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# SOIL NITROGEN CYCLE AND FLUXES OF GREENHOUSE GASES ($CH_4$ AND $N_2O$) IN MEDITERRANEAN-TYPE ECOSYSTEMS

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## ABSTRACT

Microbial processes play a fundamental role in the nitrogen (N) cycle. They make available inorganic N forms by decomposing and mineralizing organic N in soil. They also transform inorganic forms of N with different oxidation states. The available inorganic N, ammonium $NH_4^+$ and nitrate $NO_3^-$, produced by mineralization and nitrification processes from organic matter, are often in short supply in natural terrestrial ecosystems, limiting plant growth and biomass accumulation, as a consequence of quality and quantity of available substrate but also of the controlling effect of environmental factors on microbial activity and growth. Microbial N transformation thus are responsible for soil N availability and losses from the systems as leaching, or N gaseous products during the nitrification and denitrification processes, but are controlled by the environmental characteristics of the systems itself.

The purpose of this research is to improve our knowledge on natural Mediterranean ecosystems, in relation to the nitrogen cycle, and their potentiality as sources and sinks of greenhouse gases (GHGs), in particular of $N_2O$ and $CH_4$. These ecosystems can be expected to be extremely sensitive to the future predicted global changes. Land use change, fires, anthropic disturbance, climate modifications can all impact the structure of Mediterranean plant communities, the ecosystem and associated cycles of elements. The magnitude of the effect of such disturbance on nitrogen transformation, losses and GHGs fluxes in Mediterranean ecosystem is unknown. In order to improve our understanding on N cycle and estimates of GHGs fluxes in natural Mediterranean ecosystems, both in terms of natural mechanisms associated to this type of environment and in terms of response of the ecosystem to modifications and disturbance, four case studies were set up.

In first case study (chapter 3), a rainfall manipulation experiment in Mediterranean woodland of Tolfa, dominated by *Arbutus unedo* L., was carried out in order to evaluate, on the short-term, the impact of variation (both increase and decrease) of rainfall regime of about 20% on the nitrogen cycle and on GHGs emissions. In fact significant changes of rainfall regime are expected in the Mediterranean basin in the future decades, whereas variations in frequency and total amount of precipitation result in increased of aridity and drought durations. We followed over 1 and ½ year (Apr05-Feb06) soil processes related to the N cycle, mineralization, nitrification, denitrification, mineral N, emissions of $N_2O$ and fluxes of

$CH_4$. The Tolfa site demonstrate to have a quite unusual N cycle for Mediterranean woodlands dominated by organic and $NH_4^+$ forms of N, whereas nitrification and net $NO_3$ production was completely absent. Consequently also denitrification was very low, and $N_2O$ emissions were below the detection limit if the soil was not fertilized with a $NO_3^-$ salt. A 20% variation of rainfall did not influence the variation of soil water content to such an extent to modify in a significant way the biological activities in soil. Also for all the measured activities the high spatial variability associated to the measures was not allowing for clear treatment effect, although in general processes resulted faster in the wettest treatment. The site acted as a sink for $CH_4$. The treatment effect on $CH_4$ fluxes was visible only when seasonal extreme were reached and even in this case the spatial variability obscured any significant difference among treatments.

Second case study (chapter 4) investigates causes for the absence of soil nitrification activity in a Mediterranean monospecific woodland of *Arbutus unedo* L., focusing on the possible role of allelochemicals produced by this plant on the observed $NO_3^-$ production inhibition. Raw extracts of leaves and roots of *Arbutus unedo* and soil underneath its canopy, were purified using chromatographic techniques, and the structure of chemicals was defined using spectroscopic and spectrometric methods. Leaf extracts (raw and aqueous and organic fractions) were tested for their toxicity on nitrification using a "test" soil. Field and laboratory incubations showed absence of $NO_3^-$ production over the whole study period, despite the significant mineralization rates ($NH_4^+$ production). Toxicity tests indicated that 400 µg of extract $g^{-1}$ dry soil were already sufficient to have more than 50% inhibition of $NO_3^-$ production. Gallocatechin and catechin were among the most abundant chemicals in the extracts of leaves and roots. Their soil concentration was significantly higher than the annual calculated input via litter, indicating a quite high residence time, and was in the range of toxic (> EC50, more than 50% inhibition) concentrations deduced from the dose-response curve of the toxicity test. Data seem to support the hypothesis that plant produced chemicals, rather than substrate limitation, are a probable cause of nitrification inhibition in this forest.

A study (chapter 5) was set to estimated the amount of N which enters into the ecosystem in Mediterranean grasslands via $N_2$ fixation, considering that for these herbaceous species, which dominate the early stages of secondary successions following disturbance, there is a complete lack of informations in relation to this topic. Moreveor, the impact of grassland areas covered by a different covers of $N_2$ fixers on N processes and pools was

investigated. To meet this goals the following objectives were set: a) to quantify the $N_2$ fixation capacity of the most frequent leguminous species in order to estimate the N input in the ecosystem via $N_2$ fixation; b) to quantify the gain and losses of N under $N_2$ fixing and non fixing plant covers. The most abundant leguminous plants, derived by previous studies in Castel Volturno site, are *Medicago minima*, which derive around 30-47 %, and *Melilotus neapolitana,* which derive around 24-50 % of the N present in their tissue from the atmosphere. These data were obtained using either "N-difference" and "$^{15}$N natural abundance" techniques. They indicate that the leguminous plants compete for with non fixing plants to exploit the soil mineral N pool, at a great extent. Estimates of pools of N, mineralization, nitrification and denitrification rates carried out in intermixed plots with different cover density and biomass of $N_2$ fixers did not show significant differences. Probably, in N-limited ecosystems, the input of nitrogen by $N_2$-fixation was not high enough to modify N pools, losses and processes, probably because the extra N deriving from $N_2$ fixation was quickly immobilized by microbes and plants. Very low $N_2O$ emissions were in fact observed. The site acted as sink of $CH_4$.

In the last experiment, four Mediterranean woodland ecosystems with different soil characteristics and 3 successional stages within the same soil type (grassland, maquis, woodland) were compared for their potential to produced NO and $N_2O$ emissions in controlled and N excess conditions (chapter 6). The experiment was carried out incubating the soil in reconstituted cores at the same temperature (25°C) and soil water content (50%WHC) in order to get independent from these two controlling factors, which influence on gas emissions is already well known. In controlled conditions, a predominance of NO emissions respect to $N_2O$ were measured in all studied sites, despite the incubation conditions which were supposed to be more favourable for $N_2O$ production. The highest NO production was observed in the sandy soil with sub-alkaline pH and low organic content, all characteristics which are expected to favour nitrifiers over denitrifiers. The highest gas emissions in the succession were measured in the intermediate stage (maquis). Comparing the woodlands, it appeared clear the combination of many factors influences the potential of NO and $N_2O$ emissions, and not the sole water content. In all cases the addition of extra N resulted in net immobilization after 10 days and in a burst of N gaseous emissions, which only in the case of one site, Roccarespampani, resulted higher than NO emissions. In all the other cases NO emissions were always dominant also in presence of excess N. The flush of N emissions

lasted one day or maximum a week in presence of extra N. Soil with the highest mineralization rates were not necessarily those with the highest N gaseous emissions. Nitrification seemed strongly influence by soil pH, whereas this was not true for mineralization. Overall the results suggest that these Mediterranean soils tend to retain the N in the systems as much as possible and that nitrification and NO emissions are the dominant processes in the system also at intermediate water content such as the one used in the experiment (50% water saturation).

# 1 GENERAL INTRODUCTION

## 1.1 SOIL NITROGEN CYCLE

Nitrogen (N) is a fundamental macronutrient, a key element for life in terms of quantities and functions. It is involved in the energy transfer molecules ATP and ADP, it is an essential component of the amino acids, peptides, enzymes, hormones and vitamins, and thus in numerous metabolic processes. It is a constituent of the nucleic acids RNA and DNA that make up the genetic material in all living organisms.

Nitrogen high versatility is reflected by the high number of processes which constitute the N biogeochemical cycle. Firstly, nitrogen valence varies from -3 to +5 and most of the transformations are carried out by few soil organisms, at normal temperatures and pressure. Secondly, nitrogen is abundant on earth, but only a very small proportion of it enters into the biogeochemical nitrogen cycle at significant rates. Most of the nitrogen is thus found in the lithosphere (especially in primary rocks of the mantle, *Stevenson, 1972*) and only 0.001 per cent occurs in the biosphere *(Sweeney et al., 1978)*. Nitrogen accounts for about 78% of the atmosphere as elemental dinitrogen ($N_2$) gas. The dinitrogen molecule, $N_2$, is a very stable substance and a considerable quantity of energy is needed to break its inter-atomic bonds, so it is not directly available for plant uptake and metabolism. This means that biologically available nitrogen is often in short supply in natural terrestrial ecosystems, limiting plant growth and biomass accumulation (*Binkley & Hart, 1989; Paul & Clark, 1989;Vitousek and Howarth, 1991*).

Since soil has a solid, a liquid and a gas phase, all forms of nitrogenous compounds may be present. The soil pool N is dominated by N found in organic structures. In soils with significant contents of $K^+$-containing clay minerals, capable of fixing ammonium ($NH_4^+$-N), approximately 90% of the soil N is contained in organic structures (*Kelley & Stevenson,*

*1996*), 8% exists as fixed $NH_4^+$-N, and 1-3% can be found in the inorganic fraction ($NO_3^-$-N and $NH_4^+$-N). In soils with little capacity to fix $NH_4^+$-N in clay minerals, the proportion of organic N is >97% and the inorganic fraction is 1-3%. Only a small fraction of the soil N exists as soluble $NO_3^-$-N and exchangeable $NH_4^+$-N, the main forms of inorganic soil N, this pool is thus very small in size but plays a key role since it is the main form available for plants and micro-organisms.

On a global scale, *Söderlund and Svensson (1976)* estimated that the organic N fraction of soils accounted for 95% of the total soil N pool, which is equivalent to the average value presented by *Bremner (1967)*. Soil organic N has been traditionally divided into the following five fractions based on a variety of acid hydrolysis procedures: (1) acid insoluble N; (2) ammonia N recovered after hydrolysis; (3) amino acid N, (4) amino sugar N and (5) hydrolysable unidentified N. Data summarized by *Stevenson (1994)* for 11 studies where acid hydrolysis procedures were applied to different soil types showed that there was as much variation in the contents of each form of N within similar soils as between different soil types. The proportions of each form of organic N were 7-44% acid insoluble N, 9-37% ammonia N, 13-50% amino acid N, 1-14% amino sugar N, and 4-40% hydrolysable unidentified N. Researchers, using such analytical pyrolysis, found a larger presence of heterocyclic compounds with values comprised between 27% and 34% of the total N (*Schulten, 1994; Schulten et al., 1997; Schulten & Schnitzer, 1998*) and proposed that those would represent the main part of unidentified organic N pool.

