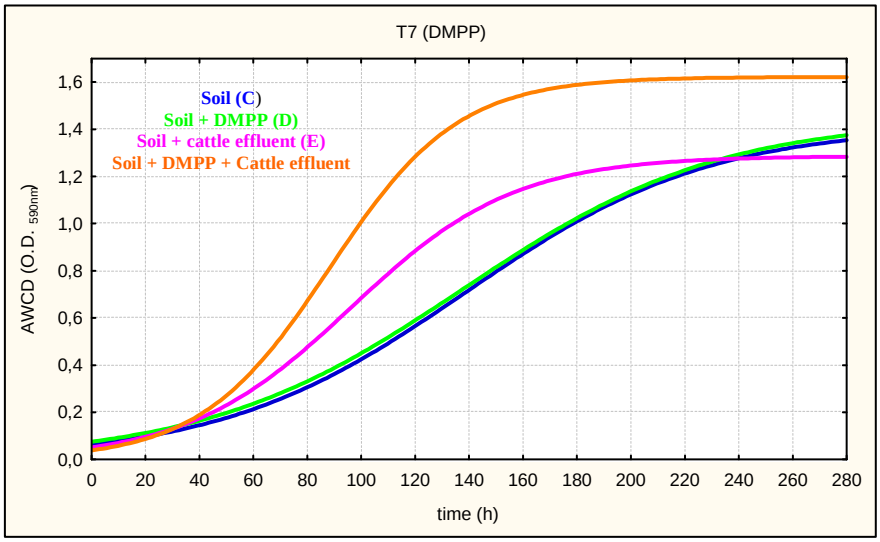

Fig. 3.3. AWCD curves during the plates incubation period after 7 days of microcosms incubation. Treatments: soil only (C), soil + DMPP (D), soil + cattle effluent (E), soil + cattle effluent + DMPP (ED).

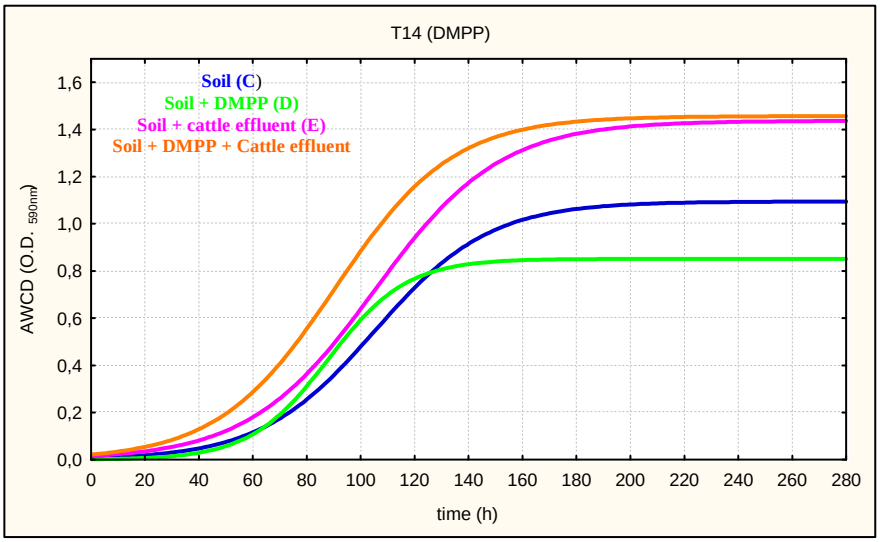

Fig. 3.4. AWCD curves during the plates incubation period after 14 days of microcosms incubation. Treatments: soil only (C), soil + DMPP (D), soil + cattle effluent (E), soil + cattle effluent + DMPP (ED).

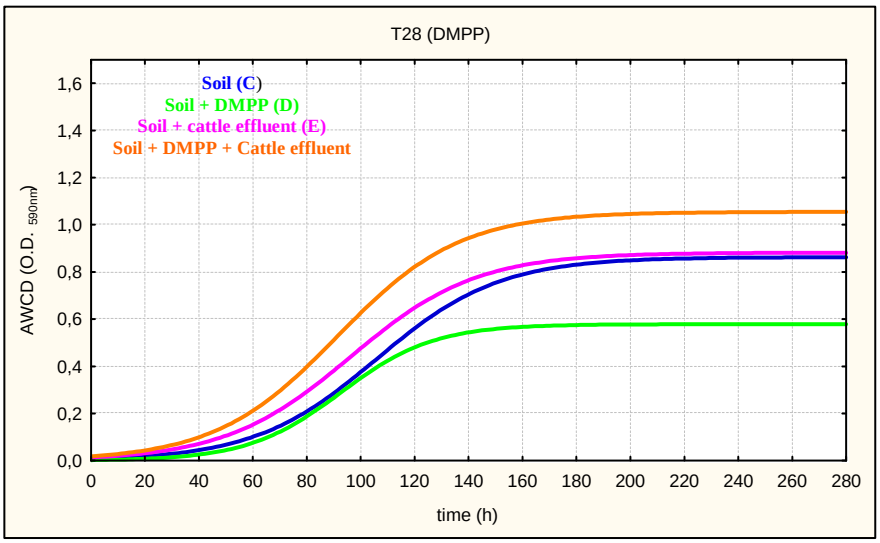

Fig. 3.5. AWCD curves during the plates incubation period after 28 days of microcosms incubation. Treatments: soil only (C), soil + DMPP (D), soil + cattle effluent (E), soil + cattle effluent + DMPP (ED).

was detected in D and E when compared to the control at day 1, while the inflection point (s), which is the time (expressed in hours) when the mid point of the exponential portion of the curve (i.e., when y = K/2) is reached (Insam and Goberna, 2004), indicates that heterotrophic culturable microrganisms were significantly more reactive in treatments where cattle effluent was added.

Potential microbial activity remained higher in ED when compared to the control during the entire incubation period, while this increase was effective in E treatment starting from day 7. On the contrary, heterotrophic culturable metabolism was lower in D treatment during the entire incubation period except in day 7. In the same way, the reactivity of the microbial community was positively affected by the simultaneous

application of cattle effluent and DMPP (ED) from the beginning to the end of microcosms incubation, whereas the positive effect was visible in D treatment starting from day 14.

Catabolic versatility (VC) is a measure of functional diversity and estimates the ability of a microbial community to utilize different substrates. At day 1, 7 and 14 ED treatment showed a significant higher VC values when compared to the control (Tab. 3.1), thus indicating a greater range of substrates being metabolised by microbial community. These conditions, however, are temporarily, and after 28 days of incubation no difference in functional diversity could be detected.

Soil microbial communities showed a metabolic diversification in the carbon sources oxidation (Tab. 3.2). After 1 day of microcosms incubation, D-Glucosaminic Acid, D,L-α-Glycerol Phosphate, D-Galacturonic Acid, D-Malic Acid, Phenil-ethyl-amine and Putrescine consumption was inhibited in D treatment with respect to the control ($p<0,05$), whereas N-Acetil-D-Glucosamine only was more metabolised.

During the incubation several substrates in D treatment were less (Pyruvic Acid Methyl Ester, I-Erythritol, D-Mannitol, D-Galactonic Acid γ-Lactone, γ-Hydroxybutyric Acid) or more metabolised (Tween 40, Tween 80, Glycogen, α -D-Lactose), while both D,L-α -Glycerol Phosphate and Putrescine were inhibited at day 1 and more consumed at day 14. In E treatment, 6 and 9 substrates were inhibited at day 1 and day 7, respectively, while after 4, 14 and 28 days the number of substrates induced was greater of that of substrates repressed. In ED treatment only few substrates (I-Erythritol, D,L-α -Glycerol Phosphate, D-Galactonic Acid γ-Lactone, D-Galacturonic Acid and γ-Hydroxybutyric Acid) were less utilized by heterotrophic bacteria, while most of the other ones (21 out of 31) were more metabolised at least once during the incubation period.

In most of the cases it seems that the cattle effluent played a big role in the substrates consumption patterns, since it has its own microbial population being added to the microcosms, while in some other cases the nitrification inhibitor could have driven the selection of specific biota responsible of the degradation of specific C-sources.

In conclusion, DMPP treatments induced rapid changes in microbial heterotrophic metabolism showing significant differences after 24h of incubation: DMPP seemed to

	C T_1	C T_4	C T_7	C T_{14}	C T_{28}	D T_1	D T_4	D T_7	D T_{14}	D T_{28}	E T_1	E T_4	E T_7	E T_{14}	E T_{28}	ED T_1	ED T_4	ED T_7	ED T_{14}	ED T_{28}
Pyruvic Acid Methyl Ester	1,496	1,012	1,943	1,666	1,723	1,538	1,102	1,868	1,357	1,013	1,307	1,111	1,080	1,682	1,586	1,840	1,949	2,218	2,268	1,752
Tween 40	0,800	0,209	0,957	0,434	0,373	0,656	0,549	1,032	0,778	0,425	0,791	1,010	0,864	0,624	0,403	1,235	0,983	1,351	1,606	1,074
Tween 80	0,955	0,381	0,803	0,128	0,154	0,696	0,385	0,838	0,516	0,353	1,041	1,094	0,856	0,696	0,351	1,104	1,050	0,784	0,965	0,428
Cyclodextrin	0,307	0,000	0,263	0,042	0,055	0,157	0,051	0,323	0,147	0,090	0,339	0,015	0,017	0,054	0,023	0,128	0,160	0,147	0,579	0,287
Glycogen	0,344	0,000	0,664	0,532	0,476	0,387	0,390	0,949	0,745	0,344	0,423	0,601	0,487	0,410	0,502	0,460	1,354	1,293	1,234	1,339
D-Cellobiose	0,854	1,163	1,478	1,218	0,702	1,087	0,909	1,299	1,085	0,603	0,959	0,561	0,926	0,905	1,134	1,434	1,803	2,048	1,772	1,641
α -D-Lactose	0,737	1,054	1,420	0,895	0,638	1,031	0,860	1,656	1,442	0,402	0,555	0,341	0,676	1,207	0,976	1,687	0,928	1,663	1,236	0,986
β- Methyl-D-Glucoside	0,696	0,733	1,126	0,806	0,548	0,944	0,500	1,212	0,819	0,516	0,420	0,162	0,499	0,728	0,634	0,706	1,071	0,977	1,188	0,355
D-Xylose	1,026	0,576	0,769	0,622	0,642	0,641	0,522	0,899	0,501	0,559	1,150	0,955	1,006	1,266	1,067	1,229	0,869	1,140	0,786	0,877
I-Erythritol	0,295	0,141	0,793	0,384	0,233	0,416	0,241	0,600	0,243	0,167	0,353	0,344	0,685	0,441	0,304	0,414	0,328	0,353	0,230	0,173
D-Mannitol	1,349	0,809	1,962	1,096	0,552	0,868	0,565	1,329	1,142	0,668	1,031	0,744	1,553	0,890	0,725	1,408	1,479	1,498	0,603	0,563
N-Acetil-D-Glucosamine	0,440	0,410	1,237	1,005	0,532	1,173	0,897	1,070	1,597	1,004	0,931	0,662	0,871	1,364	0,536	0,936	1,657	2,126	2,070	1,671
D-Glucosaminic Acid	0,785	0,256	0,955	0,238	0,104	0,286	0,321	1,092	0,285	0,154	0,419	0,522	0,474	0,407	0,312	0,774	0,793	0,768	0,450	0,213
Glucose-1-Phosphate	0,772	0,955	0,463	0,335	0,247	0,342	0,484	0,428	0,362	0,013	0,703	0,791	0,219	0,232	0,284	0,895	1,122	0,849	0,046	0,074
D,L- α -Glycerol Phosphate	0,300	0,068	0,083	0,011	0,022	0,083	0,069	0,038	0,136	0,021	0,214	0,159	0,097	0,084	0,081	0,069	0,026	0,123	0,079	0,094
D-Galactonic Acid γ-Lactone	1,313	1,148	1,333	1,352	1,075	1,124	0,605	1,338	0,614	0,614	0,835	0,773	0,733	1,307	1,241	1,012	1,055	1,287	0,787	0,504
D-Galacturonic Acid	1,874	1,106	1,156	0,614	0,568	1,323	0,543	1,249	0,480	0,212	0,942	1,024	0,719	0,711	0,767	1,301	0,712	0,812	0,243	0,257
2-Hydroxy Benzoic Acid	0,132	0,100	0,191	0,059	0,130	0,283	0,083	0,220	0,130	0,286	0,118	0,069	0,070	0,225	0,121	0,249	0,104	0,205	0,190	0,288
4-Hydroxy Benzoic Acid	0,806	0,331	0,521	0,231	0,294	0,425	0,179	0,598	0,340	0,143	0,924	1,023	0,752	0,297	0,115	0,919	0,467	1,154	0,775	0,566
γ-Hydroxybutyric Acid	1,555	0,649	0,144	0,234	0,689	1,321	0,509	0,422	0,193	0,032	0,441	0,478	0,307	0,816	0,111	0,556	0,251	1,247	0,115	0,084
Itaconic Acid	0,366	0,217	0,620	0,159	0,177	0,187	0,175	0,675	0,174	0,047	0,830	0,431	1,028	0,660	0,357	0,588	0,447	0,768	0,602	0,379
α -Ketobutyric Acid	0,259	0,075	0,217	0,082	0,053	0,160	0,145	0,402	0,098	0,082	0,201	0,381	0,124	0,322	0,050	0,129	0,044	0,866	0,131	0,221
D-Malic Acid	0,649	0,630	0,744	0,413	0,353	0,368	0,186	0,812	0,477	0,631	0,365	0,614	0,902	0,764	0,384	0,736	0,445	0,465	0,602	0,724
L-Arginine	1,048	0,616	0,997	0,899	0,388	0,753	0,275	0,771	0,849	0,144	1,024	1,149	1,280	0,700	0,427	1,077	0,702	0,885	0,385	0,256
L-Asparagine	1,609	1,173	1,496	1,430	0,718	1,683	0,810	1,553	1,102	0,699	1,427	1,469	1,553	1,598	1,186	1,824	1,938	2,128	1,894	1,320
L-Phenilalanine	0,337	0,223	0,877	0,131	0,123	0,316	0,110	1,175	0,105	0,053	0,158	0,313	0,263	0,528	0,259	0,655	0,512	0,758	0,933	0,338
L-Serine	0,305	0,434	0,702	0,046	0,102	0,180	0,218	0,780	0,478	0,482	0,290	0,835	0,262	0,454	0,059	1,496	0,370	0,642	0,599	0,506
L-Threonine	0,096	0,055	0,288	0,065	0,029	0,052	0,079	0,223	0,218	0,099	0,060	0,119	0,037	0,092	0,028	0,251	0,291	0,198	0,192	0,119
Glycyl-L-Glutamic Acid	0,185	0,053	0,256	0,131	0,085	0,181	0,086	0,442	0,149	0,044	0,199	0,278	0,261	0,402	0,258	0,221	0,104	0,323	0,830	0,737
Phenil-ethyl-amine	0,171	0,058	0,098	0,079	0,080	0,039	0,149	0,225	0,193	0,066	0,167	1,409	0,975	0,294	0,098	0,141	0,262	0,205	0,309	0,102
Putrescine	0,777	0,002	0,288	0,075	0,126	0,186	0,054	0,175	0,304	0,111	0,317	0,917	0,416	0,074	0,118	0,748	0,844	0,783	0,710	0,333

Tab. 3.2. OD_{590nm} values at the end (170 hours) of plates incubation in soil microcosms. Green and red colors represent induced or repressed substrate consumption, respectively. Comparisons of means were conducted using one way ANOVA and Duncan test at level of $p<0.05$.

decrease metabolic activity with respect to the negative control when applied without a N source but AWCD values were increased in ED treatment, suggesting that a few effluent strains were the main responsible of substrates consumption. Soils are thought to be an oligotrophic and carbon limited habitat (Killham, 1994). Incubating soil extracts at high concentrations of readily decomposable organic substrates may favour copiotrophic microbes able to grow explosively (r-strategists) (Buyer *et al.*, 2002), outcompeting the oligotrophic species (K-strategists) in the wells.

Biolog CLPPs do not reflect the functional abilities of the entire soil microbial community, but only that of a very limited subset of microbial genera. Therefore, CLPP data should be carefully interpreted and always accompanied by complementary techniques.