However, nitrogen is an incredibly versatile element and also forms some of the most mobile compounds in the soil-plant-atmosphere system. The concept of an N cycle was first formulated by *Löhnis (1913)* following identification of the forms of N in soil and the role of microorganisms in the transformations between organic and inorganic N (**Fig. 1.1**). Microorganisms, particularly bacteria, play a major role in all of the principal nitrogen transformations.

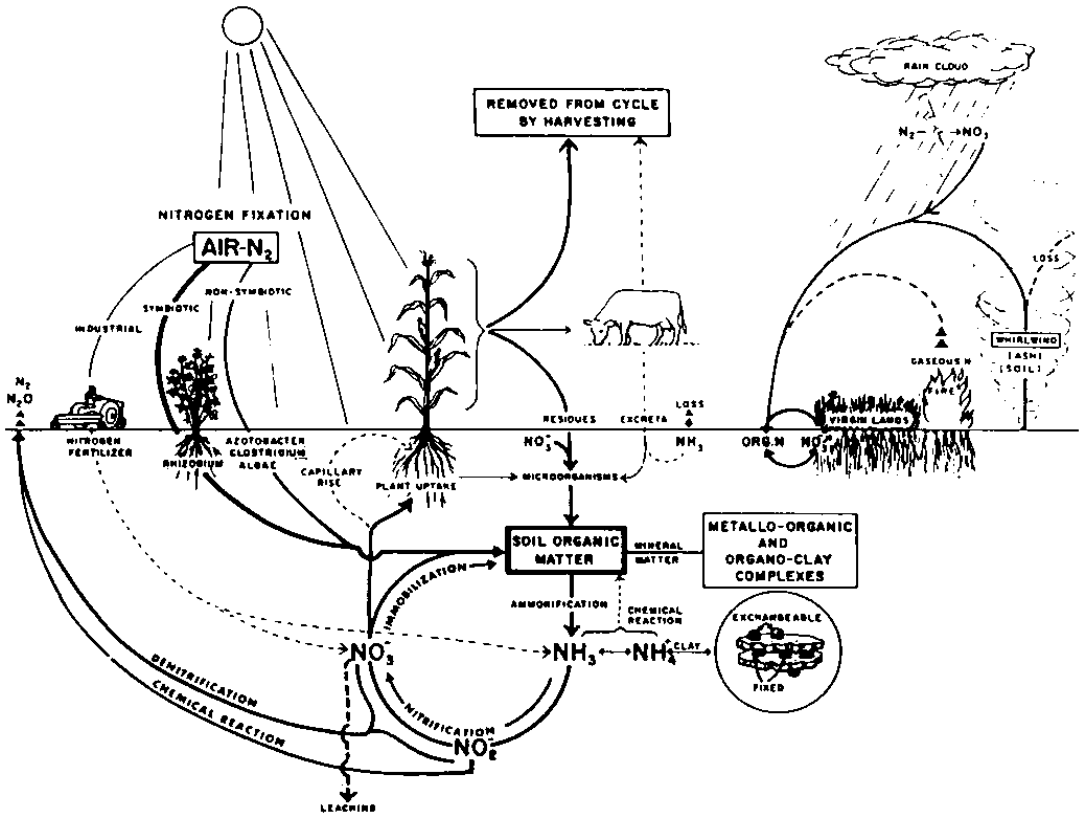


**Figure 1-1.** Nitrogen cycle in soil (From *Stevenson, 1982*).

The amounts of energy that is required by, or lost during, the major reactions of the nitrogen cycle are listed in **Table 1.1**.

**Table 1-1.** Free energy changes in inorganic nitrogen metabolism reactions (from *Rosswall,1981*)

| | $\Delta G'_o$ (kJ $mol^{-1}$) |
|---|---|
| Nitrate respiration *Escherichia coli* | |
| $NO_3^- + [H_2] \rightarrow NO_2^- + H_2O$ | -161 |
| **Denitrification** Pseudomonas aeruginosa | |
| $2\ NO_3^- + 2H^+ + 5[H_2] \rightarrow N_2(g) + 6H_2O$ | - 1121 |
| Other possible reactions | |
| $N_2O(g) + [H_2] \rightarrow + N_2(g) + H_2O$ | - 340 |
| $NO_2^- + ½\ [H_2] + H^+ \rightarrow NO(g) + H_2O$ | - 76 |
| $2NO(g) + [H_2] \rightarrow N_2O(g) + H_2O$ | - 306 |
| $2NO_2^- + 2H^+ + 2[H_2] \rightarrow N_2O(g) + 3H_2O$ | - 459 |
| Assimilatory nitrate reduction | |
| $NO_3^- + 2H^+ + H_2O \rightarrow NH_4^+ + 2O_2$ | + 348 |
| **Nitrate fermentation** Clostridium perfringens | |
| $NO_3^- + 2H^+ + [4H_2] \rightarrow NH_4^+ + 3H_2O$ | - 591 |
| Nitrification *Nitrosomonas* | |
| $NH_4^+ + ½\ O_2 \rightarrow NH_2OH + H^+$ | + 15 |
| $NH_2OH + O_2 \rightarrow NO_2^- + H_2O + H^+$ | - 289 |
| *Nitrobacter* | |
| $NO_2^- + ½\ O_2 \rightarrow NO_3^-$ | - 77 |

In order to known whether reactions are likely to occur, it is necessary to considerate the more complex actual energetic. First, the rate of reactions must be considered - if a reaction is thermodynamically possible, but occurs very slowly, it is generally of little use to living organisms. Biological catalysts, enzymes, are the common way of speeding up reactions: their synthesis and maintenance require energy. Additionally, some of the available energy may be used to drive the reaction. Second, where reactions are required to occur at low substrate concentration (if the substrate is toxic, for example) it may be more advantageous to use energy to help to drive that reaction, so that a more rapid reaction rate is achieved. Third, the partial reactions may have different energies associated with them, so that an overall reaction which is energetically feasible may not occur at finite rates in practice. Fourth, living organisms have various potential energy sources available to them and these sources vary considerably in the fraction of their energy available to drive reactions. Fifth, energy available for oxidation-reduction reactions will vary according to whether the environment is oxidative or reducing. Some of these aspects are considered in *Sprent (1987)*.

As microbially mediated processes, nitrogen transformations tend to occur at quite fast rate. Rates are affected by environmental factors that influence microbial activity, such as temperature, moisture, and resource availability. Each steps of soil nitrogen cycle is subject to constraints related to the physical and biological environment, thus the actual pathway followed and its rate-limiting steps vary with geographical location and season.

### 1.1.1 DINITROGEN FIXATION

Inert atmospheric nitrogen naturally enters in the active pool through two processes:

• spontaneously by lightning, forest fires, even hot lava flows and photochemical reactions; probably about 10% of the nitrogen fixed by natural processes is by this route (*Sprent et al., 1990*);

• biologically by specific 'nitrogen-fixing' microorganisms known collectively as diazotrophs, for about 60%,

Biological dinitrogen fixation (BNF), can be regarded as second in importance only to photosynthesis for the maintenance of life on Earth. Under natural conditions it is the main pathway mediated by micro-organisms able to convert the most abundant but relatively inert

form of N into biologically available substrates, in fact the covalent triple bond of the $N_2$ molecule (N≡N) is highly stable and can be broken chemically only at elevated temperatures and pressures.

Input of nitrogen to soil from biological fixation vary widely from one ecosystem to another, tropical legumes such as alfalfa have been shown, on occasion, to fix at rates as high as over 400 kg N $ha^{-1}$ $yr^{-1}$ (*Tisdale, Nelson & Beaton, 1985*), while in soils of upland pastures in the UK, free-living and symbiotic $N_2$-fixation may only account for an input of 5-30 kg N $ha^{-1}$ $yr^{-1}$ (*Batey, 1982*). On a global scale, biological fixation by natural terrestrial ecosystems in the early-1990s was estimated 107 Tg N $yr^{-1}$, decreasing by about 15% respect to 1860 *(Galloway et al., 2004)*.

The ability to fix $N_2$ is restricted to prokaryotic organisms that are taxonomically diverse including heterocystous (*Anabaena, Nostoc*) and nonheterocystous (*Trichodesmium, Gloeocapsa* [*Bergman et al., 1997*]) cyanobacteria, actinomycetes (*Frankia*), and heterotrophic (*Azotobacter, Bacillus*), autotrophic (*Thiobacillus*), aerobic (*Pseudomonas, Methylosinus*), anaerobic (*Clostridium, Desulfovibrio*) and phototrophic (*Chlorobium, Rhodospirullum*) bacteria. No eukaryote is known to have this capability. *Young (1992)* provides a detailed listing of $N_2$-fixing species. They are generally grouped on the basis of their lifestyle, in fact $N_2$-fixing prokaryotic can (1) live free in nature (*Azotobacter, Trichodesmium*), (2) occur in loose or associative symbiosis with other plants or animals (*Acetobacter or Herbaspirillum* and sugar cane [*Boddey and Dobereiner, 1995*]), or (3) enter into symbiosis with their host and be housed within specialized structures such as *Rhizobium* and the legume nodule (*Graham, 1997*) and *Anaeba* and the water fern, *Azolla* (*Wagner, 1997*). Generally, in a symbiotic relationship, one organism contains chlorophyll and uses light energy to produce carbohydrates. The other organism receives some of the carbohydrates and uses them as an energy source to enzymatically fix atmospheric $N_2$ into the ammonia ($NH_3$) form of N and thence into amino acids and other nitrogenous compounds that are nutritionally useful to the chlorophyll-containing organism.

As evident from the equation (*Allen et al., 1994*):

$$N_2 + 10H^+ + nMgATP + 8e^- \rightarrow 2NH_4^+ + H_2 + nMgADP + nP_i \qquad (n \geq 16)$$

$N_2$ fixation is an energy requiring process; biological systems utilize chemically bound energy in organic matter (symbiotic and asymbiotic non-photosynthesizing bacteria) or light energy (blue-greenalgae and photosynthetic bacteria; *Stewart, 1973a,b; Fogg, 1974*).

All organisms which reduce dinitrogen to ammonia do so with the aid of an enzyme complex, nitrogenase. The nitrogenase enzyme system is composed of two oxygen labile and separable metalloproteins (*Allen et al., 1994; Vance, 1997*):

component I is dinitrogenase, the site of $N_2$ reduction, and is a molybdo-ferro-protein (MoFe protein);

component II is dinitrogenase reductase, an iron protein (Fe protein), which provides electrons to component I for $N_2$ reduction.

The Fe protein is a γ2 homodimer (Mr, approximately 60 kDa) encoded by *nifH*, incorporating two binding sites for MgATP. The two identical subunits are bridged by a single [4Fe–4S] cluster. During catalysis, the Fe protein is an agent of electron transfer that sequentially delivers single electrons to the protein in a process coupled to MgATP hydrolysis (*Zehr et al., 2003*). In addition, the Fe protein has at least two, and possibly three, other functions: (i) it is required for the biosynthesis of FeMo cofactor (FeMoco) and apodinitrogenase maturation (*Rangaraj et al. 1997*); and (ii) it has been implicated as being possibly important in the regulation of the alternative systems (*Burgess and Lowe 1996*).

The MoFe protein is an α2β2 heterotetramer encoded by *nifDK* with a Mr of approximately 240 kDa. Associated with MoFe protein are two novel metalloclusters, called P-cluster and FeMoco. The P-cluster is an [8Fe–7S] cluster that can be described in its reduced state as the covalent attachment of two regular [4Fe–4S] clusters through a shared corner S atom (*Kim and Rees 1992; Peter et al. 1997; Mayer et al. 1999*). Electron transfer is believed to proceed from the Fe protein [4Fe–4S] cluster to the P-cluster and then to FeMoco, which provides the substrate reduction site (Dos Santos et al. 2004). The structure of the FeMoco can be described as two bridged partial cubanes: a [Mo–3Fe–3S] partial cubane bridged to a [4Fe–3S] partial cubane via three sulfur atoms that bind to the Fe atoms of each cluster fragment (*Drennan and Peters et al., 2004*).

At the protein level, the basic mechanism of nitrogenase is commonly described as follows: (i) formation of a complex between the reduced Fe protein with two bound ATP molecules and the MoFe protein; (ii) electron transfer between the two proteins coupled to the hydrolysis of ATP; (iii) dissociation of the Fe protein accompanied by re-reduction and

exchange of ATP for ADP; and (iv) repetition of this cycle until sufficient numbers of electrons (and protons) have been accumulated so that available substrates can be reduced (*Rees and Howard 2000*).

Both two components of nitrogenase, dinitrogenase and deinitrogenate reductase, are irreversibly inactivated by oxigen, the latter having a half-life in air of 0.5 to 0.75 sec. (*Zuberer, 1997*). Conseguently, free-living, nitrogen-fixing bacteria only fix nitrogen in anaerobic or low oxygen environments (*Eady, 1992*). Aerobic $N_2$-fixing organisms have developed a number of mechanisms to avoid exposure of these proteins to $O_2$. In the legume-rhizobia symbiosis, these include physical barriers by nodule cortex to oxygen diffusion (*Witty and Minchen, 1990*) and use of leghemoglobin, which binds oxygen and transports it to respiratory sites while excluding it from nitrogenase (*Gallon and Chaplin, 1987; Witty and Minchin, 1994; Iannetta et al., 1995; James et al., 1996*). Leghaemoglobin is the red pigment which colours the interiors of legume root nodules. Other heterocyst formation, dark phase $N_2$ fixation and other structural changes in cyanobacteria (*Wolk, 1996; Fredickson and Bergman, 1997*), and respiratory protection in *Azotobacter* and other organisms (*Prosperi, 1994*).

To agriculture, the most important type of BNF is symbiotic fixation between the root of a leguminous plant (i.e. alfalfa, clovers, peas, beans, etc.) and the soil bacterium *Rhizobium* and it is of considerable ecological significance. The association between legumes and rhizobial bacteria starts as an infection of the root tissues which later becomes a mutualistic relationship. Nodule development and subsequent $N_2$ fixation is a complex process involving a large number of genes.

*Rhizobia* occur in the soil as free-living in rhizosphere soil, stimulated by the flow of carbon from root plant. Nodulation process in legumes results from molecular signalling, the secretion of lipopolysaccharide, between host and *rhizobia*. The *rhizobia* are attracted and bound to the surface of root hairs by this specialized glycoprotein molecule, lectin, produced by the plant. *Rhizobia*, that are compatible with the legume root, exude acidic polysaccharide, named recadhesin which facilitates selective adsorption to the plant leucine. The presence of the *rhizobia* causes the root hair to curl prior to invasion. This invasion is facilitated by invagination of the wall of the root hair into tube or infection thread, and is rapid. Having entered the host's root cytoplasm, both the bacterial cells and the surrounding host cells multiply to enable development of the nodule. Inside the nodule, the rhizobial cells cease their motile habit and take on a non-motile "bacteroid" habit. Nitrogen fixation is carried on by

these specialized symbiontic cells, the bacteroids enclosed within the plant-derived peribacteroid membrane. This membrane control nutrient flow in the form of malate and succinate as well as the iron, molybdenum, and sulphur required in large quantities. Fixed N, as $NH_3$, move to the plant.

Infection with *Rhizobium* also results in the formation numerous nodulins, proteins expressed during symbiosis. Early nodulins function in infection, and in nodule development and morphogenesis; later nodulins (leghemoglobin and the enzyme uricase) play a role in nodule function and $N_2$ fixation (*Pawlowski, 1997*). Nodulins expressed during legume/*Rhizobium* symbiosis have also been detected in mycorrhizal plants (*Wyss et al., 1990*).

### *1.1.2 ECOLOGICAL FACTORS ON BIOLOGICAL FIXATION*

Interactions between the microsymbiont and the plant are complicated by edaphic, climatic, and management factors. A legume-Rhizobium symbiosis might perform well in a loamy soil but not in a sandy soil, in the subhumid region but not in the Sahel, or under tillage but not in no-till plots. These factors affect either the microsymbiont, the host-plant, or both.

Main ***edaphic factors*** limiting biological nitrogen fixation are related to soil:

**excessive moisture and waterlogging** prevent the development of root hair and sites of nodulation, and interfere with a normal diffusion of $O_2$ in the root system of plants. *Sesbania rostrata* and *Aeschynomene* sp. can actively fix $N_2$ under these conditions because they are located on the plant stems, rather than on the roots.

**drought** reduces the number of rhizobia in soils, and inhibits nodulation and $N_2$ fixation. Prolonged drought will promote nodule decay. Deep-rooted legumes exploiting moisture in lower soil layers can continue fixing $N_2$ when the soil is drying. Mycorrhizal infection has also been found to improve tolerance of plants to drought (e.g., *Acacia auriculiformis* inoculated with the ectomycorrhizal *Baletus suillus*). Mycorrhiza are symbiotic associations between fungi and plant roots. Some mycorrhizal fungi develop exclusively outside the roots; these are called ectomycorrhiza (e.g., *Baletus suillus*). Others, called endomycorrhiza, grow inside the roots with their vesicles and arbuscules inside the roots and

with their fungal filaments extended outside (e.g., *Glamus* sp.). These are the vesicular-arbuscular mycorrhiza, usually referred to as VAM.

**soil acidity** and related problems of Ca deficiency and aluminum and manganese toxicity adversely affect nodulation, $N_2$ fixation and plant growth (*Wood, Cooper & Holding, 1983*). Research work on the identification of symbioses adapted to acid soil should focus on the host plant, because effective rhizobia adapted to- soil acidity can be found naturally and can be produced through genetic manipulations.

**phosphorus deficiency** is commonplace in tropical Africa and reduces nodulation, $N_2$ fixation and plant growth. Identification of plant species adapted to low-P soils is a good strategy to overcome this soil constraint. The role of mycorrhizal fungi in increasing plant P uptake with beneficial effects on $N_2$ fixation has been reported. Dual inoculation with effective rhizobia and mycorrhizal fungi shows synergistic effects on nodulation and $N_2$ fixation in low P soils (Trees are usually infected by mycorrhizal fungi in natural ecosystems in the tropics. The significance of this symbiosis in nature should be better recognised). The use of local rock phosphate has been recommended, particularly in acid soils, as an inexpensive source of P. The addition of P-solubilizing microorganisms, particularly of the general *Psemdamaias*, *Bacillus*, *Penicillium*, and *Aspergillus* can solubilize rock phosphate and organically bound soil P (which constitutes 95-99% of the total phosphate in soils). However, the use of these microorganisms is not widespread. Some reports show nodulation response to K under field conditions. However, other investigators consider the K effect to be indirect, acting through the physiology of the plant.

**mineral N** inhibits the Rhizobium infection process and also inhibits $N_2$ fixation. The former problem probably results from impairment of the recognition mechanisms by nitrates, while the latter is probably due to diversion of photosynthates toward assimilation of nitrates. Some strains of Rhizobium, and particularly stem-nodulating *Azarhizobium caulinodans*, fix $N_2$ actively even when plants are growing in high-N soils (e.g., in the presence of 200 kg fertilizer N $ha^{-1}$). Application of large quantities of fertilizer N inhibits $N_2$ fixation, but low doses (<30 kg N ha-1) of fertilizer N can stimulate early growth of legumes and increase their overall $N_2$ fixation. The amount of this starter N must be defined in relation to available soil N.

**various microelements (Cu, Mo, Co, B)** are necessary for $N_2$ fixation. Some of these are components of nitrogenase for example Mo.

The two important ***environmental determinants*** affecting BNF are:

**temperature** affect $N_2$ fixation adversely. This is easy to understand because $N_2$ fixation is an enzymatic process. However, there are differences between symbiotic systems in their ability to tolerate high (>35°C) and low (<25°C) temperatures.

**light**, the availability of light regulates photosynthesis, upon which biological nitrogen fixation depends. This is demonstrated by diurnal variations in nitrogenase activity. A very few plants can grow and fix $N_2$ under shade (e.g., *Flemingia congesta* under plantain canopy). In alley farming if hedgerows are not weeded, or if trees are planted with food crops like cassava, their nitrogen fixation and growth will be reduced due to shading. Early growth of legume trees is slow and they cannot compete successfully for light.

Among ***biotic factors***, the absence of the required rhizobia species constitute the major constraint in the nitrogen fixation process. The other limiting biotic factors could be:

excessive defoliation of host plant;

crop competition;

insects and nematodes.