3.4.2. Abundance and expression of N-cycling genes

Figures 3.6-3.11 show the changes in the bacterial 16S rRNA, bacterial *amoA*, archaeal *amoA*, *nirK*, *nirS* and *nosZ* gene copy numbers quantified using qPCR assays. The number of ammonia oxidizers in certain environments is relatively low (about 10^4 to 10^6 cells g $soil^{-1}$), making real-time PCR, which is highly sensitive, a convenient method for enumeration of these microrganisms. Real-time PCR can also be used to determine a broad range of starting molecule template concentrations spanning at least 5 orders of magnitude (Heid *et al.*, 1996). Thus, it is also suitable for quantifying soil microrganisms with population dynamics radically different than those of ammonia oxidizers.

The 16S rRNA gene copy numbers during the first 7 days of incubation range from 4.19 x 10^8 for D at day 1 to 2.56 x 10^9 for ED at day 7; however, these differences were not significant (Fig. 3.6). There was no significant change in bacterial and archaeal *amoA* gene copy numbers either, throughout the course of the experiment, which ranged from 2.38 x 10^5 for C at day 1 to 1.84 x 10^6 for ED at day 7, and from 1.71 x 10^5 for ED at day 7 to 5.74 x 10^5 for E at day 1, respectively. It has been found that AOB population size increased in seven days after applying of N-fertilizer (Okano *et al.*, 2004). Regardless of treatments, copy numbers of archaeal amoA genes were considerably more stable than their bacterial counterparts (Fig. 3.6, 3.7). Other quantitative studies

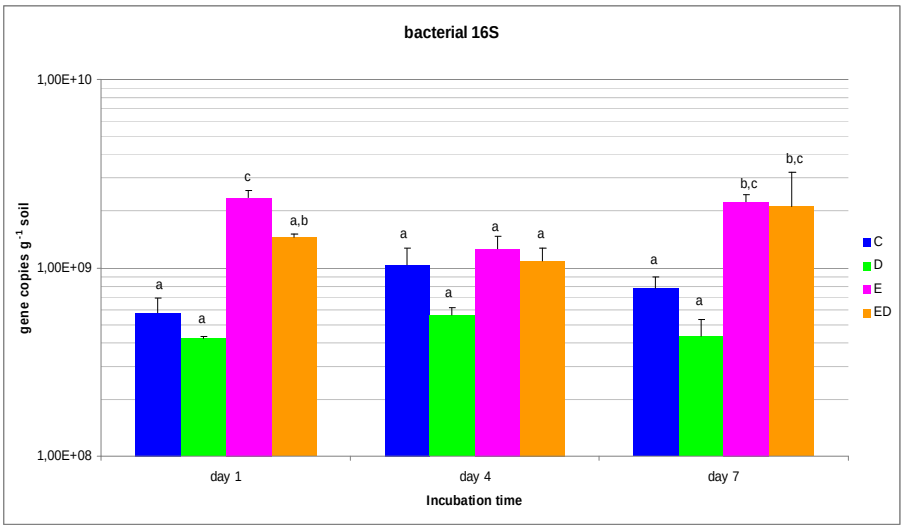

Fig. 3.6. Bacterial 16S *rRNA* gene copy numbers g^{-1} dry weight (dw) soil after 1, 4, 7 days of incubation by qPCR. Comparisons of means were conducted using one way ANOVA and Duncan test at level of $p<0.05$.

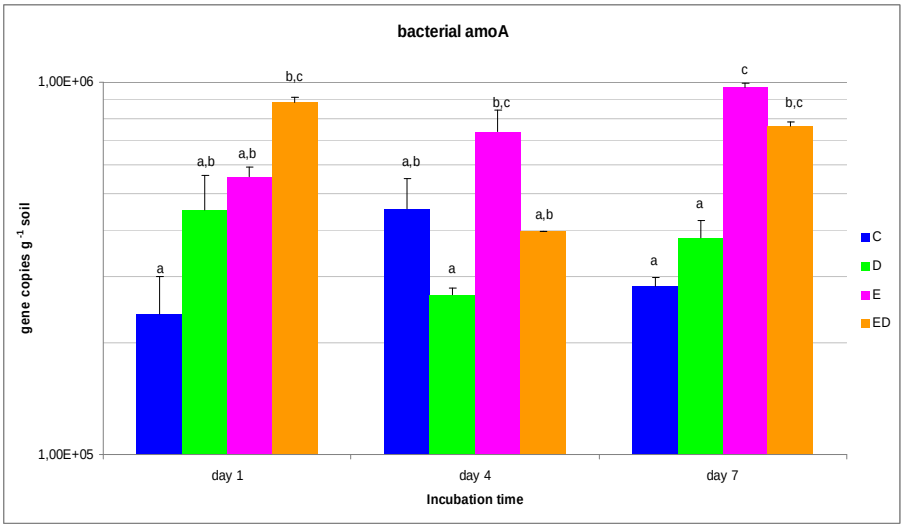

Fig. 3.7. Bacterial *amoA* gene copy numbers g^{-1} dry weight (dw) soil after 1, 4, 7 days of incubation by qPCR. Comparisons of means were conducted using one way ANOVA and Duncan test at level of $p<0.05$.

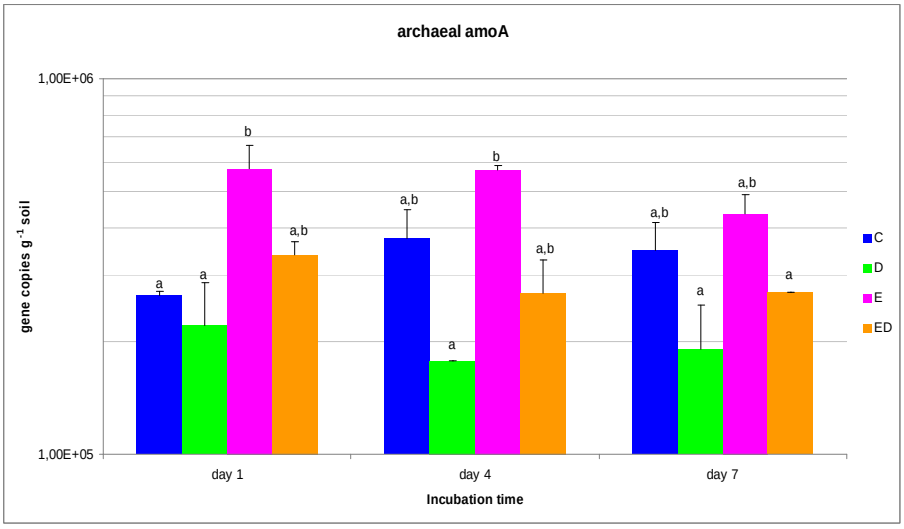

Fig. 3.8. Archaeal *amoA* gene copy numbers g^{-1} dry weight (dw) soil after 1, 4, 7 days of incubation by qPCR. Comparisons of means were conducted using one way ANOVA and Duncan test at level of $p<0.05$.

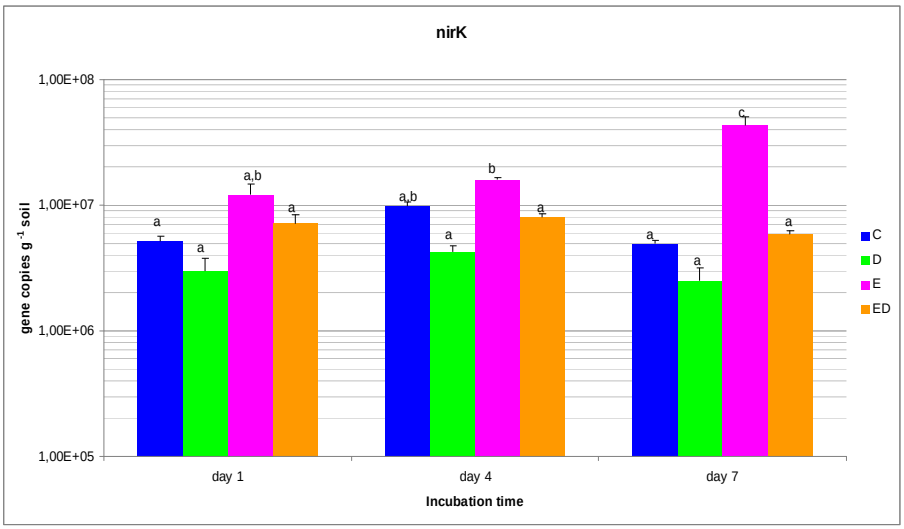

Fig. 3.9 *nirK* gene copy numbers g^{-1} dry weight (dw) soil after 1, 4, 7 days of incubation by qPCR. Comparisons of means were conducted using one way ANOVA and Duncan test at level of $p<0.05$.

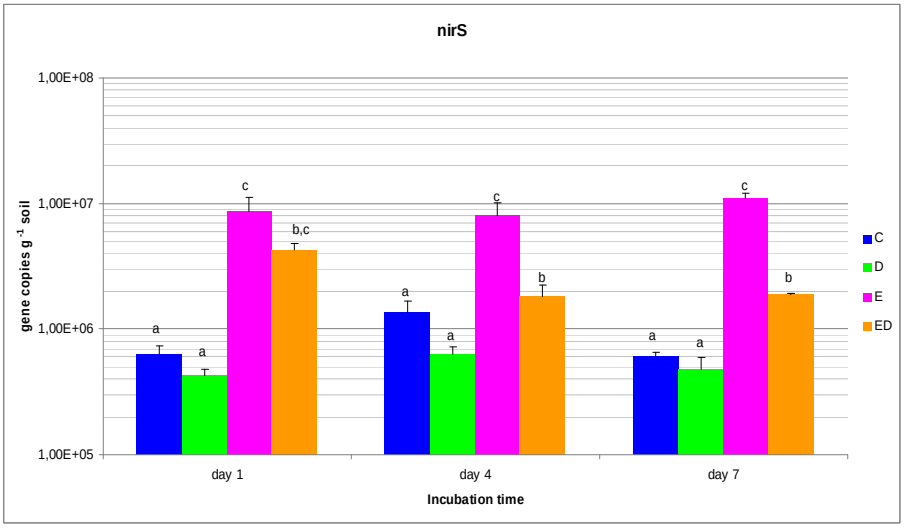

Fig. 3.10. *nirS* gene copy numbers g^{-1} dry weight (dw) soil after 1, 4, 7 days of incubation by qPCR. Comparisons of means were conducted using one way ANOVA and Duncan test at level of $p<0.05$.

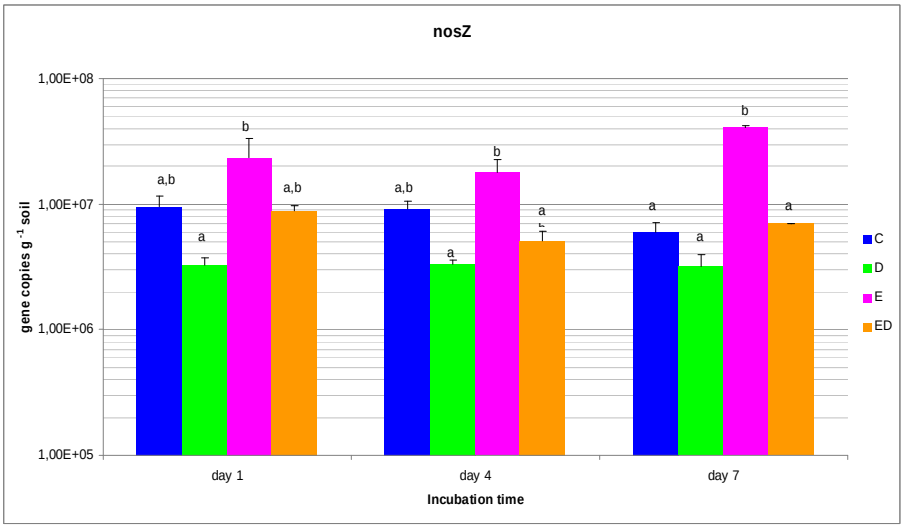

Fig. 3.11. *nosZ* gene copy numbers g^{-1} dry weight (dw) soil after 1, 4, 7 days of incubation by qPCR. Comparisons of means were conducted using one way ANOVA and Duncan test at level of $p<0.05$.

(Francis *et al*., 2005; Hai *et al*., 2009) revealed that the AOA are more stable and less responsive to environmental differences than AOB. Several recent studies have suggested that archaea may play a significant role in ammonium oxidation in soil (Leininger *et al*., 2006; Prosser and Nicol, 2008). Although several studies have identified archaeal amoA genes in soils, and transcriptional activity has been demonstrated (Leininger *et al*., 2006; Mertens *et al*., 2009; Nicol *et al.*, 2008; Treusch *et al.*, 2005), the actual role of AOA in N-cycling remains unclear and may vary between soil types and conditions. Schauss *et al.* (2009) showed AOA were potentially responsible for up to half of nitrification activity in manured soil. Both Nitrosospira- and Nitrosomonas-like 16S rDNA have been detected in swine manure (Ceccherini *et al.*, 1998); thus, cattle effluent contained its own microbial populations and AOB were found at a concentration of 3.91×10^4 gene copy number per g effluent detected by real-time PCR.

However, the high numbers in various ecosystems and at greater soil depths indicate that AOA are adapted to a broad range of growth conditions and might therefore have a more versatile metabolism than AOB, perhaps being able to grow mixotrophically.

However, the relative abundance of genes involved in denitrification showed a different pattern: *nirK* gene copy numbers were $1.6 \times 10^7/8.02 \times 10^6$ (day 4) and $4.32 \times 10^7/5.95 \times 10^6$ (day 7) in E/ED treatments, respectively; *nirS* gene copy number were $8.03 \times 10^6/1.8 \times 10^6$ (day 4) and $1.1 \times 10^7/1.88 \times 10^5$ (day 7) in E/ED treatments, respectively, whereas *nosZ* gene copy numbers varied even at day 1: $2.37 \times 10^7/8.75 \times 10^6$ (day 1), $1.8 \times 10^7/5.08 \times 10^6$ (day 4), and $4.05 \times 10^7/6.95 \times 10^6$ (day 7) in E/ED treatments, respectively. This indicates that denitrifiers microrganisms were significantly affected by the application of cattle effluent and cattle effluent combined with DMPP.

It is known that N fertilization can change the structure and activity of denitrifying community, and subsequently affect the N_2O emission (Dambreville *et al*., 2006; Kramer *et al.*, 2006). The ratio N_2O/N_2 rises if NO_3^- is abundant in the soil because the latter is preferred to N_2O as electron acceptor (Schlegel, 1992). Hence, if nitrate production is inhibited by DMPP, even denitrification genes will be found at lower concentrations where the inhibitor was added.

Figures 3.12-3.15 show the changes in the bacterial 16S rRNA, bacterial *amoA*, archaeal *amoA*, and *nosZ* mRNA levels quantified using RT-qPCR assays. In contrast to

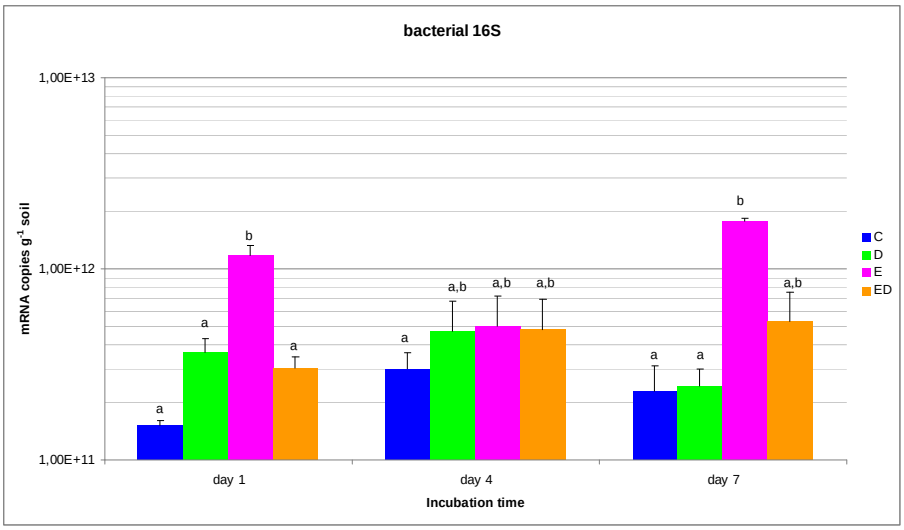

Fig. 3.12. Bacterial 16S *rRNA* mRNA copy numbers g^{-1} dry weight (dw) soil after 1, 4, 7 days of incubation by qPCR. Comparisons of means were conducted using one way ANOVA and Duncan test at level of $p<0.05$.

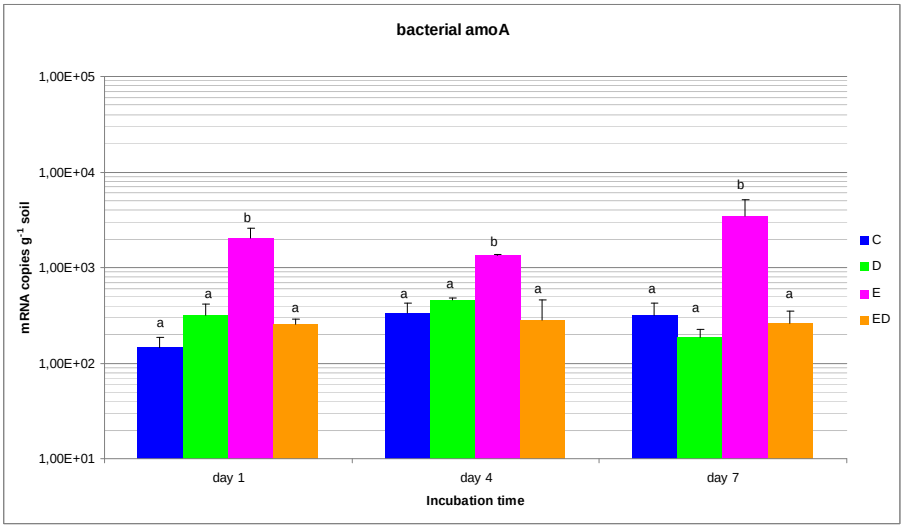

Fig. 3.13. Bacterial *amoA* mRNA copy numbers g^{-1} dry weight (dw) soil after 1, 4, 7 days of incubation by qPCR. Comparisons of means were conducted using one way ANOVA and Duncan test at level of $p<0.05$.

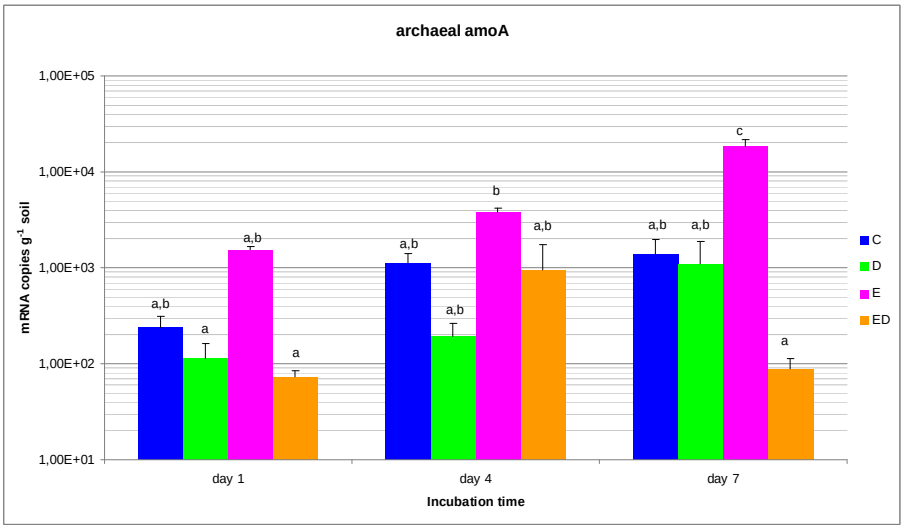

Fig. 3.14. Archaeal *amoA* mRNA copy numbers g^{-1} dry weight (dw) soil after 1, 4, 7 days of incubation by qPCR. Comparisons of means were conducted using one way ANOVA and Duncan test at level of $p<0.05$.

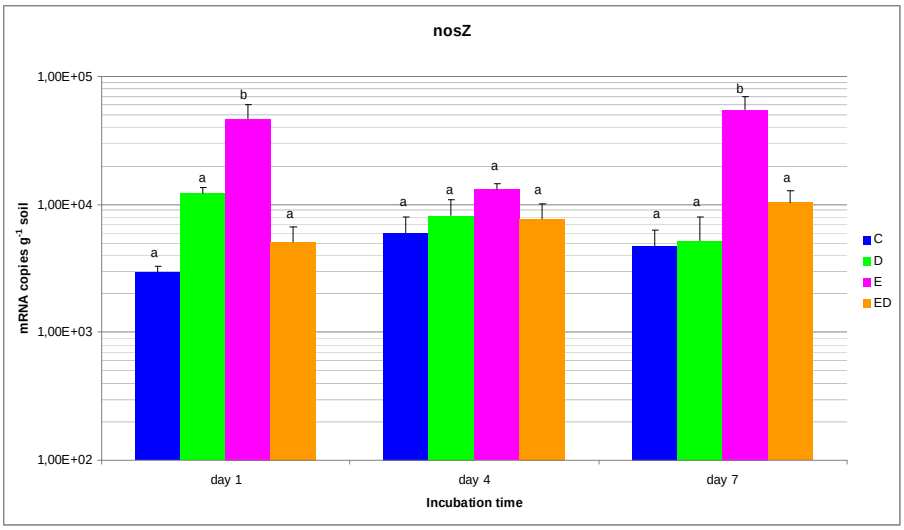

Fig. 3.15. *nosZ* mRNA copy numbers g^{-1} dry weight (dw) soil after 1, 4, 7 days of incubation by qPCR. Comparisons of means were conducted using one way ANOVA and Duncan test at level of $p<0.05$.

gene copy number, gene expression showed significant differences in mRNA levels for all the genes detected at 1 and 7 days but not ad 4 days of microcosms incubation: 16S rRNA mRNA levels decreased from from 1.17 x 10^{12} to 3.04 x 10^{11} at day 1 and from 1.77 x 10^{12} to 5.32 x 10^{11} mRNA copy number in E/ED treatments; bacterial *amoA* mRNA levels decreased from 2.02 x 10^{3} to 2.54 x 10^{2} at day 1 and from 3.43 x 10^{3} to 2.62 x 10^{2} mRNA copy number in E/ED treatments; archaeal *amoA* mRNA levels decreased from 1.56 x 10^{3} to 7.27 x 10^{1} at day 1 and from 1.82 x 10^{4} to 8.90 x 10^{1} mRNA copy number in E/ED treatments; *nosZ* mRNA levels decreased from 4.66 x 10^{4} to 5.09 x 10^{3} at day 1 and from 5.52 x 10^{4} to 1.04 x 10^{4} mRNA copy number in E/ED treatments. Rapid changes in gene expression occurred in DMPP + cattle effluent treatment: after 24h hours and after 7 days of incubation the molecule inhibited ammonia oxidizers population in soil microcosms. Hence, it was demonstrated for the first time that the nitrification inhibitor DMPP, that is known to inhibit AOB population, showed its effectiveness even against archaeal ammonia oxidizers.

The inhibitor was found to be active against denitrifiers (*nosZ* mRNA levels) after 1 and 7 days of incubation too, showing a positive effect towards N_2 production and consequently a positive implication both from economically and environmental perspective.

However, even bacterial 16S gene expression was reduced where DMPP was applied (Fig. 3.12), suggesting a possible effect on non-target populations by the molecule.

3.4.3. Structural changes of metabolically active microbial communities

The structural differentiation of metabolically active microbial communities during the first 7 days of microcosms incubation were analyzed by T-RFLP.

Molecular fingerprinting techniques target the most dominant microbial community members (Muyzer and Smalla, 1998) and can be biased due to the formation of heteroduplex, PCR selection and PCR drift (Suzuki and Giovannoni, 1996; Head *et al.*, 1998). Therefore, it might be possible that the contribution of less abundant species were not included in this approach. Hence, both archaeal and bacterial community structures were profiled by T-RFLP of 16S rRNA (rather than DNA) to increase sensitivity and potentially target the predominantly active members of the microbial community (Felske and Akkermans, 1998). Great sensitivity will arise through the high copy number of 16S rRNA per cell and growth and activity may be detected through increase in ribosome content prior to cell division.

Figures 3.16 and 3.17 show the number of bacterial and archaeal T-RFs, respectively, found in soil microcosms after 1, 4 and 7 days of incubation. Bacterial T-RFs were more impacted by treatments when compared to their archaeal counterparts: at day 1 bacterial T-RFs in only-DMPP microcosms were significantly lower than the other treatments, and after 4 and 7 days a significant difference between E and ED microcosms was detected. In line with the greater level of stability of the archaeal

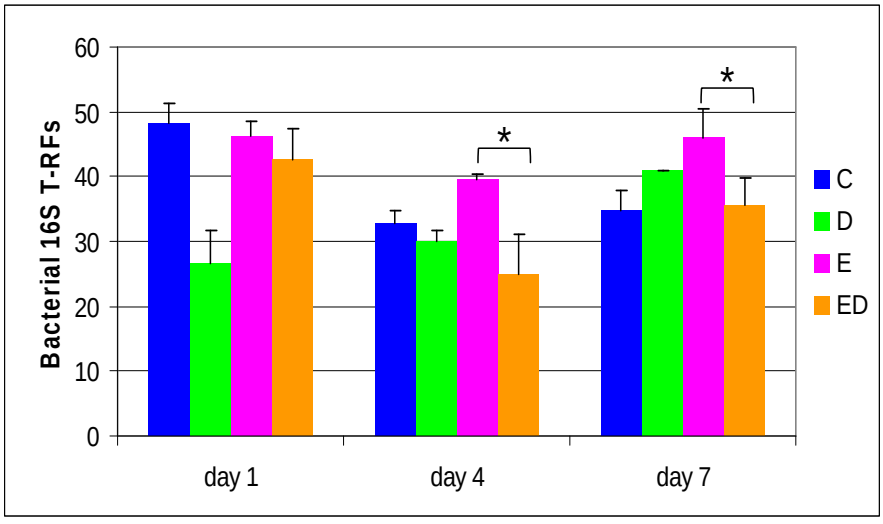

Fig. 3.16. Number of bacterial T-RFs after 1, 4, 7 days of incubation by qPCR. Comparisons of means were conducted using one way ANOVA and Duncan test at level of $p<0.05$. *Represents values significantly different.

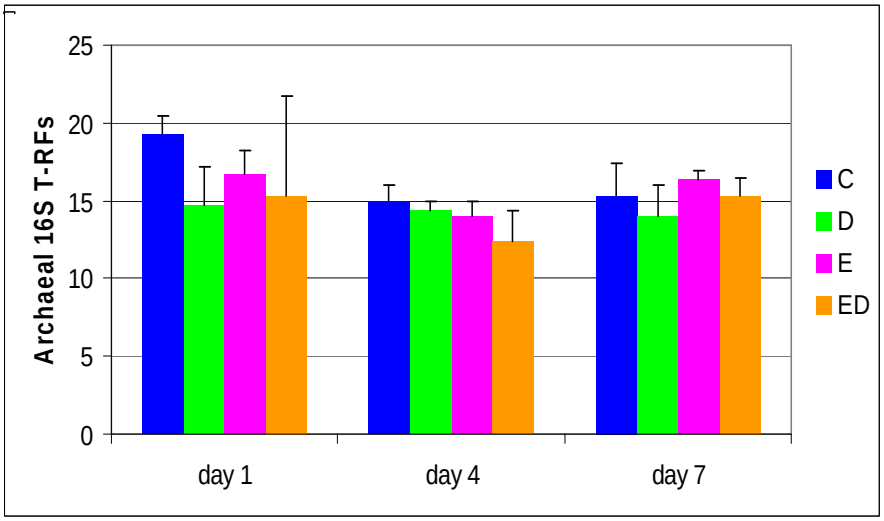

Fig. 3.17. Number of archaeal T-RFs after 1, 4, 7 days of incubation by qPCR. Comparisons of means were conducted using one way ANOVA and Duncan test at level of $p<0.05$. *Represents values significantly different.

community, the number of archaeal 16S T-RFs fluctuated less than those of bacteria throughout the present experiment, and no statistical difference could be detected.

Principal Component Analysis (PCA) was conducted for analysis of species abundance data: the analysis compared to set of descriptor, T-RFs versus treatments and reduced the multidimensional relationship between them to two principal components. In all cases on the first principal component loadings (PC1) a strong laddering occurred, and this was due to the fact that a major fraction of the T-RFs found were not affected to any of the microcosm treatments (Figg. 3.18-3.23); furthermore, most of the time PC1 explained a great percentage of variance, ranging from 44 to 50% and from 75 to 96% for bacterial and archaeal population, respectively. Hence, most of the total metabolically active microbial population was insensitive to the treatments. However, significant differences in metabolically active microbial community structure were evident across treatments. At day 1, the bacterial community structure was more similar in the two microcosms where cattle effluent was applied, whether or not the presence of the nitrification inhibitor, and was distinct from that without effluents (Fig. 3.18). Some T-RFs were specifically associated with the effluent (157.7, 417.7, 155.5, 101), whereas some others were associated with the control without effluent (D) (113.5, 111.7, 45.3, 124.6, 120.8) or C (424.4, 456.6). The first two components explained the 66% of the total variance; microcosms with DMPP were negatively affected by PC2, and “C” and “D” microcosms were negatively affected by PC3 (which explained 17% of the total

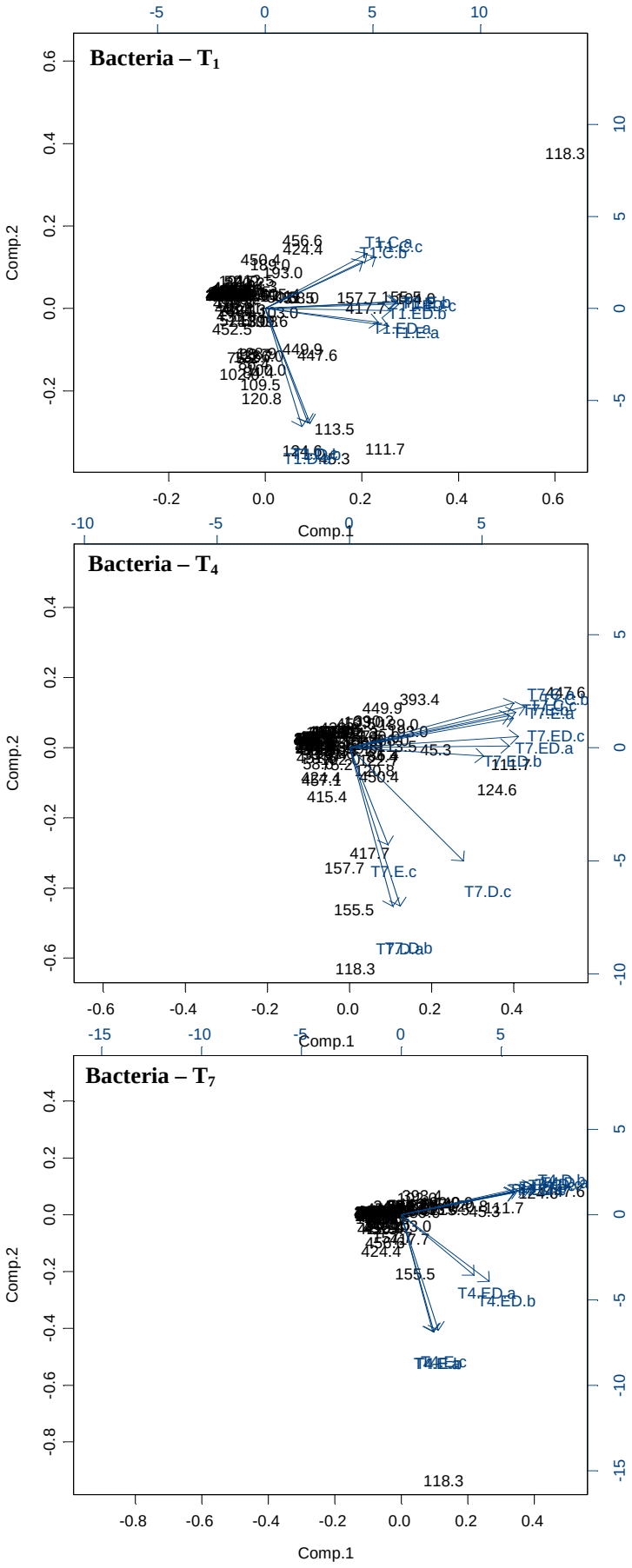

Fig. 3.18. Principal component analysis (PCA) biplot of metabolically active bacterial community structure in soil microcosms after 1 day of incubation. Only the first principal component (PC1) and the second principal component (PC2) were shown. Treatments: soil only (C), soil + DMPP (D), soil + cattle effluent (E), soil + cattle effluent + DMPP (ED).