### 1.1.3 NITROGEN IMMOBILIZATION

The process of immobilization involves the incorporation of inorganic N, ammonium and nitrate, into organic N, microbial protein and nucleic acids mediated by microorganisms. The incorporation of N into the microbial biomass and organic N occurs through numerous enzymatic pathways. In general, the preferred inorganic N source for assimilation by bacteria and fungi is $NH_3/NH_4^+$ and microbial assimilation of $NO_3^-$ is minimal (*Tiedje et al., 1981; Myrold and Tiedje, 1986*). Nevertheless, substantial microbial assimilation of $NO_3^-$ has been observed in several forest soils (*Davidson et al., 1992; Hart et al., 1994; Stark and Hart, 1997; Berntson and Aber, 2000*). In addition, it was shown that $NH_4^+$ (*Johnson et al., 2000*) and $NO_3^-$ (*Davidson et al., 2003*) can be immobilized by abiotic processes as well. Typically, $NH_3$ enters microbial cells by rapid diffusion across cytoplasmic membranes although there is now evidence of $NH_4^+$ active transport in several bacteria (*Merrick and Edwards, 1995*).

Biological $NH_4^+$ immobilization by soil microorganisms is mainly accomplished by two enzymatic pathways, either through glutamate dehydrogenase (GDH) or through glutamine

synthetase/glutamate synthase (GS/GOGAT). The glutamate and glutamine formed by these pathways serve as central N donors for transamination reactions yielding the amino acids and nucleotides which are the building blocks for proteins and nucleic acids. Although GDH was previously considered the major enzyme system involved in ammonium assimilation, it now seems clear that, at the low $NH_4^+$ concentration typical of most soils, GS/GOGAT is the major enzyme involved both in microorganisms and in plants (*Miflin and Lea, 1977; Brown and Johnson, 1977; Lee and Stewart, 1978*). The GS/GOGAT system has a much higher affinity for the substrate than glutamate dehydrogenase, then is operative requiring the input of energy in the form of ATP (*Paul and Clark, 1996*) while the GDH pathway is not ATP-dependent and immobilizes N at relatively high $NH_4^+$ concentrations (>1mM) (*Neidhardt et al., 1990*).

Assimilation of nitrogen as $NH_4^+$ by microorganisms leads to proton production and soil acidification while assimilation as $NO_3^-$ results in hydroxyl or bicarbonate ion production.

Nitrate may be immobilized directly by both bacteria and fungi by assimilatory $NO_3^-$ reduction (ANR) (*Paul and Clark, 1996; Berntson and Aber, 1999; Zogg et al., 2000; Perakis et al., 2001*). The occurrence of nitrate assimilation in bacteria seems to be more common than in fungi, although it is in no way ubiquitous (*Hall, 1978*). Of the 2500 genera of fungi described, only 20 have been reported to assimilate nitrate (*Payne, 1973; Downey, 1978*). The enzymes responsible for reduction are assimilatory nitrate reductase and assimilatory nitrite reductase. The pathway of ANR is:

$$NO_3^- \rightarrow NO_2^- \rightarrow NH_2OH \rightarrow NH_4^+ \rightarrow R\text{-}NH_2 \quad (\textit{Paul and Clark, 1996})$$

The synthesis of the ANR enzymes (nitrate and nitrite reductases) is regulated by the global reactive nitrogen system, inducible by $NO_3^-$ (*Merrick and Edwards, 1995*) but repressed by $NH_4^+$ (*Gottschalk, 1979; Rice and Tiedje, 1989; Recous et al., 1990*), or glutamine (*McCarty, 1995*).

There is evidence that amino acids may form an important source of nitrogen for plants which may therefore compete with soil microorganisms (*Chapin et al., 1993; Jones and Darrah, 1994; Kaye and Hart, 1997*). Microorganisms may also uptake nitrogen in organic form. For example, all fungi appear able to utilise some organic nitrogen sources while certain plant pathogenic fungi may be unable to utilize inorganic forms (*Jennings, 1989*). Ericoid, some ectomycorrhizal and other fungi produce diffusible enzymes around the roots and

assimilate the lower molecular weight breakdown products of protein degradation (*Read et al., 1989*) to meet their nitrogen requirements.

### 1.1.4 NITROGEN MINERALIZATION

The amount of N produced by mineralization is a major control on N availability to plants especially in unfertilized systems (*Binkley and Hart, 1989; Powlson and Barraclough, 1993; Mengel, 1996*). Nitrogen mineralization is the microbial process of converting organic nitrogen to an inorganic form, usually ammonium ($NH^+_4$-N), which gives rise to their cellular respiration, coupled with energy (ATP) production and is therefore the opposite process of immobilization. Ammonium can then be further transformed into nitrate ($NO_3^-$-N). The first step, called *ammonification*, is the oxidation process of organic nitrogen to $NH_4^+$, and is carried out by both aerobic and anaerobic microbes, while the second step, named *nitrification* is the process that further oxidizes $NH^+_4$-N to $NO_3^-$-N and only aerobic microbes, mainly *Nitrosomonas* and *Nitrobacter*, are involved in nitrification. In most soils the ammonification rate is slower than the nitrification rate.

Organic N ➔ *ammonification* ➔ $NH_4^+$ ➔ *nitrification* ➔ $NO_3^-$

### *1.1.5 AMMONIFICATION*

When plants or animals die they contribute organic nitrogen to the soil. As the organic N is assimilated into the microbial biomass, N in excess of microbial requirements is released as $NH_4^+$ or mineralized. The major biological forms of this organic nitrogen include a broad range of proteins, microbial cell wall constituents such as amino-sugars and their polymers, and nucleic acids, such as DNA and RNA. Nitrogen is also mineralized from soil organic matter (SOM) which contains various N compounds including a large amount of heterocycling and phenolic N which is only slowly decomposed. Since proteins and peptides are a major source of mineralizable N (*Mengel, 1996*), a variety of enzymes (*Ladd and Jackson, 1982*) and microbes are involved. Mineralization of organic N is basically a

sequence of enzymatic reactions. Proteins are macro-molecules consisting of long chains of amino acids. Proteins are broken down by proteinases and peptidases to amino acids. Then $NH_3$ is released from the amino acids through dehydrogenases and oxidases (*Stevenson, 1986).*

Proteins ➔ *proteinases* ➔ Amino Acids ➔ *oxidases dehydrogenase* ➔ $NH_4^+$

The polymers of amino sugars, that are important components of bacterial and fungal cell walls, are hydrolyzed by *glycosidases.* Other compounds follow a similar enzyme mediated path from the intact macro-molecule to $NH_4^+$. DNA and RNA are broken down rapidly by the action of nucleases to mono-nucleotides. *Nucleotidases* break down the mono-nucleotides to nucleosides and $PO_3^{4-}$. Other enzymes (nucleosidases) break these nucleosides down to purine and pyrimidine bases, which are in turn hydrolyzed to ammonium. Due to the phosphate ester bond, the energy content of nucleic acids are relatively high, and therefore they do not persist in soils (*Paul and Clark, 1989*), moreover the observation that free nucleic acids are degradated rapidly substantiates the ubiquitous presence of the nucleases in the soil environment.

Nucleic Acids ➔ *nucleases* ➔ Mononucleotides
Mononucleotides ➔ *nucleosidases* ➔ Purines
Purines ➔ *hydrolyases* ➔ $NH_4^+$

### *1.1.6 CONTROLS ON MINERALIZATION/IMMOBILIZATION*

Mineralization is always accompanied by immobilization. The two processes operate simultaneously in the soil within relatively small volumes of soil and are mutually dependent. Most of the N released during mineralization is quickly assimilated in the microbial biomass and immobilized in the microbial tissue, if enough C is available. The immobilized N will be newly available at the end of the growing phase of the microbial population, as the turnover of the microbial biomass is very fast (few days) and the microbial N represents one of the main components of the "available N pool" in the soil (*Schnürer et al., 1985*).

Mineralization and immobilization are widely distributed because they are so fundamental — all heterotrophic soil organisms consume organic materials for energy and C and immobilize and mineralize N as a by-product. The widely distributed nature of mineralization and immobilization means that the environmental regulation of these processes is relatively straightforward.

Rates of activity increase with temperature and are optimal at intermediate water contents, similar to respiration in **Fig. 1.2**, although it is important to recognize that significant activity is likely to occur at extremes of both temperature and moisture. In most soils the quantity and quality of detrital inputs are the main factors that control the rates and patterns of mineralization and immobilization. When moisture and temperature are favourable, large inputs of organic matter lead to high rates of microbial activity and the potential for high rates of mineralization and immobilization.

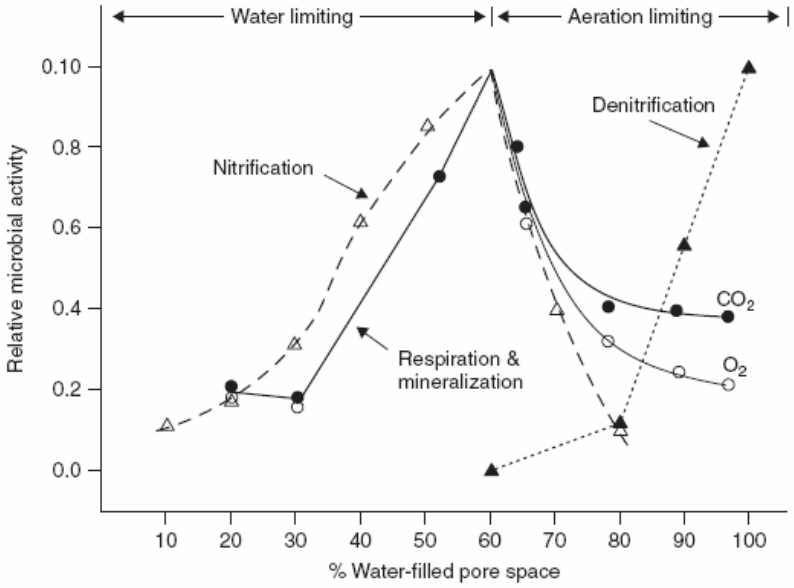


**Figure 1-2.** The relationship between water-filled pore space (a measure of soil moisture availability) and relative amount of microbial activities. (From *Linn and Doran, 1984*).

As a result of the simultaneous nature and small scale of these processes, it is also important to make a distinction between gross and net mineralization and immobilization. Gross N mineralization is the total amount of soluble N produced by microorganisms, and gross N immobilization is the total amount of soluble N consumed. *Net mineralization* is the difference between gross mineralization and gross immobilization. When gross mineralization

exceeds gross immobilization, inorganic N in the soil is increasing, i.e., there is net mineralization. When gross immobilization exceeds gross mineralization, inorganic N in the soil is decreasing, i.e., there is net immobilization.

One of the critical points in the net balance mineralization/immobilization is the C/N ratio of the substrate. To be able to measure a net mineralization, it is usually necessary that the substrate to be decomposed has a C:N ratio lower than 30 (more than 1.18 % of N), the microbes have no trouble obtaining N and as a result mineralization dominates over immobilization, and plant-available N increases in soil. On the other hand, when adding a material with a high C:N ratio (625:1) to soil, the microbes are keen to obtain the energy and C but cannot degrade this material without additional N because it does not have sufficient N to allow the microbes to build proteins. So the microbes must immobilize N from their environment, resulting in a decrease in plant-available N in the soil. As a general rule of thumb, materials with a C:N ratio >30:1 stimulate immobilization, while those with a C:N ratio <30:1 stimulate mineralization. However a generalization is very difficult to make, as other factors such as the quality of the substrate (lignin and pholiphenolic content) can influence the mineralization rates (*Sequi, 1989*). Highly decomposed substances with a low C:N ratio, for example., soil organic matter (humus or compost) in which labile C and N have been depleted and the remaining C is in complex forms are inherently resistant to decomposition and therefore resistant to mineralization.

The balance between mineralization and immobilization is also affected by organism growth efficiency. Fungi generally have a higher C/N ratio in their tissues than bacteria and may therefore immobilize less N per unit of substrate, and mineralize N more readily. However this difference can be offset by fungi having a higher efficiency for C assimilation (less C lost as $CO_2$) (*Wood, 1995*).

### 1.1.7 NITRIFICATION

The process of nitrification is a major control point in the N cycle and rates have been intensively studied in a variety of ecosystems.

Nitrification is the soil microbial process responsible for conversion of $NH_4^+$-N into nitrite ($NO_2^-$-N) and then into $NO_3^-$; gaseous nitrogen compounds (NO, $N_2O$, $N_2$) can be

produced as a by-product of nitrification. Nitrification is an aerobic process performed both by autotrophs and heterotrophs microorganisms in soils. Autotrophic nitrification is by far the most studied process (*Prosser, 1986; Umarov, 1990*) and the main process recognized in most soils, although heterotrophic nitrification may be of importance in specialized situations (*van Neil et al., 1993*).

### *1.1.8 AUTOTROPHIC NITRIFICATION*

The most diffused pathway of nitrification is the chemoautotrophic oxidation of ammonium ($NH_4^+$) to nitrate ($NO_3^-$). A specific group of microorganisms; gram-negative bacteria of the family Nitrobacteraceae are responsible for autotrophic nitrification (*Bock et al., 1992; Schmidt and Belser, 1994*). For these groups of chemoautotrophic bacteria the oxidation of $NH_4^+$ to $NO_2^-$ and to $NO_3^-$ is the sole energy source available (*Wood, 1986*). With the exception of some strains of Nitrobacter, nitrifying bacteria utilise $CO_2$ as the major carbon source (*Bock, 1978; Matin, 1978*). Few strains are able to grow mixotrophically, assimilating organic compounds, but the rate of growth on these substrates is quite limited (*Matin, 1978; Krummel and Harms, 1982*). Nitrifiers have a very slow growth rate as, for each carbon fixed in the chemosynthesis, they have to oxidise about 35 molecules of $NH_4^+$ or 100 molecules of $NO_2^-$ (*Baas Becking and Parks, 1927*). The $NH_4^+$-N can originate from mineralization of soil organic material by other organisms or from fertilizer:

$$NH_4^+ + 2O_2 \rightarrow NO_3^- + 2H^+ + H_2O + \text{energy}$$

No bacteria have been found which can convert $NH_3$ to $NO_3^-$ directly (*Hooper et al., 1997*). In autotrophic nitrification, the conversion of N takes place in two separate steps (*Haynes, 1986*).

In the first one step, bacteria called ammonium-oxidizers convert $NH_4^+$ to $NO_2^-$ with $NH_2OH$ as an intermediate. They are given names with the prefix Nitroso-, and belong to the genera *Nitrosomonas, Nitrosoccocus, Nitrosospira* e *Nitrosolobus*. Species of *Nitrosomonas* are the best known in this group. The oxidation of $NH_4^+$ to $NO_2^-$ is obtained through several reactions. The first key reaction is the transformation of ammonia ($NH_4^+$) to hydroxylamine

($NH_2OH$), which involves $NH_4^+$ (*Suzuki et al., 1974*), molecular $O_2$ (*Hollocker et al., 1981*) and reductant (*Hooper, 1969; Suzuki et al., 1976*) and yields hydroxylamine as a product (*Lees, 1952, Hofman and Lees, 1953; Nicholas and Jones, 1960*):

$$NH_4^+ + O_2 + H^+ + 2e^- \rightarrow NH_2OH + H_2O$$
$$NH_2OH + O_2 \rightarrow NO_2^- H^+ H_2O$$
$$NH_4^+ + 2e^- 2O_2 \rightarrow NO_2^- 2H_2O$$

The reaction is catalysed by an enzyme known as ammonia monooxygenase, which is located in the cellular membrane (*Suzuki and Kwok, 1981; Tsang and Suzuki, 1982*). Acetylene ($C_2H_2$) acts as an irreversible inhibitor of this enzyme (*Hynes and Knowles, 1978*). It seems that the enzyme, in the attempt to oxidise the $C_2H_2$, becomes covalently bound to the substrate and remains permanently modified and destroyed (*Hyman and Wood, 1985*) and thereby provides a means for experimentally differentiating autotrophic from heterotrophic nitrification in soil.

The oxidation of $NH_4^+$ to $NH_2OH$ is energetically unfavourable ($\Delta G^{\circ\prime} = +17$ kJ/mol) (*Wood, 1986*). For the reaction to proceed, there is the need for a parallel reaction which provides electrons. It seems that monooxygenase accepts electrons from the ubiquinone-cytochrome b region of the transport chain (**Fig. 1.3**), with NADH acting as a donor (*Wood, 1986*).

In a second step $NH_2OH$ is converted to $NO_2^-$. *Andersson and Hooper (1983)* have found that water contributes one oxygen to the synthesis of $NO_2^-$ in a mechanism described as follows:

$$E + H_2NHO \rightarrow E\text{-}NO^+ + 3\,H^+ + 4\,e^-$$
$$E\text{-}NO^+ + H_2O \rightarrow E + NO_2^- + 2\,H^+$$

Electrons which are produced in the course of these subsequent oxidations flow through a electron transport chain, which is completely reversible except for the terminal oxidase (**Fig. 1.3**). As can be seen from **Figure 1.3**, hydroxylamine oxidoreductase feeds electrons to a point close to the ubiquinone, while ammonia monooxygenase abstracts electrons, probably at

the same point. It also can be seen that monooxygenase and terminal oxidase are competing sinks for the electrons and the balance between them needs to be carefully controlled.

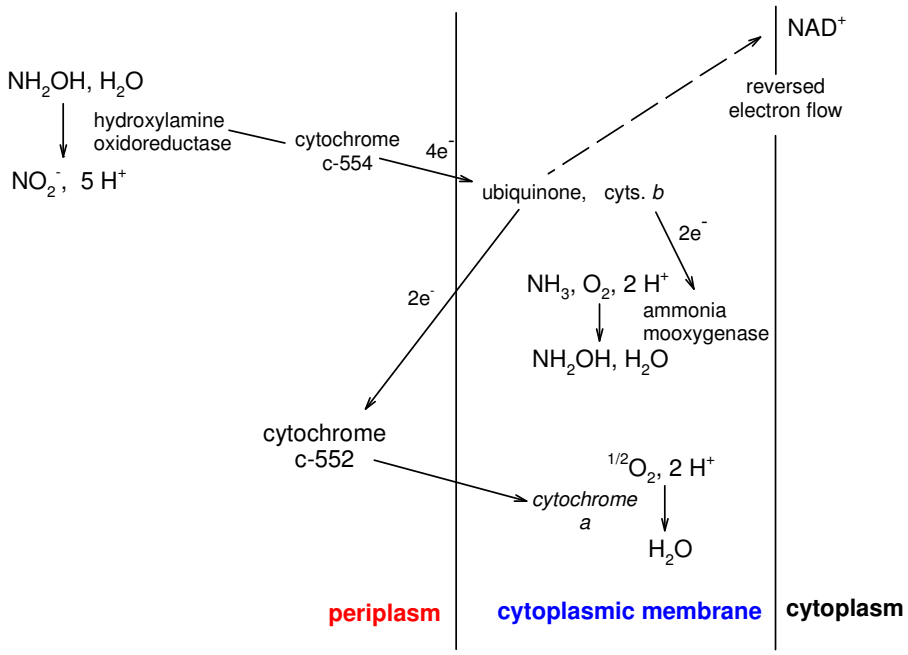


**Figure 1-3.** Electron transport in Nitrosomonas (from *Wood, 1986*).

Intermediary compounds formed during the oxidation of hydroxylamine to nitrite can result in the formation of NO (**Fig. 1.4**), which can escape to the atmosphere and influence the photochemical production of ozone ($O_3$) and the abundance of hydroxyl (OH) radicals in air, primary oxidants for a number of tropospheric trace gases including methane. Ammonia oxidizers also appear able to produce NO via $NO_2^-$ reduction, which results in the production of $N_2O$, an important greenhouse gas that can also escape to the atmosphere. Nitrite reduction occurs when ammonia oxidizers use $NO_2^-$ as an electron acceptor when $O_2$ is limiting—effectively becoming denitrifying nitrifiers.

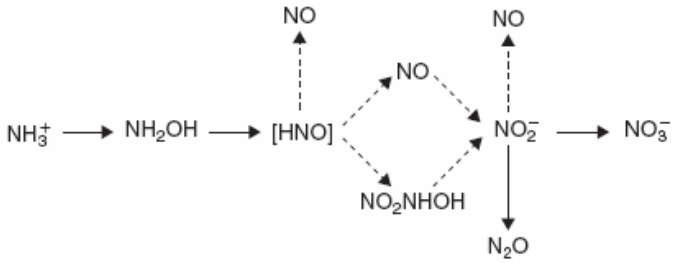


**Figure 1-4.** Autotrophic nitrification pathways including pathways for gas loss. Broken lines indicate unconfirmed pathways (from *Firestone and Davidson, 1989).*

In most soils the nitrite produced by ammonia oxidizers does not accumulate but is quickly oxidized to nitrate by the nitrite-oxidizing bacteria when they perform nitrite oxidation.