Fig. 3.19. Principal component analysis (PCA) biplot of metabolically active bacterial community structure in soil microcosms after 4 days of incubation. Only the first principal component (PC1) and the second principal component (PC2) were shown. Treatments: soil only (C), soil + DMPP (D), soil + cattle effluent (E), soil + cattle effluent + DMPP (ED).

Fig. 3.20. Principal component analysis (PCA) biplot of metabolically active bacterial community structure in soil microcosms after 7 days of incubation. Only the first principal component (PC1) and the second principal component (PC2) were shown. Treatments: soil only (C), soil + DMPP (D), soil + cattle effluent (E), soil + cattle effluent + DMPP (ED).

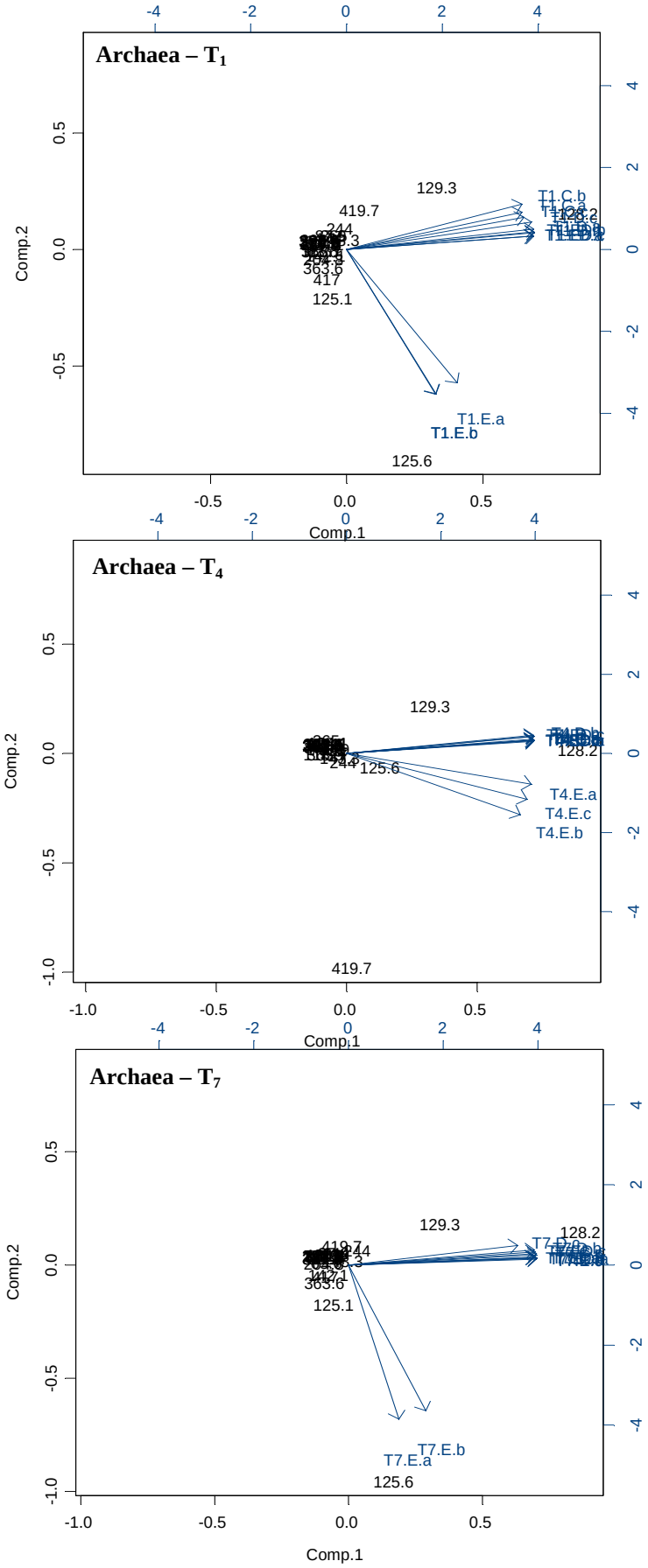

Fig. 3.21. Principal component analysis (PCA) biplot of metabolically active archaeal community structure in soil microcosms after 1 day of incubation. Only the first principal component (PC1) and the second principal component (PC2) were shown. Treatments: soil only (C), soil + DMPP (D), soil + cattle effluent (E), soil + cattle effluent + DMPP (ED).

Fig. 3.22. Principal component analysis (PCA) biplot of metabolically active archaeal community structure in soil microcosms after 4 days of incubation. Only the first principal component (PC1) and the second principal component (PC2) were shown. Treatments: soil only (C), soil + DMPP (D), soil + cattle effluent (E), soil + cattle effluent + DMPP (ED).

Fig. 3.23. Principal component analysis (PCA) biplot of metabolically active archaeal community structure in soil microcosms after 7 days of incubation. Only the first principal component (PC1) and the second principal component (PC2) were shown. Treatments: soil only (C), soil + DMPP (D), soil + cattle effluent (E), soil + cattle effluent + DMPP (ED).

variance) whereas PC4, which explained the 12% of the total variance, was able to discriminate E (positively) and ED (negatively) treatments. Archaeal community structure after 1 day of incubation was relatively stable: C, D and ED microcosms didn't show any differences, only cattle effluent microcosms were affected in a different way when compared to the others on PC2, but it explained only the 20% of the total variance occurred; peak 125.6 seems to be strongly associated to E.

After 4 days of incubation metabolically active bacterial community structure of E and ED was affected differently from their controls without cattle effluent and the inhibitor, and differently from each other on PC2. T-RFs 118.3 and, to a lesser extent, 155.5 were associated with E. These results indicate that application of cattle effluent alone or in conjunction with DMPP was the dominant force driving shifts in the genetic diversity of the active soil bacterial communities. As for day 1, at day 4 archaeal population was stable across treatments, except for E treatments which were lightly separated by PC2 explaining only 3% of the variation.

After 7 days of microcosms incubation PCA discriminated in a similar way of what found for day 4, even if ED seems to be less separated from C and D when compared to day 4. Archaeal populations didn't show any differences, confirming that there is minimal metabolic response of archaea to treatments.

It has been suggested that higher species richness increases ecosystem functionality and its functional stability (Tilman, 1996; Bell *et al.*, 2005). In this study, high active species richness was found by a high number of TR-Fs spread over the profile. However, it has been suggested that population diversity alone does not drive ecosystem stability. Redundancy of function may be much more important for understanding the stability of microbial communities, and of the ecosystem functions they perform (Fernandez *et al.*, 1999; Briones and Raskin, 2003; Franklin and Mills, 2006).

Over time, it was observed that a small percentage of the TR-Fs corresponded with a high percentage of the total area of peaks, indicating a medium functional organization according to Marzorati *et al.* (2008). This internal structuring demonstrated that at each moment only a small group of metabolically active species was numerically dominant in the microcosms. The remaining less dominant TR-Fs represent a pool of species that can proliferate to replace the current dominant species, according to the concept of functional redundancy (Fernandez *et al.*, 1998; Wittebolle *et al.*, 2008).

3.5. Conclusions

As expected, total bacteria population was higher where cattle effluent was applied, as it cointains its own microbial population, but no massive differences were found between treatments in qPCR. On the contrary, rapid changes in gene expression occurred in DMPP + effluent treatment: after 24h bacteria and archaea *amoA* mRNA were lower with respect to the effluent treatment, due to a high efficacy in the inhibition of the ammonia-oxidizing populations, but even bacteria 16S gene expression was reduced, suggesting a possible effect on non-targeted populations by DMPP. These results were confirmed by profiling analysis on active microbial community being separated in different ways on PCA, although application of cattle effluent combined with DMPP did not cause a massive change in the structure of the active general archaeal community. It's important to identify the parameters influencing AOA and AOB populations in soils and to quantify and compare their specific activities under varying environmental conditions. Given that archaea contribute significantly to nitrification, as their abundance now suggests, estimates of the ecological impact of ammonia oxidation, including greenhouse gas emissions, based on bacterial ammonia-oxidizing activity should be re-assessed.

The use of artificial chemical inhibitors to disrupt natural soil processes initially seemed contrary to wide ecological concepts of soil quality and conservation of biological diversity (Cuttle, 2008). However, the high level of environmental hazard imposed by ongoing land use intensification and fertilizer application necessitates the use of remedial measures to prevent further environmental degradation. Given the importance of maintaining soil quality and function, it is essential that remedial soil treatments such as nitrification inhibitors do not impact negatively on non-target soil microbial population. The findings of the current work highlight the need to understand how different treatments impact on soil properties, soil functions and the microbial communities responsible, in order to optimise crop cultivation and land management.

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Chapter 4 – Application of subsurface liquid manure and nitrification inhibitor 3,4-Dymetylpyrazole Phosphate (DMPP) to reduce nitrates leaching and gaseous N emissions.

4.1. Introduction

The efficiency and sustainability of agriculture production is highly dependent on interactions within the soil–water–atmosphere–plant–animal ecosystem. Soil, water, and the atmosphere provide a growth medium that enables sustained plant and animal production and play critical roles in recycling processes. While modern agricultural systems have provided considerable benefits to the growing world population, these activities have also produced some undesirable environmental consequences including damage to soil, water, and atmospheric resources and deleterious effects on ecosystem and human health (Dockery and Pope, 1994; Dockery *et al*., 1996; Künzli *et al*., 2000).

Soil is a quite heterogeneous system, with aerobic and anaerobic microsites not homogeneously distributed along its profile, consequently nitrification and denitrification can occur simultaneously and N_2O can be evolved from soil via both these processes (Nielsen *et al*., 1995; Abbassi and Adams, 1998; 2000). As illustrated by the "hole in the pipe" model of Davidson (Davidson, 1991) besides being influencd by rates of nitrification and denitrification, N_2O emissions depend on how soil physico-chemical parameters affect the ratio of end-products via both the processes and on how much fast, after being produced, N_2O gas can diffuse through gaseous phase of soil to the atmosphere. (Fig. 4.1) (Davidson *et al.*, 2000).

Manure is a valuable resource that needs to be managed effectively and efficiently. Land application of manure should not be considered simply a disposal system. Manure provides nutrients for crops and helps build and maintain soil fertility. Manure can also improve soil structure, increase water-holding capacity, lessen wind and water erosion, improve aeration, and promote beneficial organisms. Available land for manure application is an important consideration for all livestock operations. When planning a new operation or expanding an existing operation, adequate land area for manure

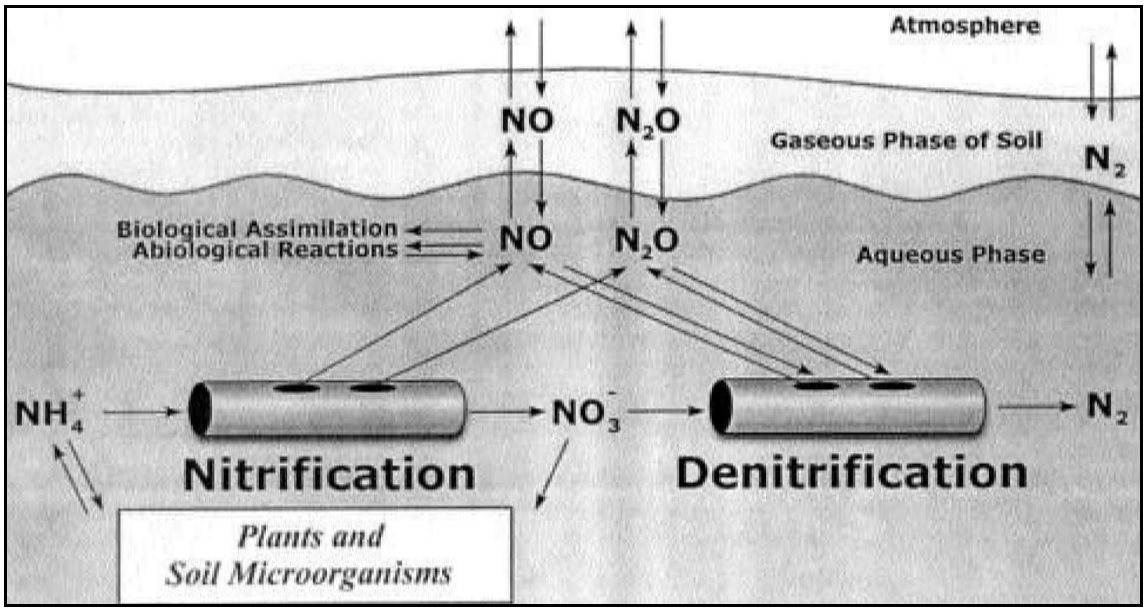

Fig. 4.1. Diagram of the hole-in-the-pipe conceptual model (revised from Davidson, 1991). Soil emissions of NO and N_2O are regulated at two levels: First, the rate of nitrogen cycling through ecosystems, which is symbolized by the amount of nitrogen flowing through the pipes, affects total emissions of NO and N_2O; second, soil water content and perhaps other factors affect the ratio of N_2O:NO emissions, symbolized by the relative sizes of the holes through which nitric oxide and nitrous oxide "leak" (from Davidson *et al*., 2000).

application must be included in the plan. A conservative approach in determining the amount of land required is to consider the removal of the nutrient by the harvested crop. This will ensure that enough land area is available in future years to prevent nutrient buildup in the soil beyond recommended agronomic and environmental levels. The whole farm nutrient management should consider procedures for balancing nutrient inputs and outputs on the farm; furthermore, best management practices (BMPs) for applying manure to crops should be used. In order to maximize manure nutrients while minimizing potential environmental impacts, manure application must consider nutrient losses during handling and storage, runoff and preferential flow, and timing and rate of application; additional issues should be considered when applying liquid manure and wastewater. To minimize the risk of runoff or preferential flow, site inspection and preparation are important; in addition, hourly application rates must be controlled.

The application of liquid manure could offer several advantages:

- ✓ large amounts of effluent can be spread in a relatively short time;
- ✓ waste effluent can be used to supplement irrigation water and to supply plant nutrients where regular crop irrigation is practical;
- ✓ irrigation may cost less than other land-application methods, and requires less work for equivalent volumes of application;
- ✓ a high degree of automation is possible with some types of irrigation equipment;
- ✓ manure guns (nozzles) can handle slurry directly from confinement and wash-down operations.

However, liquid manure applied to tile drained fields presents a risk of the liquid manure following preferential flow paths, such as worm holes, cracks, old root channels, some of which connect directly to subsurface drains and are a direct route to surface water. Anything that promotes good drainage will increase the risk of preferential flow of liquid manure to subsurface drains.

Animal feeding operations (AFOs) can have a significant effect on gaseous emissions. The primary pollutants of concern include volatile organic compounds (VOCs), particulate matter (coarse particulates, PM_{10} and fine particulates, $PM_{2.5}$), and ammonia (NH_3), which also serves as a secondary particulate matter (NRC, 2003). Other problematic emissions include methane (CH_4), nitrous oxide (N_2O), various nitrogen oxides (NO_x), and hydrogen sulfide (H_2S).

Ammonia emissions have been recently estimated from confined and rangeland AFOs in the United States (Fig. 4.2; USEPA, 2004), and are projected to be nearly 2400 Gg per year nationally by 2030, an increase of 12% over 2010. Dairy and beef production is the largest contributor to the national ammonia inventory, with approximately 50% of the total emissions (Fig. 4.2; NRC, 2003; USEPA, 2004, Yates *et al.*, 2011).