Nitrite oxidation is carried out by a different bacterium, Nitrobacter is regarded as the dominant (*Watson et al., 1981; Laanbroek and Woldendorp, 1995*), with detectable intermediates and the extra atom of oxygen which is derived from water (*Aleem et al., 1965, Kumar et al., 1983*):

$$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^-$$
$$2H^+ + 2e^- + \tfrac{1}{2} O_2 \rightarrow H_2O$$
$$NO_2^- + \tfrac{1}{2} O_2 \rightarrow NO_3^-$$

The oxidation is coupled to ATP synthesis (*Cobley, 1976a*) via a mechanism which could involve some direct chemical intermediate (*Cobley, 1976a,b*) or some other pathway involving a proton pump (*Ferguson, 1982*).

### *1.1.9 HETEROTROPHIC NITRIFICATION*

Heterotrophic nitrification may be broadly defined as the oxidation of reduced N compounds producing $NO_2^-$ and $NO_3^-$. So-called heterotrophic nitrification is not linked to cellular growth, as it is for autotrophic nitrification. Heterotrophic organisms use organic substances as both a carbon and an energy source. *Eylar and Schmidt (1959)* isolated 978 cultures of heterotrophic organisms from twelve actively nitrifying soils and tested them for

their ability to form $NO_2^-$ and $NO_3^-$ in glucose peptone broth. Though the yields of $NO_2^-$ were very low, fungus isolates were the most numerous and active $NO_2^-$ producers and fifteen of the fungi formed $NO_3^-$ in addition to $NO_2^-$ (**Table 1.2**).

**Table 1-2.** Microbial forms capable of producing $NO_2^-$ and $NO_3^-$ when grown on glucose and peptone medium (from *Eylar and Schmidt., 1959*).

| *Isolates* | Total number | Isolates forming $NO_2^-$-N in excess of | | Isolates forming $NO_3^-$-N in excess of |
|---|---|---|---|---|
| | | *0.2 μg/ml* | *0.5 μg/ml* | *5.0 μg/ml* |
| ***Actinomycetes*** | 222 | 16 | 1 | 0 |
| ***Bacteria*** | 341 | 24 | 8 | 1 |
| ***Fungi*** | 415 | 26 | 14 | 15 |
| Total | 978 | 66 | 23 | 16 |

Most of the work done to investigate heterotrophic nitrification has been done on *Aspergillus flavus*. *Eylar and Schmidt. (1959)* identified most of the fungi which produced nitrate as *Aspergillus flavus* (16 of the 18 active cultures). However, it has been shown that numerous other fungi isolated from coniferous forest soils have the ability to nitrify (*Remacle, 1977a; Remacle, 1977b; Johnsrud, 1978)*.

Though fungi have been found to be the most efficient heterotrophic nitrifiers (*Odu and Adeoye, 1970*) a number of heterotrophic bacteria (*Tate, 1977; Castignetti and Hollocher, 1982; Kuenen and Robertson, 1988; Papen et al., 1989*) and an actinomycete (*Remacle, 1977b*) have been identified as potential nitrifiers in soil.

Different pathways have been postulated, the substrate for heterotrophic nitrification is generally amino N although some organisms have been identified that can oxidize $NH_4^+$ through an inorganic pathways involving $NH_2OH$ (*Aleem, 1975; Prosser, 1989*):

$$NH_4^+ \rightarrow NH_2OH \rightarrow NOH \rightarrow NO_2^- \rightarrow NO_3^-$$

The second heterotrophic pathway is organic and appears limited to fungi. Organic pathway involve oxidation of an amine or amide in place of hydroxylamine, with subsequent oxidation to a nitroso and then to a nitro-compound (*Doxtader, 1965*):

$RNH_2 \rightarrow RNHOH \rightarrow R\text{-}NO \rightarrow R\text{-}NO_2 \rightarrow NO_3$

Heterotrophic nitrification has not been extensively studied, the rate of production of $NO_2^-$ or $NO_3^-$ by heterotrophic nitrification have generally been much lower than autotrophic nitrification (*Prosser, 1989; Jetten et al., 1997*). From the results reported in the literature it seems that heterotrophic nitrification does not yield any significant quantity of energy. The heterotrophic bacterium *Tiosphera pantotropha,* has been found able to catalyse the oxidation of $NH_4^+$ to $NO_2^-$ only if an organic electron donor is present (acetate in the specific case) (*Kuenen and Robertson, 1987; Robertson et al., 1988*). Also, other heterotrophic microorganisms have been found to be able to nitrify, but only if a source of energy is supplied (*Castignetti, 1988*). Heterotrophic nitrification may be important in view of the possible ecological significance of some of the products identified. Nitrification led by the fungus *Aspergillus flavus* has been proposed as a mechanism which could function as an endogenous metabolism for the organism (*Van Gool and Schmidt, 1973*). *Focht and Verstraete (1977)* suggested that heterotrophic nitrifiers could utilize certain intermediates of nitrogen oxidation (hydroxamic acids) as growth factors, possibly linked to iron uptake, or as biocidal factors to assist in their competition and survival.

Heterotrophic nitrification may dominate over autotrophic under certain conditions. A low pH is one factor that seems to strongly restrict autotrophic nitrification (*Kuenen and Robertson, 1988*), so heterotrophic nitrification may be of significance in acidic forest soils (*Killham, 1987,1990; Duggin 1991; Papen and von Berg, 1998*). However, recent studies have shown that autotrophic nitrification does occur in acidic coniferous forest soils (*De Boer and Kowalchuk, 2001*), although the exact mechanisms by which nitrification occurs at low pH are not well understoodand, and that heterotrophic nitrification does not play an important role (*De Boer et al., 1992; Martikainen et al., 1993; Rudebeck and Persson, 1998*). Heterotrophic nitrification thus appears important in some soils and microenvironments, perhaps particularly where autotrophic nitrifiers are chemically inhibited, but are thought now to rarely dominate the soil nitrifier community.

### *1.1.10 CONTROLS ON NITRIFICATION*

The single most important factor regulating nitrification in the majority of soils is ammonium supply (**Fig. 1.5**). Where decomposition and thus N mineralization is low or where $NH_4^+$ uptake and thus N-immobilization by heterotrophs or plants is high, nitrification rates will be low. Conversely, any ecosystem disturbance that increases soil $NH_4^+$ availability will usually accelerate nitrification unless some other factor is limiting. Tillage, fire, clear-cutting, waste disposal, fertilization, atmospheric N deposition—all have well-documented effects on nitrate production in soils, mostly due to their effects on soil $NH_4^+$ pools.

The fact that nitrification usually accelerates only when the $NH_4^+$ supply exceeds plant and heterotroph demand implies that nitrifiers are relatively poor competitors for $NH_4^+$ in the soil solution. In fact this is the case: nitrification rates are typically low in midsuccessional communities and aggrading forests because of high plant demand for N, and also following the addition of high C:N residues to agricultural soils because of high microbial (heterotroph) demand for N. In old-growth forests and mature grasslands, plant N demand has diminished and consequently nitrification is usually higher than in midsuccessional communities in which plant biomass is still accumulating, but not usually as high as in early successional communities, in which N supply often greatly exceeds demand (*Robertson and Vitousek, 1981*).

Oxygen is another important regulator of nitrification in soil. All known nitrifiers are obligate aerobes, and nitrification proceeds very slowly if at all in submerged soils. In flooded environments such as wetlands and lowland rice, nitrifiers are active only in the oxidized zone around plant roots and at the water–sediment interface, usually only a few millimeters thick. And although some nitrifiers have the capacity to use nitrite rather than $O_2$ as an electron acceptor during respiration, $O_2$ is still required for ammonia oxidation.

Nitrifiers are little different from other aerobic microbes with respect to their response to temperature, moisture, and other environmental variables (see **Fig. 1.5**). Nitrification occurs slowly but readily under snow and in refrigerated soils, and soil transplant experiments (e.g., *Mahendrappa et al., 1966*) have demonstrated an apparent capacity for nitrifiers to adapt to different temperature and moisture regimes. For many decades nitrifiers were thought to be inhibited in acid soils, probably because in many cases and especially in soils from cultivated fields, raising soil pH with calcium or magnesium carbonate stimulates nitrification, and

culturable nitrifiers exhibit a pH optimum of 7.5–8 (*Prosser, 1989*). We now recognize that nitrification can be high even in very acid forest soils (pH< 4.5; *Robertson, 1989*), although the physiological basis for this is still not well understood (*DeBoer and Kowalchuck, 2001*).

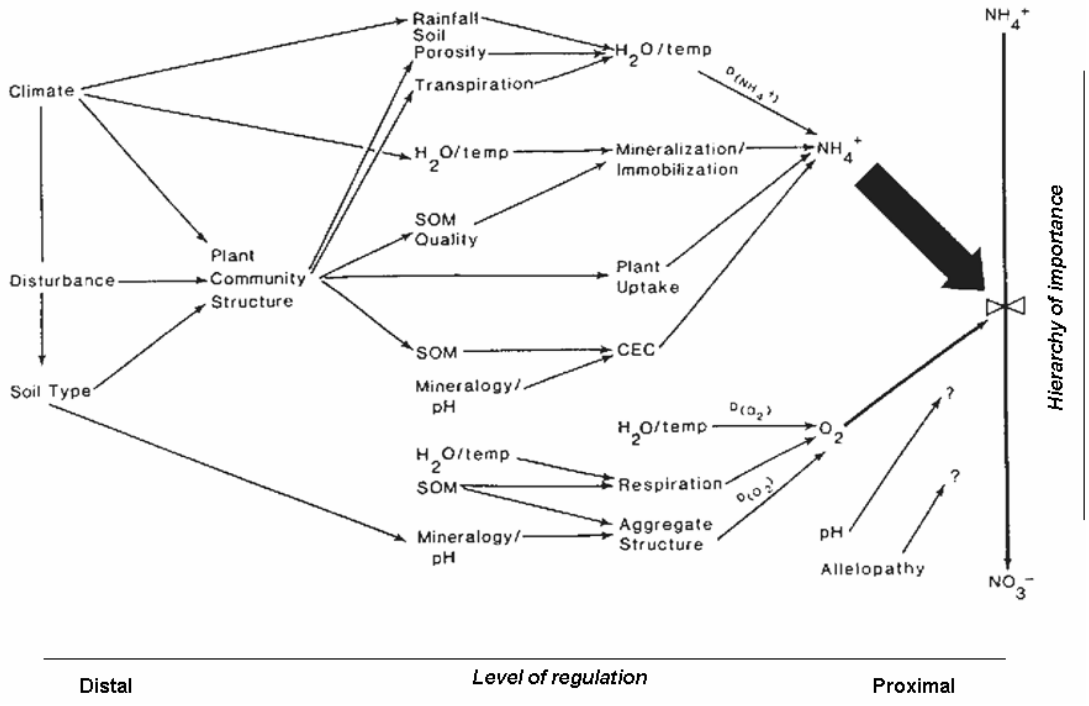


**Figure 1-5.** Environmental controls on nitrification (from *Robertson, 1989,* after *Groffman et al., 1988*). The most proximal scale (right side) is at the cellular level.