Agricultural operations can contribute significantly to the emissions of PM to the atmosphere. Here, NH_3 primarily reacts to form ammonium sulphate and ammonium nitrate aerosols, which contribute to particulate matter with an aerodynamic diameter < 2.5 μm formation ($PM_{2.5}$). Exposure to PM has serious health consequences and is linked to increased heart and lung disease and to premature mortality (Samet *et al.*, 2000). Particles with diameters of 10 μm (PM_{10}) or less can penetrate the lungs deeply,

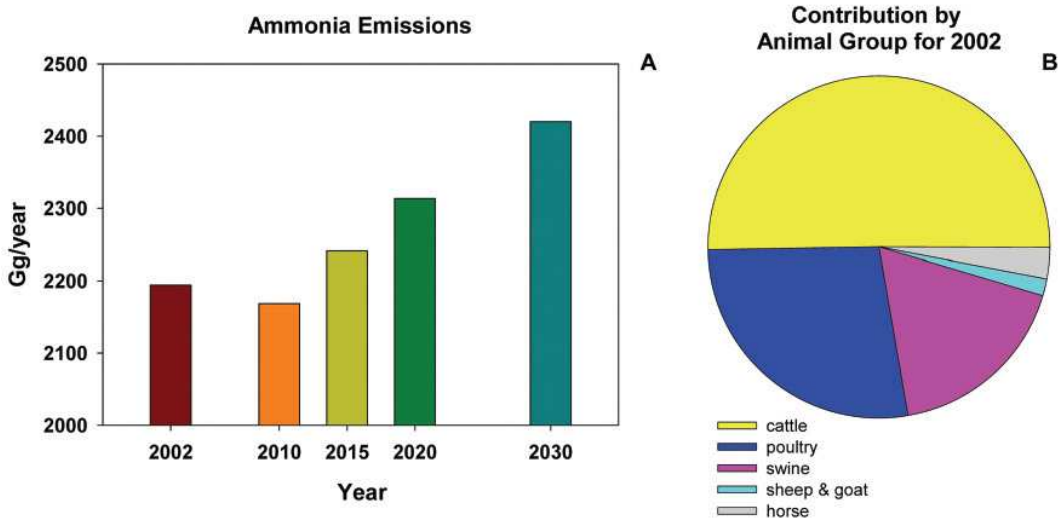

Fig. 4.2. (A) Estimated ammonia emissions (Gg/yr) from 2002 until 2030 from animal feeding operations; the data include cattle, swine, sheep, goat, and poultry. (B) The contribution (%) from each animal group using data from 2002. (From Yates *et al.*, 2011, adapted from USEPA, 2004) .

and very small particles can pass through the lungs and affect other organs. Exposure to PM may even have the potential to affect non respiratory systems such as the central nervous system (Ngo *et al.*, 2010). The particle-size fraction less than 2.5 μm has received much more regulatory attention and associated research due to its potential to cause severe health effects compared with larger-sized particles.

Besides, NH_3 concentrations in poultry rearing facilities can reach very high levels, causing poor poultry performance (Carlisle, 1984). Anderson *et al.* (1964) showed that NH_3 levels as low as 20 μl L^{-1} compromised the immune system of chickens and damaged the respiratory system of the birds. This is particularly important at present due to the threat posed by avian influenza.

Emission of PM from land preparation exhibits a seasonal pattern that varies depending on field activities associated with specific crops, and emissions related to AFOs depend on the structure of the facility and the activity of animals (Cambra-López *et al.*, 2010; McGinn *et al.*, 2010). Physical characteristics of emission sources, particle-size distributions of the PM emitted, and natural dispersion of emissions into the environment cause difficulties in quantifying PM emissions from agricultural operations

(Wanjura *et al.*, 2009). There is still a significant need for researching accurate methods to quantify particulate emissions from agricultural operations.

Most of NH_3 losses occur in the first 24 h following the manure application, and they are dependent on weather variables such as temperature and wind speed, and on pH, moisture content, ammonium content, and soil properties, such as cation exchange capacity and surface residue cover (Reddy *et al.*, 1979; Meisinger and Jokela, 2000). Of the manure characteristics, the most important is manure pH, with higher losses of NH_3 occurring at pH values above 7.0.

There is very good evidence to show that substantial reductions in NH_3 losses can be expected with subsurface application compared with surface broadcasting. These reductions are achieved by limiting the manure surface area exposed to the air and by increasing immobilisation of ammonium because of greater contact of manure with soil. This can be accomplished with soil incorporation of solid manures by tillage soon after application or by direct soil-injection of liquid slurries. Increased N recovery from manures when they are applied by improved technologies is generally attributed to decreased NH_3 volatilization (Dell *et al*., 2011). Although the extent of NH_3 reduction varies among studies, values from 40 to over 90% have commonly been reported (Dosch and Guster, 1996; Meisinger and Jokela, 2000; Wulf *et al*., 2002).

A substantial number of best management practices (BMPs) associated with manure applications, ranging from riparian buffers to diet modification aimed at decreasing nutrient concentrations in manure (Maguire *et al.*, 2007), are designed to lower nutrient losses to surface waters. One BMP that has not been heavily studied, and has not been widely adopted, is manure placement below the soil surface. Kronvang *et al*. (2009) reported that future research should be focused on filling the knowledge gap of novel manure management, including manure injection. There is hope that improved manure management, such as manure injection, can improve N capture through limiting NH_3 volatilization as well as decreasing negative impacts of N and P loss on surface waters. Surface application of manure is currently favoured by most farmers because it is fast and relatively cheap, whereas technologies such as manure injection require more expensive equipment and are typically slower (Maguire, 2008). However, improved N use efficiency by crops will help to compensate for some of these constraints since

manure injection almost eliminates NH_3 volatilization losses (Sommer and Hutchings, 2001), substantially increasing manure N availability to crops (Maguire, 2008).

Although subsurface manure applications can almost eliminate NH_3 volatilization, they are theoretically susceptible to greater denitrification losses due to the close juxtaposition of the available C that drives microbial activity with nitrate resulting from nitrification of manure ammonium (Wulf *et al.*, 2002), and could offset as much as half of the N conserved in some cases (Dell *et al.*, 2011). On a global basis, the estimated agricultural emissions of CH_4 and N_2O are about 47 and 58% of the total anthropogenic sources (Smith *et al.*, 2007). For N_2O, emissions are primarily associated with N fertilization and application of animal manure. In regions associated with large livestock numbers, high levels of CH_4 occur due to enteric fermentation (USEPA, 2006). Large emissions of CH_4 are also associated with rice production. Agricultural emissions of CH_4 and N_2O are likely to increase significantly by 2030 due to a combination of increased fertilizer use, increased livestock and rice production, and increased land application of animal manure (Smith *et al.*, 2007). The USEPA (2006) estimated that N_2O emission will increase from approximately 8 Tg in 1990 to over 11 Tg by 2020, with emissions from soil representing the largest source (Fig. 4.3).

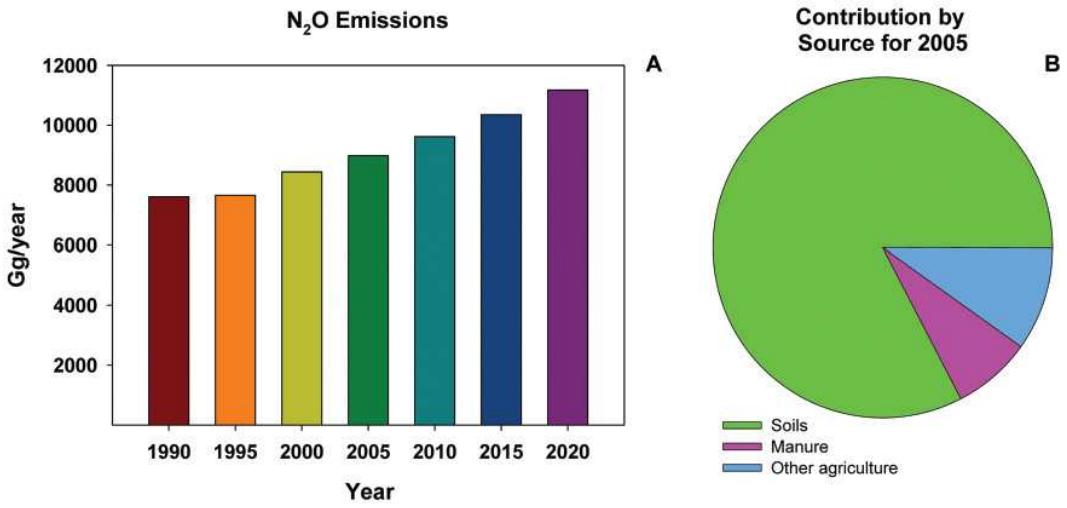

Fig. 4.3. (A) Estimated N_2O emissions (Gg/yr) from 1990 until 2020 from agricultural sources. (B) The contribution (%) from each source group using data from 2005 (From Yates *et al.*, 2011, adapted from USEPA, 2006).

Future estimates have relatively high levels of uncertainty because the effects of potential mitigation methodologies to decrease emissions remain largely unknown (Yates *et al.*, 2011).

Concentrations of N_2O in the troposphere continue to increase at 0.26% yr^{-1} (Forster *et al.*, 2007). Countries that are signatories to the Kyoto Protocol have committed to reducing their GHG emissions. The first commitment period (2008-2012) requires these Kyoto participant countries to take responsibility for their excess emissions over and above a base level set in 1990. Such action may involve offsetting the surplus emissions against carbon credits or the mitigation of the GHG emissions. To take such measures, countries must construct national GHG inventories using clearly defined methodologies defined by the Intergovernmental Panel on Climate Change (IPCC) (IPCC, 1997).

Global agricultural N_2O emissions are thought to equate to 6.3 Tg N yr^{-1}. The IPCC distinguishes between direct and indirect N_2O emissions. Direct emissions include the microbial conversion of fertilizer or excreta nitrogen to N_2O in agricultural soils or animal waste management systems, whereas indirect emissions involve the production of N_2O from agricultural N that is transported off the agricultural site, such as leaching. Indirect emissions account for approximately one third of global N_2O emissions, with N leaching and N runoff accounting for over 75% of estimated indirect emissions (Mosier *et al.*, 1998; Nevison, 2000). Uncertainty in the total agricultural N_2O source is dominated by a paucity of information and the wide range of estimates for indirect N_2O emissions. (Nevison, 2000).

Although reduction in N fertilizer application rates may be an effective means of reducing N_2O emissions, this may come at the cost of decreased yields depending on the level of fertilizer input before any change (Millar *et al*., 2010). Alternative practices that could reduce N_2O emissions without necessarily reducing N inputs or crop yields have also been considered, such as optimization of fertilizer source (Halvorson *et al.*, 2010), or modification of tillage regime (Omonode *et al*., 2011). However, the effectiveness of these practices in reducing N_2O emissions may vary depending on local soil and other conditions (Akiyama *et al*., 2010).

Subsurface manure applications may also increase leaching losses due to possible channelling of preferential flow through the zone of high nitrate produced from

nitrification and the mineralization of organic-N within the manure band (Shipitalo and Gibbs, 2000). Leaching N losses can be highly site specific because they are greatly influenced by precipitation, soil properties, topography, and land management. Placement of concentrate bands of manure belowground could increase N leaching if manure bands intercept preferential flow pathways. There are only a few reports of N leaching in conjunction with subsurface manure application. Ball-Coelho *et al.* (2006) found increased leaching to tile drains with swine manure injection at high application rates, but they saw no impact of application method when manure N additions did not exceed crop N requirement. Wielsen *et al.* (1998) found that nitrate leaching to tile drainage was not affected by shallow, closed trench injection of swine slurry. These available data suggest that in most cases manure injection is not likely to substantially increase leaching losses if manure is applied at rates consistent with crop needs. However, additional research is required to fully understand how these conditions affect N leaching after subsurface manure applications.

The use of nitrification inhibitors like nitrapyrin, dicyandiamide (DCD) and 3,4-dimetylpyrazole phosphate (DMPP) with injected manure has the potential to decrease denitrification losses and N_2O emissions by causing a disconnect between the nitrate production and the peak of metabolism of readily metabolized manure C. Most readily available manure C can metabolize within 2 weeks of application (Comfort *et al.*, 1990); therefore, delaying nitrification until after that time can reduce the quantity of nitrate that is present during the period when high rates of microbial metabolism of manure C can lead to conditions that favour denitrification. Thompson *et al.* (1987) reported that the addition of the nitrification inhibitor nitrapyrin to injected manure resulted in denitrification losses that were comparable to those from surface-applied manure in their winter experiment; however, nitrapyrin addition only reduced loss about 20% in the spring experiments. NIs have been shown to decrease leaching and denitrification from both mineral fertilizers and urine patches in grazed pastures (Di *et al.*, 2007; Monaghan *et al.*, 2009) and, under some conditions, can lead to improved pastures productivity (Moir *et al.*, 2007; Sprosen *et al.*, 2008; Zaman and Blennerhasset, 2010).

Estimating the N balance for a complex N source like manure is challenging because manure interacts with many pathways in the soil-crop N cycle. Dell *et al.* (2011) reported that the range of estimates of the N losses to the various pathways for injected manure slurries is quite wide (Fig. 4.4), due to cumulative uncertainties in the estimation of the total N balance for a system.

The biggest challenge to N research with subsurface manure application is understanding and managing the soil-manure transformation to improve crop N use efficiency. Improved N use efficiency for manure requires a holistic management approach that involves improving the N rate recommendations and allowing for all N sources, the timing of manure applications in relation to the hydrologic cycle, and the placement of manure to reduce N losses through NH_3 volatilization, denitrification, and leaching.

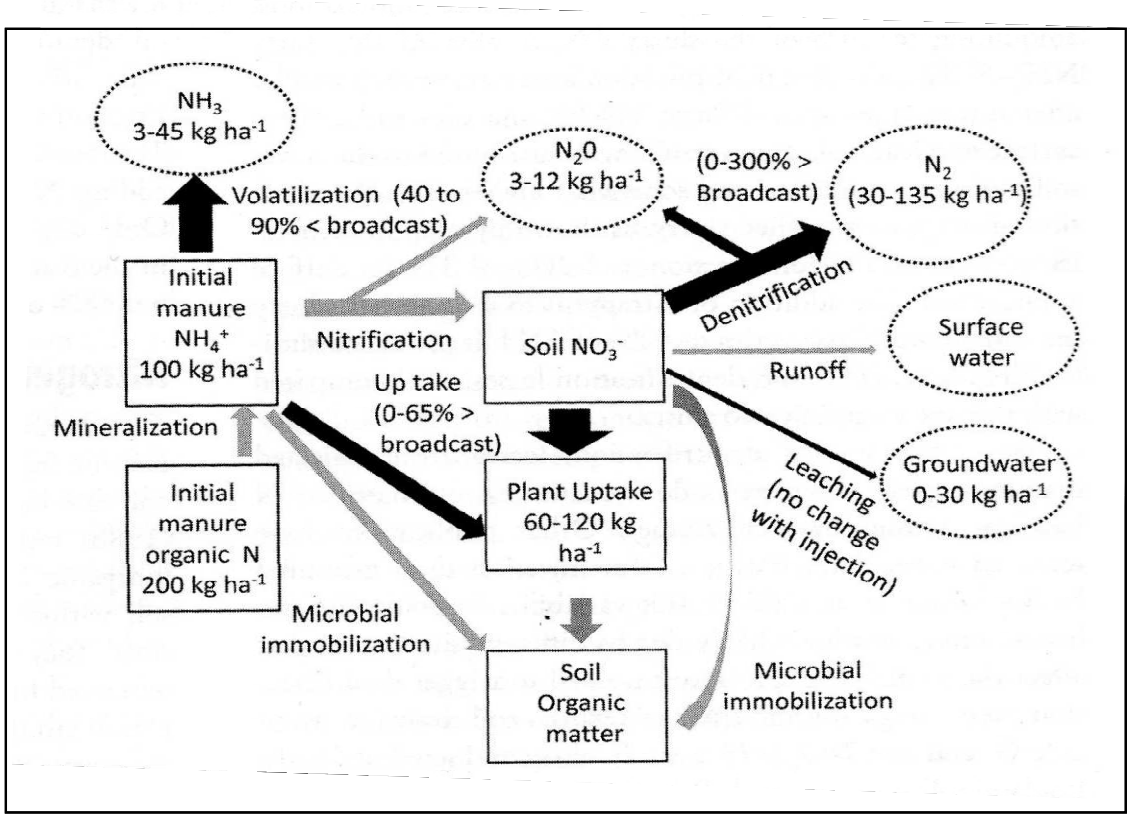

Fig. 4.4. A conceptual model of the nitrogen cycle for the injection of liquid dairy slurry with a total application rate of 300 kg N ha^{-1}. Black arrows represent transformation where data exist to estimate the impact of injection. Estimates of total losses from all pathways would range from about 36 to 222 kg N ha^{-1}. (from Dell *et al.*, 2011).