### 1.1.11 DENITRIFICATION

When oxygen concentration in the environment is not sufficient to supply for the demand of microbial respiration, a wide variety of mostly heterotrophic bacteria are able to use $NO_3^-$ rather than oxygen ($O_2$) as a terminal electron acceptor during respiration. Because nitrate is a less efficient electron acceptor than $O_2$, most denitrifiers undertake denitrification only when $O_2$ is otherwise unavailable. In most soils this occurs mainly following rainfall as soil pores become water-saturated and the diffusion of $O_2$ to microsites is slowed drastically. Typically denitrification starts to occur at water-filled pore space concentrations of 60% and higher (Fig.). In wetland and lowland rice soils diffusion may be restricted most of the time. Oxygen demand can also exceed supply inside soil aggregates and in rapidly decomposing

litter. Denitrification is a crucial part of the overall N cycle. It is the only point in the N cycle at which fixed N re-enters the atmosphere as $N_2$; it thus serves to close the global N cycle. From a management perspective, denitrification is advantageous when it is desirable to remove excess $NO_3^-$ from soil prior to its movement to ground or surface waters (*Lowrance et al., 1984*). However, in managed ecosystems it is usually desirable to minimize denitrification in order to conserve N further for plant uptake; in regions with ample rainfall ecosystem N losses due to denitrification can compare or exceed losses by nitrate leaching.

The process is generally referred as dissimilatory nitrate reduction as, starting from the form of $NO_3^-$, N can be reduced, in subsequent steps, until it is finally transformed into molecular N ($N_2$), and can be lost from the system as NO, $N_2O$ or $N_2$ (*Knowles, 1981; Tiedje, 1988*). Globally, denitrification in soil may account for > 60% of total $N_2 + N_2O$ production (*Bowden, 1986; Aulakh et al., 1992*).

It is carried out by a broad array of soil bacteria, including organotrophs, chemo- and photolithotrophs, $N_2$ fixers, thermophiles, halophiles, and various pathogens. Over 50 genera with over 125 denitrifying species have been identified (*Zumft, 1992*). In soil, most culturable denitrifiers are facultative anaerobes from only 3–6 genera, principally *Pseudomonas* and *Alcaligenes* (*Focht and Verstraete, 1977*) and to a lesser extent *Bacillus*, *Agribacterium*, and *Flavibacterium* (*Tiedje, 1994*). Denitrification is usually thought of as a bacterial process, but *Shoun et al. (1992)* reported that many fungi are capable of evolving $N_2O$ under anaerobic conditions.

Heterotrophic bacteria use $NO_3^-$ as their primary electron acceptor for obtaining energy from organic compounds when low $O_2$ availability restricts their metabolism according following equation:

$$5(CH_2O) + 4NO_3^- + 4H^+ \quad 5CO_2 + 7H_2O + 2N_2 + \text{energy}$$

Some microorganisms can be obtain energy by using $NO_3^-$ for oxidation of inorganic compounds, $S^{2-}$, $Fe^{2+}$ (autotrophic denitrification). This occurs where $NO_3^-$ diffuses into zones rich in FeS.

However, heterotrophic denitrification is the most important of the two processes into which organisms obtain energy (ATP) by electron transport phosphorylation via the cytochrome system (*Tiedje, 1982*).

The general pathway is:

$2NO_3^- \rightarrow .2NO_2^- \rightarrow 2NO \rightarrow N_2O \rightarrow N_2$

All steps within this metabolic pathway are catalyzed by complex multisite metalloenzymes with characteristic spectroscopic and structural features (*Berks, 1995*). In the overall process (**Fig. 1.6**) $NO_3^-$ is transported through the cellular membrane into the cytoplasm (1). It is then reduced in 4 steps by nitrate (2), nitrite (3), nitric oxide (4) and nitrous oxide (5) reductases (*Payne, 1981; Hochstein & Tomlinson, 1988*). The electrons necessary for these reductions are obtained from organic matter via an electron transport chain (Figure 1.3). $O_2$ and $H_2O_2$ can be used as an alternative electron acceptors. At each step the process can be inhibited by different substances; acetylene ($C_2H_2$) at high concentrations can inhibit step (5), while oxygen can inhibit both denitrifying enzyme activity and the synthesis of new denitrifying enzymes (*Payne, 1973; Smith and Tiedje, 1979*).

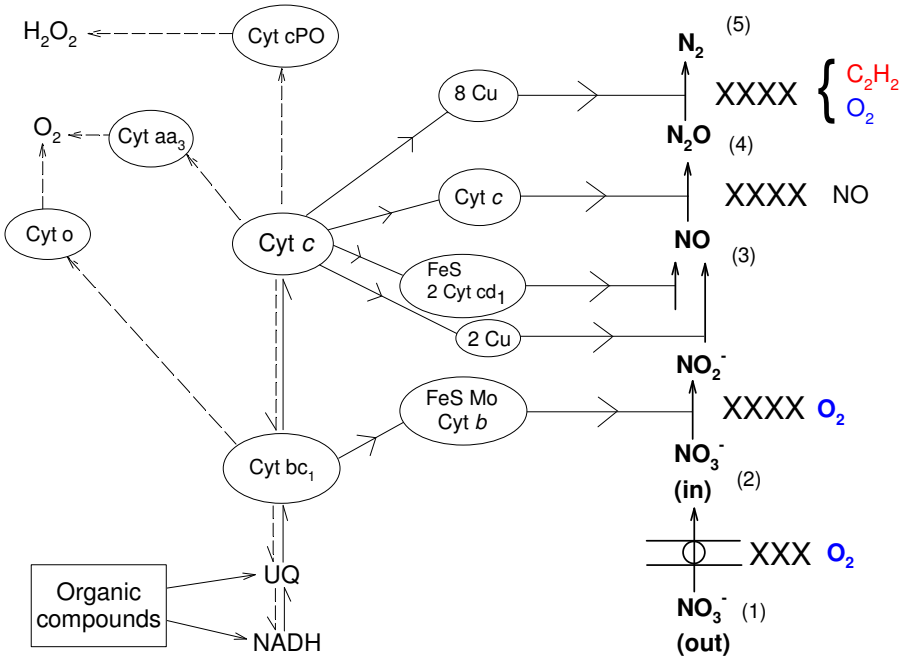


**Figure 1-6.** The process of denitrification. Reductase redox-active centres are shown encircled. (From *Lloyd, 1993*).

Some organisms possess the overall pathway, while others may carry out only a few of these steps. Some bacteria produce only $N_2$, while others give a mixture of $N_2O$ and $N_2$, and some only $N_2O$ (*Kaplan & Wofsey, 1985; Stouthamer, 1988; L.A. Robertson & Kuenen, 1991*). Depending on conditions, at any step in this process, intermediate products can be exchanged with the soil environment, making denitrifiers a significant source of $NO_2^-$ in soil solution and important sources of the atmospheric gases NO and $N_2O$.

Each denitrification enzyme is inducible, primarily in response to the partial pressure of $O_2$ and substrate (C) availability. Because enzyme induction is sequential and substrate dependent, there is usually a lag between the production of an intermediate substrate and its consumption by the next enzyme. In pure culture, these lags can be on the order of hours (**Fig. 1.7**); in the field lags can be substantially longer, and differences in lags among different microbial taxa may significantly affect the contribution of denitrifiers to fluxes of NO and $N_2O$ to the atmosphere. That induced enzymes degrade at different rates, and more slowly than they are induced, also leads to a complex response to the environmental conditions that induce denitrification; whether a soil has denitrified recently (whether denitrifying enzymes are present) may largely determine its response to newly favorable conditions for denitrification. Rainfall onto soil that is moist, for example, will likely lead to a faster and perhaps stronger denitrification response than will rainfall onto the same soil when it is dry (*Groffman and Tiedje, 1988; Bergsma et al. 2002*).

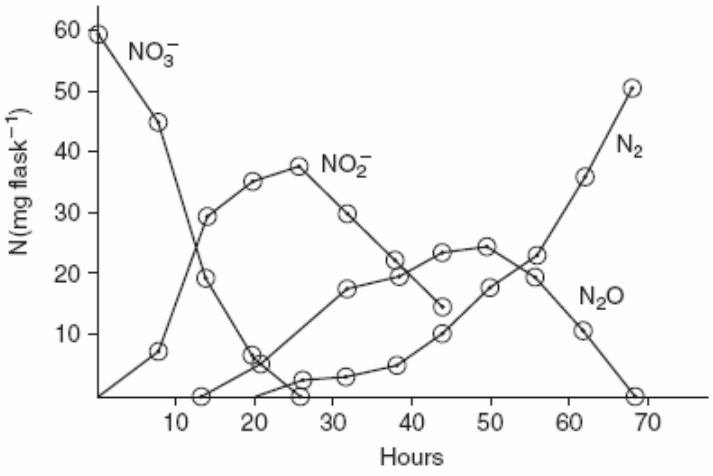


**Figure 1-7.** The sequence of products formed during denitrification. (From *Cooper and Smith,1963*)

### *1.1.12 CONTROLS ON DENITRIFICATION*

For decades after its discovery as an important microbial process, denitrification was assumed to be important only in aquatic and wetland ecosystems. It was not until the advent of whole-ecosystem N budgets and the use of $^{15}N$ to trace the fate of fertilizer N in the 1950s that denitrification was found to be important in unsaturated soils. These studies suggested the importance of denitrification in fertilized agricultural soils, and with the development of the acetylene block technique in the 1970s (*Yoshinari and Knowles, 1976; Smith et al., 1978*), the importance of denitrification in even forest and grassland soils was confirmed. Acetylene selectively inhibits nitrous oxide reductase (**Fig. 1.6**), allowing the assessment of $N_2$ production by following $N_2O$ accumulation in a soil core (*Tiedje, 1994*) or monolith treated with acetylene (*Ryden and Dawson, 1982; Rolston et al., 1982*). Unlike $N_2$, small changes in $N_2O$ concentration are easily detected in air.