4.2. Experimental field

The main goal of this study was to evaluate the efficacy of the simultaneous application of subsurface liquid manure and the nitrification inhibitor DMPP in reducing nitrogen losses from a Mediterranean short-rotation forestry field.

This study was conducted in a *Eucalyptus*, short rotation, high-density plantations field managed by the research unit for intensive wood production (CRA-PLF) situated in Rome (Italy). The 20 year-old experimental field with initial low-density plant spacing (1100 plants/ha) was converted in 2007 into a high-density plant spacing (5000 plants/ha) (Fig. 4.5). Main physico-chemical and microbiological characteristics of the soil are reported in Table 2.1 (Chapter 2).

Fig. 4.5. *Eucalyptus*, short rotation, high-density plantations field managed by CRA-PLF, Rome. The 20 year-old experimental field with initial low-density plant spacing (1100 plants/ha) was converted in 2007 into a high-density plant spacing (5000 plants/ha).

In Figure 4.6 a schematic organization of the *Eucalyptus* field is reported. At the bottom of each box *Eucalyptus* clone names are indicated (*E. camaldulensis*, *E. bridgesiana*, *E.* clones number 7, 20, 40…) whereas numbers in brakes indicate the number of individuals for each clone.

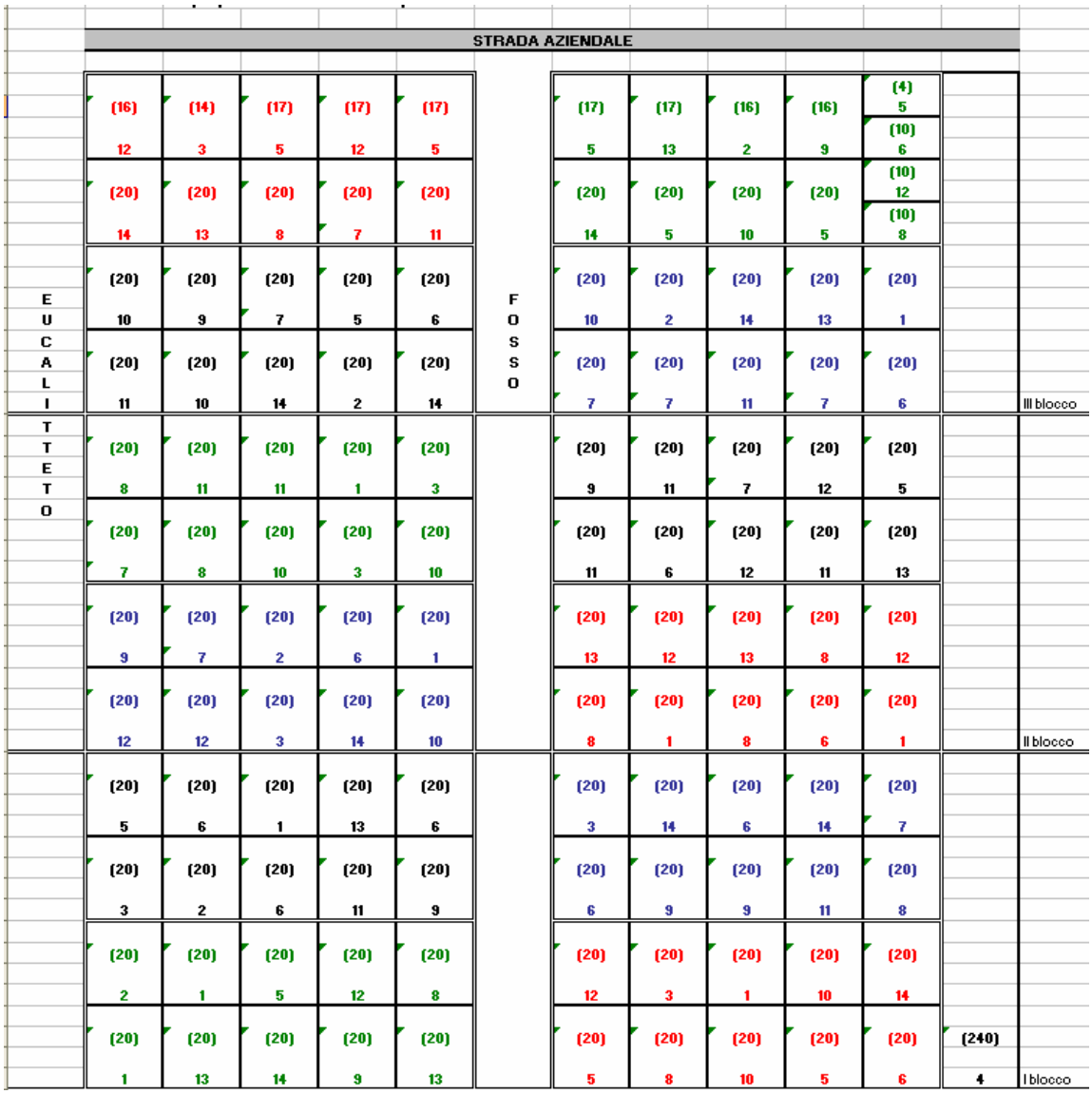

Fig. 4.6. Schematic organization of the *Eucalyptus* field. At the bottom of each box *Eucalyptus* clone names are indicated (*E. camaldulensis*, *E. bridgesiana*, *E.* clones number 7, 20, 40…) whereas numbers in brakes indicate the number of individuals for each clone. Treatments were: cattle effluent (green), cattle effluent + DMPP (red), cattle effluent + ENTEC® (black), control (blue).

The cattle effluent used was obtained from a dairy farm located in Rome; it was sampled after thorough stirring and blending and analyzed by standard laboratory methods to assess the physico-chemical properties shown in Table 2.2 (Chapter 2).

Four treatments were selected:

1) Cattle effluent
2) Cattle effluent + DMPP
3) Cattle effluent + ENTEC 46®
4) Control

Liquid formulation of DMPP (25%, provided by K+S Nitrogen, Italy) containing 1,86% mineral N (1,1% N-NO_3^-, 0,76% NH_4^+-N) was used at a final concentration of 1% according to the NH_4^+-N content, as recommended by the providers (Zerulla *et al.*, 2001). ENTEC 46®, a commercial DMPP-containing ammonium fertilizer (K+S Nitrogen, Italy) was used in combination with cattle effluent, too.
On June 2011, liquid formulation of DMPP or ENTEC 46®, combined with liquid manure, were applied to subsurface soil at a rate of 170 kg N ha^{-1} and 240 kg N ha^{-1}, respectively. Lysimeters were installed to measure nitrates leaching losses, whereas ammonia emissions were detected as reported by Pascholsky *et al*. (2006). The Dräger-Tube Method (DTM) was developed by Richter (1972) for the measurement of CO_2 evolution from soil and was adapted for the measurement of NH_3 volatilization in arable soils following mineral N fertilization (Roelcke, 1994; Roelcke *et al.,* 2002). Ambient air is sucked through a chamber by means of a hand pump (Drägerwerk AG, Lübeck, Germany). The air is then led through Teflon tubing to an NH_3 sensitive Dräger gas analysis detector tube (Drägerwerk AG, Lübeck, Germany) which immediately displays the NH_3 concentration (in volume ppm) (Figure 4.7). Ammonia fluxes were calculated according to the following equation:

$$F_{Ng} = \text{volume} * |\text{conc}| * 10^{-6} * \rho NH_3 * U_N * U_F * U_Z$$

where F_{Ng} is NH_3 flux (mg N m^{-2} h^{-1}); volume is air volume sucked through the chambers (l); |conc.|: value of NH_3 vol. concentration (volume-ppm); ρNH_3 is temperature-dependent density of NH_3 (mg l^{-1}); U_N is molecular weight conversion factor of NH_3 to N; U_F is surface area conversion factor (m^{-2}); U_Z is time conversion

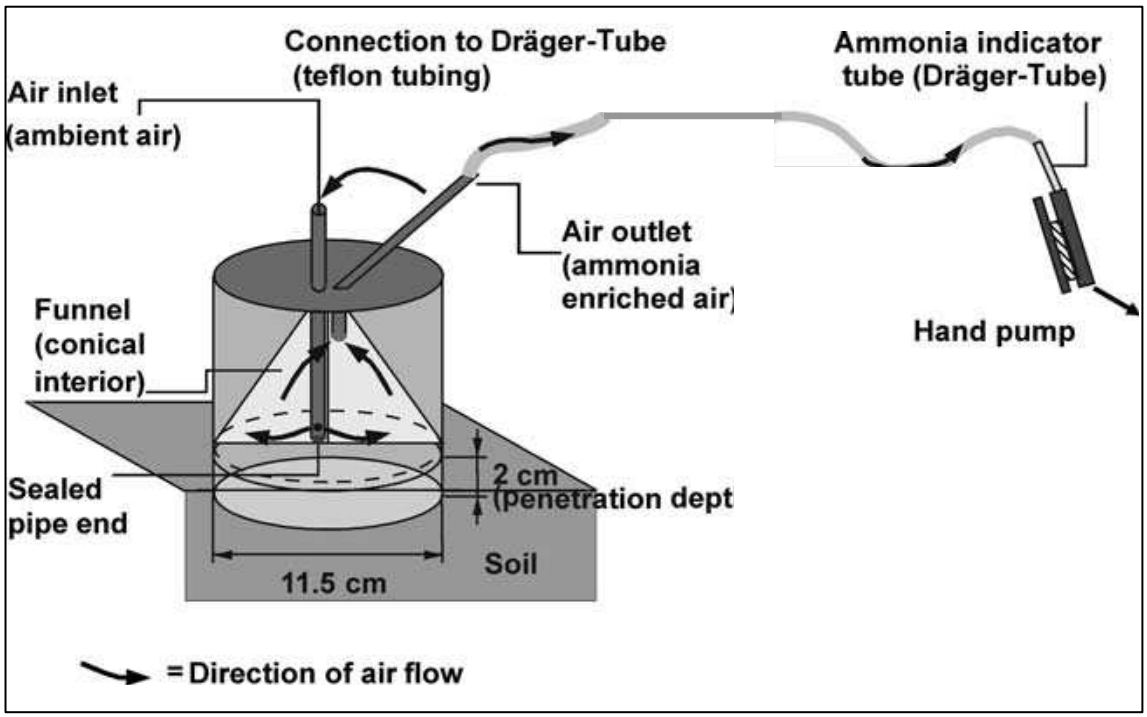

Figure 4.7. Experimental set-up of the Dräger-Tube Method applied with a hand pump. From Pascholsky *et al.* (2006), modified.

factor (h^{-1}).

The NH_3 volume concentration values displayed on the Dräger-Tubes were corrected by a factor for the barometric air pressure and air temperature. Because of the way of NH_3 enrichment involved and the limited sensitivity of the Dräger NH_3 indicator tubes employed (>0.025 vol. ppm NH_3), only fluxes higher than the quasi-natural NH_3 efflux close to the soil surface can be detected using this method.

Unfortunately, during the first three months after fertilization no enough rain occurred, therefore no leached were retrieved and nitrates losses couldn't be detected, whereas ammonia emissions and environmental data are in progress.

However, a similar experimental study was set up in Manerbio, Brescia (Italy) in a field corn crops, and nitrogen leaching data handling is in progress.

4.3. References

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Overall conclusions

The planting of short-rotation forestry can lead to several economic, territorial, social and environmental advantages, in terms of biodiversity conservation (Muys *et al.*, 1992), improvement of carbon storage (Montagnini and Nair, 2004; Lagomarsino *et al.*, 2009), enhancing the fertility of soils (Manna *et al.*, 2003), yields of goods and services to society, and providing social and economic well-being to people (Pandey, 2007). *Eucalyptus* is an ideal energy crop with certain species and hybrids having excellent biomass productivity, relatively low lignin content and a short rotation time (Hinchee *et al.*, 2009). Potential site nutrient depletion and high water use concerns have led to an interest being developed in linking SRF crop production with the recycling of nutrients from the land treatment of aqueous wastes, such as sludge and effluent arising from animal housing (cow slurry, pig slurry, poultry manure and liquid sewage sludge), municipal sewage and food and fibre processing industries.

Manure is a valuable resource that needs to be managed effectively and efficiently. Land application of manure should not be considered simply a disposal system. Manure provides nutrients for crops and helps build and maintain soil fertility. Manure can also improve soil structure, increase water-holding capacity, lessen wind and water erosion, improve aeration, and promote beneficial organisms.

Animal feeding operations (AFOs) can have a significant effect on gaseous emissions, as well as on nitrogen leaching. The primary pollutants of concern include volatile organic compounds (VOCs), particulate matter (coarse particulates, PM_{10} and fine particulates, $PM_{2.5}$), and ammonia (NH_3), which also serves as a secondary particulate matter (NRC, 2003). Other problematic emissions include methane (CH_4), nitrous oxide (N_2O), various nitrogen oxides (NO_x), and hydrogen sulfide (H_2S). Although subsurface manure applications can almost eliminate NH_3 volatilization, they are theoretically susceptible to greater denitrification losses due to the close juxtaposition of the available C that drives microbial activity with nitrate resulting from nitrification of manure ammonium (Wulf *et al.*, 2002), and could offset as much as half of the N conserved in some cases (Dell *et al.*, 2011). These potentially higher losses to denitrification and leaching could offset the reduction in NH_3 volatilization. Therefore,

it is important to develop a more complete understanding of the effects of subsurface manure applications on the fate of manure N.

The use of nitrification inhibitors like nitrapyrin, dicyandiamide (DCD) and 3,4-dimetylpyrazole phosphate (DMPP) with injected manure has the potential to decrease denitrification losses and N_2O emissions by causing a disconnect between the nitrate production and the peak of metabolism of readily metabolized manure C.

Thus, the main goal of this PhD project work was to evaluate and to establish the effectiveness of the nitrification inhibitor DMPP on a Mediterranean agroforestry system combined with a cattle effluent, and to study its effects on soil microbiota involved in N cycling processes. Firstly, a long-term incubation experiment was conducted to evaluate the influence of fertilizer (mineral or organic), temperature (30°C and 20°C), soil microbiological properties (using two soils that differ mainly in C and N content and in microbial activity and biomass) and timing of application of the inhibitor (if concomitant or prior to fertilizer addition) on the effectiveness of DMPP as nitrification inhibitor. Furthermore, experimental microcosms were set up to evaluate the effect of the addition of the nitrification inhibitor DMPP combined with a cattle effluent as organic fertilizer on culturable heterotrophic microbial metabolism, as well as on the abundance and expression both of target (ammonia oxidizers and denitrifiers populations), and non-target microbial populations. Finally, nitrogen cycle dynamics were approached in a Mediterranean agroforestry system managed with zootechnical wastes and the nitrification inhibitor DMPP.

The results of the long-term incubation study markedly show the efficacy of DMPP as nitrification inhibitor under every laboratory conditions investigated. The simultaneous application of DMPP and fertilizer resulted to be the more efficient option with respect to the application of the nitrification inhibitor prior to mineral N addition, since in the latter case more N was mineralized in both Casalotti and Celimontano soil at the end of the incubation period.

Microbiological results in microcosm studies show high efficacy in the inhibition of the ammonia-oxidizing populations and for the first time it was demonstrated the inhibition of ammonia-oxidizing archaea transcripts in soil by DMPP. Given that archaea contribute significantly to nitrification, as their abundance now suggests, estimates of

the ecological impact of ammonia oxidation, including greenhouse gas emissions, based on bacterial ammonia-oxidizing activity should be re-assessed.

The findings of the current work highlight the need to understand how different treatments impact on soil properties, soil functions and the microbial communities responsible, in order to optimise crop cultivation and land management.

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List of main publications/poster presentations

1) Florio A., Dell'Abate, M.T., Grego S., Benedetti A., 2011. Environmental Quality EQA 6, 105-114.

EFFECTS OF A NITRIFICATION INHIBITOR (3,4-DMPP) AND A BIOSTIMULANT ON POTENTIALLY MINERALIZED NITROGEN OF A SOIL AMENDED WITH BOVINE SLURRY IN A SHORT ROTATION FORESTRY (SRF) SYSTEM

EFFETS D'UN INHIBITEUR DE LA NITRIFICATION (3,4-DMPP) ET D'UN BIOSTIMULANT ADDITIONNÉS À UN EFFLUENT ZOOTECHNIQUE SUR LE POTENTIEL DE MINERALISATION DE L'AZOTE DU SOL EXPLOITÉ POUR LA SYLVICULTURE À COURTE ROTATION

EFFETTI DI UN INIBITORE DELLA NITRIFICAZIONE (3,4-DMPP) E DI UN PRODOTTO BIOSTIMOLANTE ADDITIVATI AD UN EFFLUENTE ZOOTECNICO SULLA MINERALIZZAZIONE POTENZIALE DELL'AZOTO IN UN SUOLO ADIBITO A SHORT ROTATION FORESTRY (SRF)

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Summary

The element nitrogen (N) is an essential nutrient for plant development and its intake is a key factor for crop production; however, its real availability in soil, as well as its chemical form, are crucial for the nitrogen uptake by crops. Agriculture is one of the main responsible of N compounds emission (e.g. ammonia, nitrate, nitrous oxide) causing negative impacts to the environment. N excess due to high livestock density results in intensification of environmental pollution. Hence, N emissions and leaching from agriculture to the environment have to be further reduced.

Council Directive 91/676/EEC (known as the Nitrates Directive) aims to protect water quality across Europe by preventing nitrates from agricultural sources polluting ground and surface waters and by promoting the use of good farming practices.

Nitrification inhibitors (NI) are compounds that delay the nitrification process, that consists in the oxidation of the ammonium in nitrate made by soil microorganisms such as *Nitrosomonas* and *Nitrobacter*. As a result, the delay of the oxidation of the ammonium present in the fertiliser into nitrate for a certain period of time offers the chance to reduce N losses and to increase fertilizer use efficiency.

Recently, nitrification inhibitors have been applied with livestock manures in Northern Europe to prevent N emissions and leaching and delay nitrification process in soil.
The aim of this work is to study the effects of the nitrification inhibitor DMPP (3,4 dymethylpyrazole phosphate) and a biostimulant on microbial diversity of a soil amended with bovine slurry in a Short Rotation Forestry (SRF) system. In this study, nitrate and ammonia release during the incubation has been investigated, so to establish the potentially mineralized N content.
Key words: *nitrification inhibitors; Short Rotation Forestry; DMPP; livestock manures; biostimulant*

Résumé

L'azote représente l'élément nutritif fondamental pour le correct développement végétatif des plantes et son apport est certainement un facteur clé pour la production agronomique; toutefois, la disponibilité réelle de l'élément, ainsi que sa forme chimique, constituent des aspects indispensables pour l'assimilation de l'azote par les plantes. L'usage excessif de fertilisants dans les terrains accouplé à une absorption nulle des plantes peut provoquer des problèmes de lixivation et d'érosion.
La directive 91/676/CEE du Conseil (connu comme la directive Nitrates) fixes des critères à utiliser pour contenir la pollution due aux nitrates de sources agricoles, sur la base de la connaissance de la zone pedoclimatique, des réalités des entreprises, des moyens techniques et de la prédisposition d'un plan de fertilisation sur la base de la balance de l'azote.
Les fertilisants avec des inhibiteurs de la nitrification influencent surtout la forme d' azote dans le sol: en inhibant le processus de nitrification, qui consiste en l'oxydation des ions ammonium en ions nitrate par les microorganismes du sol du genre *Nitrosomonas* et *Nitrobacter*, l'élément sera surtout présent en forme ammoniacale, et donc moins mobile que le nitrate, mais toujours en forme minérale et ainsi promptement disponible. La capacité de ces produits de relâcher l'azote dans la moyenne – longue période pourrait en permettre un emploi différent par rapport aux fertilisants traditionnels de synthèse.
Récemment, les inhibiteurs ont été redécouverts pour la possibilité d'être utilisés pour la protection de l'environnement. L'addition de inhibiteurs de nitrification a été expérimentée, en Europe, aussi dans le cas des effluents zootechniques, afin de retarder la nitrification de l'élevée quantité d'azote ammoniacal présent dans le purin, et donc en augmenter l'efficacité.
L'objectif du travail est d'étudier l'effet de la molécule antinitrifiante DMPP (3,4 dimetyl-pyrazole phosphate) et d'un modulateur de l'absorption d'azote additionné à un effluent zootechnique bovin sur le cycle de l'azote et sur la communauté microbienne nitrifiante d'un sol exploité pour la sylviculture à courte rotation. À ce propos, le relâchement d'azote nitrique et ammoniacal dans les temps a été étudié, obtenant des courbes de cession potentielle de l'azote en présence des deux produits additionnés et non au substrat organique.

Mots-clès: *inhibiteur de la nitrification ; sylviculture à courte rotation; DMPP; effluents zootechniques; biostimulants*

Riassunto

L'azoto rappresenta l'elemento nutritivo fondamentale per il corretto sviluppo vegetativo delle piante ed il suo apporto è certamente un fattore chiave per la produzione agronomica; tuttavia, la reale disponibilità dell'elemento, nonché la sua forma chimica, costituiscono aspetti imprescindibili per l'assimilazione dell'azoto da parte delle piante. L'uso eccessivo di fertilizzanti nei terreni unitamente ad un mancato assorbimento da parte delle piante può provocare problemi di lisciviazione e dilavamento, oltre che perdite per volatilizzazione.
La direttiva 91/676/CEE del Consiglio (nota come "direttiva Nitrati") fissa dei criteri da utilizzare per contenere l'inquinamento da nitrati delle acque superficiali e profonde da fonti agricole sulla base della conoscenza dell'ambiente pedoclimatico, della realtà aziendale, dei mezzi tecnici e della predisposizione di un piano di fertilizzazione basato sul bilancio dell'azoto.
La tipologia di concimi con inibitori della nitrificazione influenza soprattutto la forma di azoto nel suolo: inibendo il processo di nitrificazione, che consiste nell'ossidazione degli ioni ammonio in ioni nitrato ad opera di microrganismi del suolo del genere *Nitrosomonas* e *Nitrobacter*, l'elemento sarà presente prevalentemente in forma ammoniacale, quindi meno mobile del nitrato, ma pur sempre minerale e perciò prontamente disponibile. La capacità di questi prodotti di rilasciare azoto nel medio-lungo periodo ne potrebbe consentire un impiego diverso rispetto ai concimi di sintesi tradizionali.
Recentemente gli inibitori hanno ricevuto rinnovata attenzione per la possibilità di utilizzarli a scopi di protezione ambientale. L'addizione di inibitori di nitrificazione è stata sperimentata, in Europa, anche per gli effluenti zootecnici, al fine di ritardare la nitrificazione della elevata aliquota di azoto ammoniacale presente nei liquami, aumentandone l'efficienza.
L'obiettivo del lavoro è quello di studiare l'effetto della molecola antinitrificante DMPP (3,4 dimetil-pirazolo fosfato) e di un modulatore dell'assorbimento di azoto additivati ad un effluente zootecnico bovino sul ciclo dell'azoto e sulla comunità microbica nitrificante di un suolo adibito a produzioni legnose fuori foresta. A tal proposito è stato studiato il rilascio di azoto nitrico ed ammoniacale nel tempo, ottenendo curve di cessione potenziale dell'azoto in presenza dei due prodotti additivati e non al substrato organico.
Parole chiave: *inibitori della nitrificazione; Short Rotation Forestry; DMPP; effluenti zootecnici;, biostimolanti*

Introduzione

Il ciclo dell'azoto è un processo chimico fondamentale per la vita sulla Terra e per l'esistenza degli stessi esseri umani. L'azoto rappresenta l'elemento nutritivo fondamentale per il corretto sviluppo vegetativo delle piante ed il suo apporto è

certamente un fattore chiave per la produzione agronomica; tuttavia, la reale disponibilità dell'elemento, nonché la sua forma chimica, costituiscono aspetti imprescindibili per l'assimilazione dell'azoto da parte delle piante.
La necessità di contenere l'inquinamento da nitrati di origine agricola ha portato all'emanazione di specifiche normative in materia di fertilizzazione azotata in cui si precisa che l'apporto di fertilizzanti deve essere commisurato sulla base della conoscenza dell'ambiente pedoclimatico, della realtà aziendale, dei mezzi tecnici e della predisposizione di un piano di fertilizzazione sulla base del bilancio dell'azoto (D.L. 152/1999). In quest'ottica, la validità agronomica dei fertilizzanti assume un significato più ampio; la valutazione degli aspetti quali-quantitativi delle produzioni ottenute deve essere considerata congiuntamente alla ricaduta ambientale della fertilizzazione (Benedetti, 1998). In riferimento alla tipologia del prodotto fertilizzante, la norma agevola l'utilizzo di concimi "non a pronto effetto" o "speciali", siano essi di origine naturale (organici) o di sintesi (concimi ricoperti, concimi condensati, concimi con inibitori della nitrificazione e dell'ureasi). In particolare, la tipologia di concimi con inibitori della nitrificazione influenza soprattutto la forma di azoto nel suolo: inibendo il processo di ossidazione degli ioni ammonio in ioni nitrito ad opera di microrganismi del suolo del genere *Nitrosomonas* (Zacherl e Amberger, 1990), l'elemento sarà presente prevalentemente in forma ammoniacale, quindi meno mobile del nitrato, ma pur sempre minerale e perciò prontamente disponibile, oltre che meno esposta al rischio di lisciviazione (Watson et al., 1994). La capacità di questi prodotti di rilasciare azoto nel medio-lungo periodo ne potrebbe consentire un impiego diverso rispetto ai concimi di sintesi tradizionali. gli inibitori hanno ricevuto rinnovata attenzione per la possibilità di utilizzarli a scopi di protezione ambientale, in quanto l'inibizione della nitrificazione risulta essere l'approccio più realistico per controllare il ciclo dell'azoto nel suolo (Hauck, 1983). L'addizione di inibitori di nitrificazione è stata sperimentata, in Europa, anche per gli effluenti zootecnici, al fine di ritardare la nitrificazione della elevata aliquota di azoto ammoniacale presente nei liquami, e quindi aumentarne l'efficienza d'assorbimento.
L'orientamento di fondo delle principali azioni promosse in sede internazionale dal Protocollo di Kyoto è legato alla riduzione sostanziale dell'uso di fonti d'energia fossile o non rinnovabile, in favore delle risorse rinnovabili. Tra queste l'impiego di energia da biomasse è indubbiamente strategico sia per la disponibilità e la varietà di forme, sia per le possibilità di trasformazione, sia per l'efficienza della trasformazione dell'energia solare in energia fissata. Negli ultimi decenni si è verificato un forte incremento dell'utilizzo di suoli per colture a scopi energetici, le cui modalità di gestione sono spesso basate sul prelievo annuo di tutto o quasi l'incremento legnoso, limitando al massimo, fino ad annullare, le spese per le cure colturali durante il ciclo produttivo e per gli interventi di miglioramento di fine turno. Il suolo, pertanto, potrebbe soffrire per la indiscriminata diffusione delle colture da bioenergia, per quel che riguarda l'impoverimento di nutrienti, la contaminazione diffusa dovuta all'aumento nella distribuzione dei prodotti fitosanitari e dei reflui zootecnici e l'intensificazione delle lavorazioni.

L'impoverimento dei nutrienti dovrebbe essere supportato da una adeguata nutrizione minerale, soprattutto azotata, e l'utilizzo di effluenti, compresi quelli zootecnici, potrebbe essere una soluzione efficace dal punto di vista nutrizionale ed economico.
L'obiettivo del lavoro, che rientra nell'ambito di una tesi di dottorato di ricerca in Ecologia forestale, è quello di studiare l'effetto della molecola antinitrificante DMPP e di un modulatore dell'assorbimento di azoto additivati ad un effluente zootecnico bovino sul ciclo dell'azoto e sulla comunità microbica nitrificante di un suolo adibito a produzioni legnose fuori foresta. La molecola DMPP è stata studiata ad una dose pari all'1% sulla base dell'N totale aggiunto, in associazione ad un fertilizzante tradizionale [$(NH_4)_2SO_4$] e ad un effluente zootecnico bovino. Nel presente lavoro verranno illustrati i risultati derivanti dalle curve di cessione dell'N potenzialmente mineralizzabile.

Materiali e Metodi

Lo studio è stato effettuato utilizzando il suolo proveniente da un'azienda sperimentale situata a Casalotti (RM), presso il CRA-PLF (Centro di Ricerca per le produzioni legnose fuori foresta). Il dispositivo sperimentale è costituito da un eucalitteto di circa 20 anni di età a bassa densità d'impianto (1100 piante/ha) e gestito a ceduo, una parte del quale è stato sottoposto nel 2007 a cambio di gestione mediante espianto e reimpianto di eucalitti ad alta densità (5000 piante/ha). Sul dispositivo ad alta densità è stato effettuato un prelievo di suolo in sei punti all'interno di un'area omogenea, alla profondità di 0-20 cm; i sei campioni di suolo sono stati poi uniti in modo da ottenere un campione medio rappresentativo. Tutti i terreni sono poi stati essiccati all'aria e vagliati a 2 mm. In tabella 1 sono riportati i principali parametri chimico-fisici e biologici relativi ai campioni medi.

Caratteristiche chimico-fisiche	Suolo Casalotti	Suolo Celimontano
Sabbia (%)	41.5	67
Limo (%)	33.8	22
Argilla (%)	24.7	11
N_{tot}(%)	0.06	0.15
pH	7.5	7.9
TOC (%)	1.06	2.49
IBF	13	23

Tabella 1

Principali caratteristiche chimico-fisiche e biologiche dei suoli oggetto di studio.

L'effluente bovino da mucche da latte utilizzato proviene dall'azienda Ovile sita a Casalotti e le sue caratteristiche sono riportate in tabella 2.
La mineralizzazione potenziale dell'azoto è stata determinata seguendo il metodo biochimico di Stanford & Smith modificato da Benedetti et al. (1993): a 50g di suolo miscelato con sabbia di quarzo in rapporto 1:1 è stata addizionata una quantità di N pari a 250mg/Kg sottoforma di $(NH_4)_2SO_4$, effluente zootecnico o modulatore dell'assorbimento di N (contenente il 12% di N ammoniacale e l'8% di

N ureico) e soluzione nutritiva (1,9% N) contenente DMPP. Il DMPP è stato somministrato in quantità pari all'1% sulla base dell'N aggiunto. I campioni di suolo sono stati incubati in condizioni idriche e termiche ottimali (30°C, alla capacità di campo) e sottoposti a lisciviazione con $CaSO_4$ 0,01M ad intervalli di tempo prefissati (0, 1, 2, 4, 8, 12 settimane) dell'N mineralizzato.

Caratteristiche chimiche	Effluente zootecnico bovino
Umidità (%)	88.9
Sostanza secca (%)	11.1
N_{tot} (%)	0.32
$N\text{-}NH_4^+$ (%)	0.17
$N\text{-}NO_3^-$ (%)	0.0025
Densità (g/cm^3)	1
Rame (ppm)	0.3
Zinco (ppm)	0.2

Tabella 2

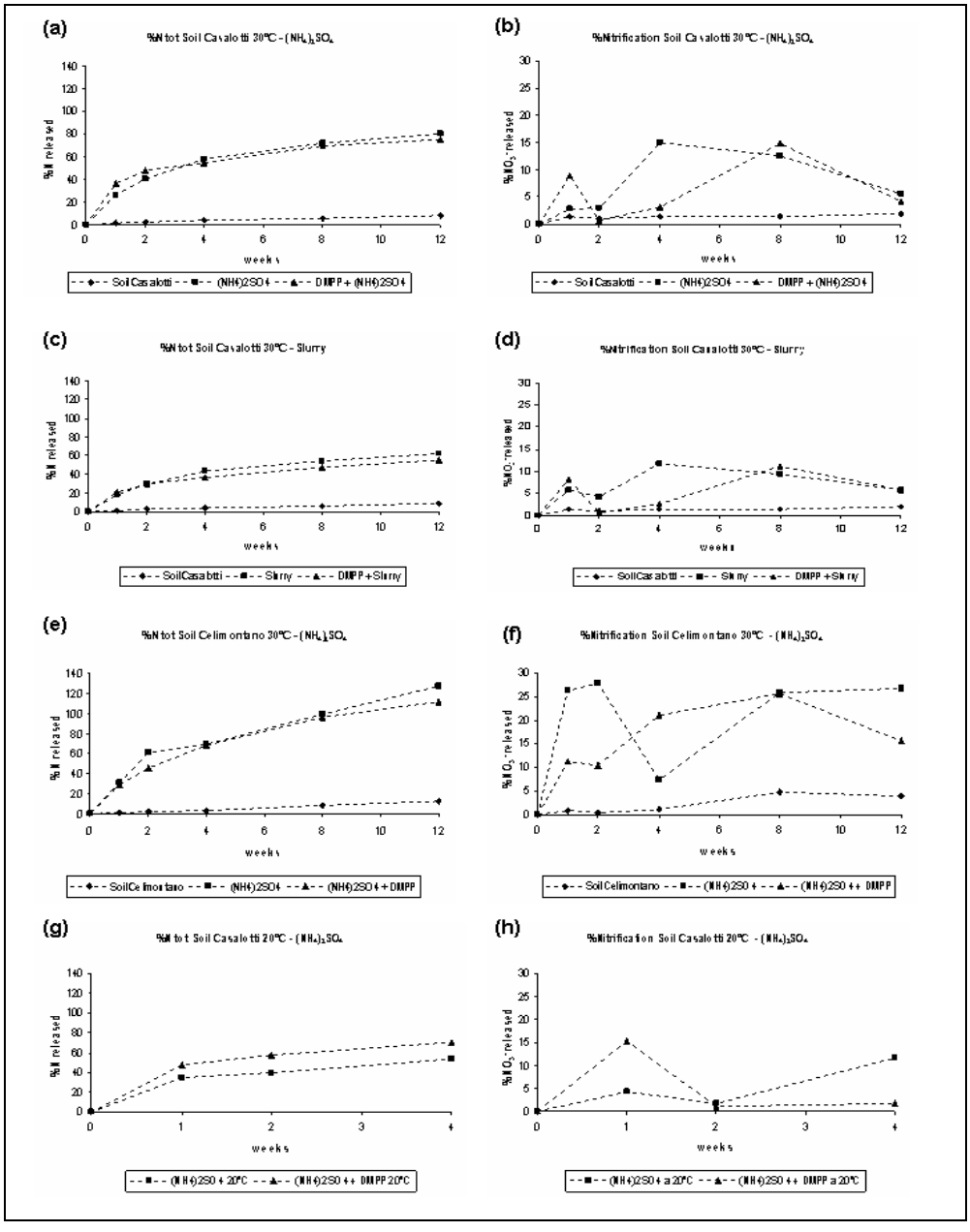
Caratteristiche chimiche del refluo bovino (Azienda Ovile, Casalotti)

Le forme minerali in soluzione sono poi state determinate mediante analizzatore automatico a flusso continuo secondo Wall et al. (1985) per l'ammonio, e secondo Kamshake et al. (1967) per i nitrati e nitriti. Al fine di indagare l'effetto della temperatura sull'inibizione della nitrificazione nel suolo descritto è stata allestita una prova anche a una temperatura inferiore (20°C). È stata inoltre effettuata una successiva prova utilizzando un suolo a fertilità medio-alta (suolo Celimontano), le cui caratteristiche chimico-fisiche sono riportate in tabella I, allo scopo di rilevare un eventuale effetto suolo. Infine, è stata aggiunta una prova sperimentale utilizzando il modulatore dell'assorbimento di azoto e l'effluente zootecnico in rapporto 1:4 di N aggiunto, per valutarne l'efficacia ed ipotizzarne l'utilizzo a scopi commerciali.

Risultati e Discussione

L'andamento delle curve cumulative di cessione dell'azoto totale potenzialmente mineralizzabile e della % di nitrificazione settimanale sono riportate in figura 1. La % di nitrificazione nella tesi con $(NH_4)_2SO_4$ e DMPP su suolo Casalotti alla I settimana di incubazione mostra un picco rispetto al controllo con solo $(NH_4)_2SO_4$ (Fig. 1b), che non si osserva nel suolo Celimontano (Fig. 1f); a partire dalla II settimana di incubazione la % di nitrificazione si riduce rispetto al controllo, e si mantiene inferiore sino almeno alla IV settimana di incubazione.
Nelle tesi con suolo Celimontano invece (Fig. 1e e 1f) l'efficacia antinitrificante del DMPP si manifesta per almeno due settimane, e cessa alla IV settimana di incubazione. La fertilità biologica del suolo influenza notevolmente il corso della mineralizzazione: nel terreno ad alta fertilità l'inibizione della nitrificazione si manifesta tempestivamente, mentre diminuisce dopo la II settimana di incubazione; nel terreno a bassa fertilità invece si osserva la massima inibizione a partire dalla II settimana sino almeno alla IV settimana di incubazione.

Figura 1 - *Curve cumulative di cessione dell'N totale potenzialmente mineralizzabile nel suolo Casalotti addizionato a $(NH_4)_2SO_4$ e DMPP (a), refluo bovino e DMPP (c), suolo Celimontano addizionato a $(NH_4)_2SO_4$ e DMPP (e), suolo Casalotti addizionato a $(NH_4)_2SO_4$ e DMPP a 20°C (g); % di nitrificazione settimanale nel suolo Casalotti addizionato a $(NH_4)_2SO_4$ e DMPP (b), refluo bovino e DMPP (d), suolo Celimontano addizionato a $(NH_4)_2SO_4$ e DMPP (f), suolo Casalotti addizionato a $(NH_4)_2SO_4$ e DMPP a 20°C (h)*



Ciò potrebbe significare che nel terreno con minor fertilità biologica i microrganismi inizialmente tenderanno ad immobilizzare la frazione azotata minerale, proveniente da $(NH_4)_2SO_4$ o da refluo, per accrescere la loro biomassa, con conseguente mineralizzazione latente dei prodotti aggiunti.
Le tesi con $(NH_4)_2SO_4$ e DMPP sul terreno Casalotti condotte a 20°C (Fig. Ig e Ih) mostrano un andamento nelle curve cumulative di cessione dell'azoto potenzialmente mineralizzabile e nella % settimanale di nitrificazione simile rispetto a ciò che avviene a 30°C (Fig. 1a e 1b), confermando questo trend.
L'applicazione al terreno Casalotti di una molecola biostimolante della comunità microbica del suolo ha comportato un forte aumento della % di ammonizzazione alla I settimana di incubazione sulla tesi con biostimolante che si riflette sull'azoto totale potenzialmente mineralizzabile (Fig. 2a).

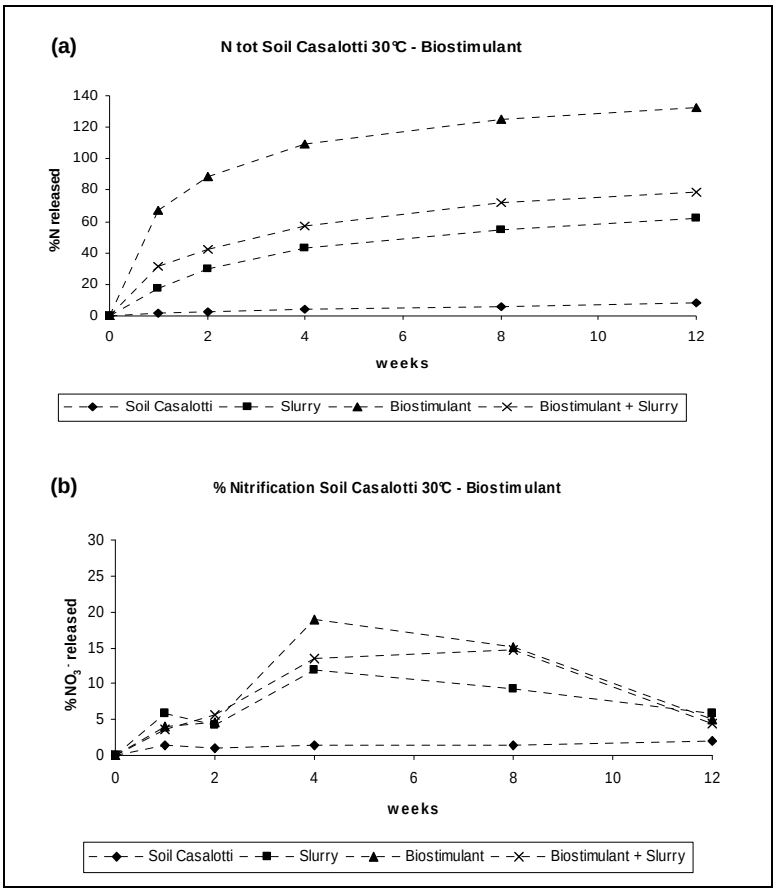


Figura 2 - *Curve cumulative di cessione dell'N totale potenzialmente mineralizzabile (a) e % di nitrificazione settimanale (b) nel suolo Casalotti addizionato al biostimolante e al biostimolante miscelato con refluo (rapporto 1:4 sulla base dell'N totale aggiunto).*

L'effetto si è manifestato, in misura più lieve, con la tesi biostimolante e refluo, presenti in un rapporto di 1:4 sulla base dell'N totale aggiunto (Fig. 2). Già alla IV settimana di incubazione la % di N potenzialmente mineralizzabile ha raggiunto un

valore lordo del 109,1%, ed un valore del 132,6% a fine prova: ciò dimostra che il prodotto applicato, agendo come modulatore dell'azoto ed incrementando la frazione assimilabile dei nutrienti, determina un'accelerazione nella mineralizzazione della sostanza organica del suolo con conseguente rilascio di N organico nativo in forma minerale.

Conclusioni

Le prove eseguite hanno dimostrato che il rilascio delle frazioni azotate assimilabili (nitrato ed ammonio) per la nutrizione dei vegetali è stato influenzato sia dal tipo di fertilizzante che dalla fertilità del terreno utilizzato. In particolare:
- durante la I settimana di incubazione del suolo Casalotti con DMPP e $(NH_4)_2SO_4$ si assiste ad un aumento della % di nitrificazione; l'effetto antinitrificante si manifesta a partire dalla II settimana ed ha efficacia fino alla IV settimana di incubazione;
- dalla prova condotta sul suolo Celimontano. a contenuto maggiore di sostanza organica. risulta che l'effetto antinitrificante del DMPP si manifesta già alla I settimana. cessando alla IV settimana di incubazione;
- utilizzando una temperatura meno elevata (20°C) il trend osservato nella prova sul suolo Casalotti viene mantenuto;
- il prodotto biostimolante applicato sul suolo Casalotti determina il rilascio di N endogeno in forma minerale, come risultato dell'accelerazione della mineralizzazione della sostanza organica.

Le successive ricerche, che rientrano nell'ambito di una tesi di dottorato di ricerca in ecologia forestale, saranno volte alla comprensione dei processi che regolano la nitrificazione nel suolo mediante approcci prevalentemente di tipo molecolari.

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Effects of the nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP) and a biostimulant on soil microbial diversity and ammonia-oxidizing bacteria after bovine effluent application in experimental microcosms

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The application of animal manure to soil can result in increased gaseous emissions such as NH_3, N_2O, CO_2 and CH_4 as well as nitrate leaching, contributing to climate warming and ground and surface water pollution.

Nitrification inhibitors are chemical compounds that delay microbial oxidation of ammonium to nitrate in soil for a certain period of time by depressing the activity of ammonia-oxidizing microorganisms. Biostimulants are products that optimise the adsorption of nutrients by plants through various different mechanisms, including stimulation of soil microbial activity. As a result, oxidation of ammonia to nitrate can be delayed, reducing N losses and increasing fertilizer use efficiency.

In this study, we assess the effects of the nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP) and a biostimulant on microbial community-level physiological profiling (CLPP) and on the abundance of ammonia-oxidizing bacteria (AOB) using RT-qPCR in soil microcosms amended with bovine effluent. DMPP reduced the nitrification rate starting from the second week of incubation until at least the fourth week, while the application of the biostimulant led to substantial increases in net N mineralization. Both DMPP and biostimulant treatments induced rapid changes in microbial heterotrophic metabolism showing significant differences after 24h of incubation, as well as in the size of ammonia-oxidizing bacterial communities, which was affected by DMPP thoughout the entire incubation period.

Keywords: Nitrification inhibitor, DMPP, biostimulant, bovine effluent, nitrogen use efficiency.

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Effectiveness of 3,4-dimethylpyrazole phosphate (DMPP) as nitrification inhibitor in soil as influenced by fertilizer, temperature and soil biological properties.

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The element nitrogen (N) is an essential nutrient for plant development and its intake is a key factor for crop production; however, its real availability in soil, as well as its chemical form, are crucial for the N uptake by crops. The main N losses in soil driven by microbial processes like nitrification, leaching and denitrification involve NO_3^- as key point, so limiting NO_3^- in the soil is a potential tool to restrict N leaching, NO_x emissions and even NH_3 volatilization from soils in one hand, and increasing N use efficiency on the other hand, obtaining both environmental and economic benefits (Jenkinson, 1982).
The application of nitrification inhibitors to soil offers the chance to reduce N losses and to increase fertilizer use efficiency. The present study therefore aimed to evaluate the influence of both inorganic and organic N fertilizer, temperature and soil biological properties on the effectiveness of DMPP in a long-term incubation experiment.
The application of ammonium sulphate and a cattle effluent as inorganic and organic N fertilzer respectively to a low biological fertility soil resulted in a reduction of the nitrification rate starting from the second week of incubation until at least the fourth week, while the nitrification inhibition occurred immediately in the higher biological fertility soil but after four weeks there was no difference with respect to the control. The temperature (20°C and 30°C) had little or no effect on potentially mineralized N.

Keywords: Nitrification inhibitor, DMPP, bovine effluent, nitrogen use efficiency.

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Effects of the nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP) on soil microbial communities after bovine effluent application in experimental microcosms

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The application of animal manure to soil can result in increased gaseous emissions such as NH_3, N_2O, CO_2 and CH_4 as well as nitrate leaching, contributing to climate warming and ground and surface water pollution. The application of nitrification inhibitors to soil offers the chance to reduce N losses and to increase fertilizer use efficiency. In this study, we assess the effects of the nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP) on microbial community-level physiological profiling (CLPP) and on the abundance of ammonia-oxidizing bacteria (AOB) using RT-qPCR in soil microcosms amended with bovine effluent. DMPP reduced the nitrification rate starting from the second week of incubation until at least the fourth week. DMPP treatments induced rapid changes in microbial heterotrophic metabolism showing significant differences after 24h of incubation: DMPP seemed to decrease metabolic activity when applied without a N source but AWCD values were increased in DMPP + bovine effluent treatment, suggesting that a few effluent strains were the main responsible of substrates consumption. As expected, total bacteria population was higher where bovine effluent was applied, as it cointains its own microbial population, but no massive differences were found between treatments in qPCR. On the contrary, rapid changes in gene expression occurred in DMPP + effluent treatment: after 24h *amoA* gene copy numbers were lower with respect to the effluent treatment, due to a high efficacy in the inhibition of the ammonia-oxidizing populations, but even bacteria *16S* gene expression was reduced, suggesting a possible effect on non-targeted populations by DMPP.

Keywords: Nitrification inhibitor, DMPP, ammonia-oxidizing microorganisms, RT-qPCR.