Most of denitrifying bacteria require anaerobic conditions, but some species continue to denitrify at varying levels of dissolved oxygen (*Lloyd et al, 1987; Jetten et al, 1997*).

There is much controversy about this point, however, as certain species have been found to denitrify in a range of dissolved oxygen concentration that goes from 90% of air saturation for *Thiosphera pantotropha* (*Robertson and Kuenen, 1984*) to 53% for *Alcaligenes sp.* (*Krul and Veeningen, 1977*) to complete anaerobic conditions for *Paracoccus denitrificans* (*Alefounder et al., 1981*). Robertson et al. (1988) have found that *Thiosphera pantotropha* is able simultaneously to respire oxygen, to denitrify and to nitrify, the latter two metabolic pathways reaching the maximum activity at 25% air saturation. This behaviour of *Tsa. pantotropha* has been explained as the presence of a "bottleneck" in the flow of electrons of the respiratory chain at the level of cytochrome c, which limits the rate at which NADH can be reoxidized by this route. This causes a reduction in the cytochrome chain which allows electrons to flow to other pathways (**Fig. 1.8**), thus allowing a faster rate of NADH oxidation (*Robertson et al., 1988*).

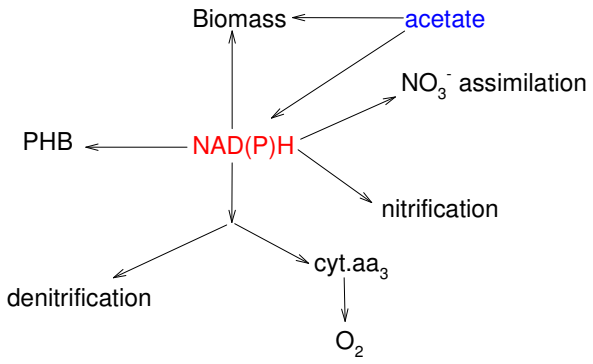


**Figure 1-8.** Simplified scheme showing the various possible options for NAD(P)H utilization available to *Tsa. pantotropha* (from *Robertson et al., 1988*).

Many other organisms have been found to have the same ability to denitrify aerobically, often associated with heterotrophic nitrification activity (*Robertson and Kuenen, 1990b*).

Denitrification activity has been reported in dried soils and in desert soils (*Virginia, 1982; Smith and Parsons, 1985; Peterjohn, 1991*), where it seems to depend on a complex interplay between soil moisture, carbon, nitrogen availability, pH, temperature and $O_2$. It is still a matter of controversy whether the combinations of environmental factors can create microsites convenient for denitrification activity in aerobic soils, or if aerobic denitrification does really occur (*Lloyd, 1993*).

Today, denitrification is known to be an important N cycle process wherever $O_2$ is limiting and C and $NO_3^-$ are available. In unsaturated soils, this frequently occurs within soil aggregates (*Sexton et al., 1985b*), in decomposing plant litter (*Parkin, 1987*), and in rhizospheres (*Nieder et al., 1989; Prade and Trolldenier, 1990*). Soil aggregates vary widely in size but in general are composed of small mineral particles and pieces of organic matter □2 mm diameter that are glued to each another by means of biologically derived polysaccharides. Like most particles in soil, aggregates are surrounded by a thin water film that impedes gas exchange. Modelling efforts in the 1970s and 1980s suggested that the centres of these aggregates ought to be anaerobic owing to a higher respiratory demand in the aggregate centre than could be satisfied by $O_2$ diffusion from the bulk soil atmosphere. This was confirmed experimentally in 1985 (*Sexstone et al., 1985*), providing a logical explanation for active

denitrification in soils that appeared otherwise to be aerobic, and an explanation for the almost universal presence of denitrifiers and denitrification enzymes in soils worldwide.

In addition to $O_2$, denitrification is also regulated by soil C and $NO_3^-$ . C is important because most denitrifiers are heterotrophs (*Poth and Focht, 1985*) and require reduced C as the electron donor, although as noted earlier, denitrifiers can also be chemo- and photolithotrophs. Nitrate serves as the electron acceptor and must be provided via nitrification, rainfall, or fertilizer. However, $O_2$ is the preferred electron acceptor because of its high energy yield, and thus must be depleted before denitrification occurs. In most soils the majority of denitrifiers are facultative anaerobes (*Knowles, 1981; Tiedje, 1988*) that will simply avoid synthesizing denitrification enzymes until $O_2$ drops below some critical threshold. In the field $O_2$ is by far the dominant control on denitrification rates. Denitrification can be easily stimulated in an otherwise aerobic soil by removing $O_2$ and can be inhibited in saturated soil by drying or otherwise aerating it.

The relative importance of C and $NO_3^-$ , the other major controls, will vary by ecosystem. Under saturated conditions, such as those found in wetlands and lowland rice paddies, $NO_3^-$ limits denitrification because the nitrifiers that provide $NO_3^-$ are inhibited at low $O_2$ concentrations. Consequently, denitrification occurs only in the slightly oxygenated rhizosphere and at the sediment–water interface, places where there is sufficient $O_2$ for nitrifiers to oxidize $NH_4^+$ to $NO_3^-$ , which can then diffuse to denitrifiers in the increasingly anaerobic zones away from the root surface or sediment– water interface. It is often difficult to find $NO_3^-$ in persistently saturated soils, not only because of low nitrification, but also because of the tight coupling between nitrifiers and denitrifiers (*Patrick, 1982; Reddy and Patrick, 1986; Mosier et al., 1990*). In wetlands with fluctuating water tables or with significant inputs of $NO_3^-$ from groundwater, $NO_3^-$ may be more available.

In unsaturated soils, on the other hand, the availability of soil C more often limits denitrification. In these soils C supports denitrification both directly by providing donor electrons to denitrifiers and indirectly by stimulating $O_2$ consumption by heterotrophs. It can be difficult to distinguish between these two effects experimentally; from a management perspective, there probably is no need to. It is well recognized that exogenous C stimulates denitrification (*Bremner and Shaw, 1958a,b; Knowles, 1981*), although the C added must be in an available form and must not lead to N immobilization sufficient to deplete $NO_3^-$ availability (*Firestone, 1982*).

*Tiedje (1988)* and *Groffman et al. (1988)* used the terms distal and proximal to describe the hierarchical nature of major controls on denitrification. In this scalar characterization (**Fig. 1.9**), different soil and ecosystem attributes affect C, $NO_3^-$ and $O_2$ availability differentially, with these attributes themselves affected by physical and biological phenomena that occur at larger spatial scales. Water, for example, affects denitrification principally via its influence on $O_2$ availability and on the diffusivity of $NO_3^-$ and C; water, in turn, is affected by (among other factors) soil porosity and transpiration rates, which are influenced, in turn, by (among other factors) the plant community and soil invertebrate activity. Eventually, of course, all controls evolve from climate and land use influences. The usefulness of this scheme is in its identification of how ecosystem disturbance, whether delivered by management or by nature, might affect denitrification fluxes.

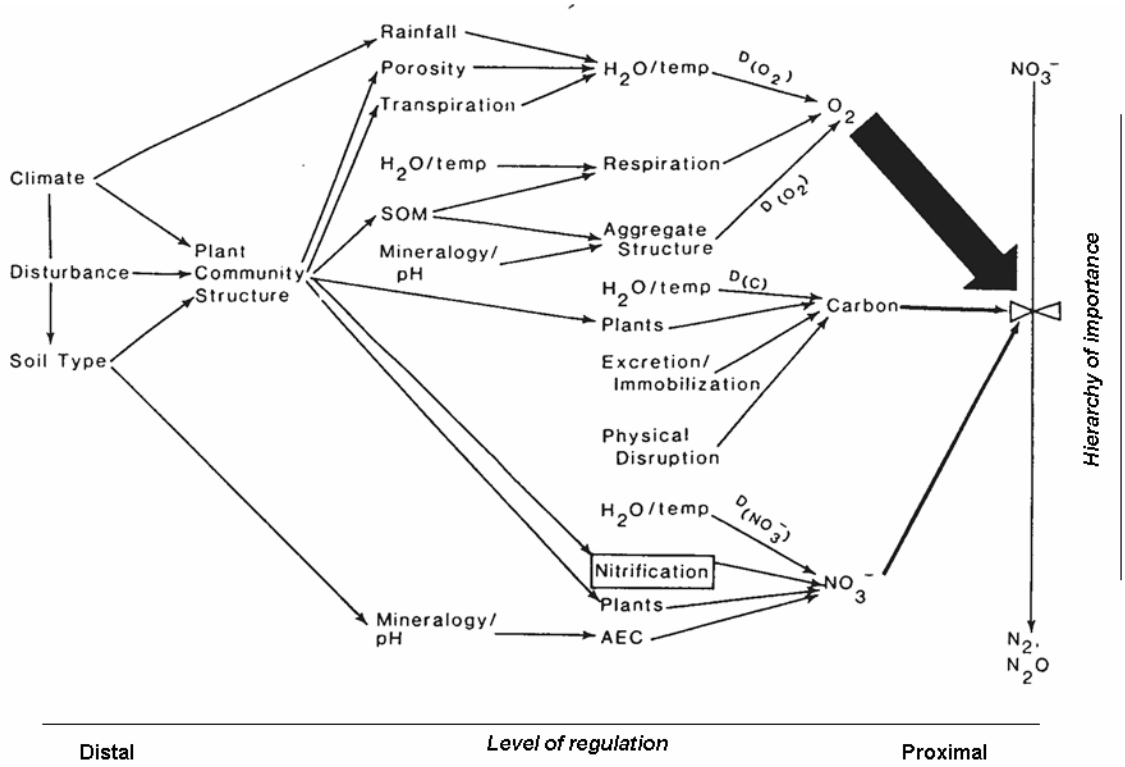


**Figure 1-9.** Major from the cellular (right) to landscape scales (from *Robertson, 1989*).

## 1.2 GREENHOUSE GASES NON-$CO_2$

The earth's surface with its atmosphere acts as a greenhouse. Most of the Solar radiation of wavelengths between 0.3 and 4 μm (short-wave radiation) is transmitted through the atmosphere to the surface of the Earth and absorbed, warming it. Only about one third of it is reflected. The energy absorbed is balanced (in the long term) by outgoing thermal radiation (4 to 100 μm wavelength) in the long-wave band (infrared radiation). Radiatively-absorbing particles and molecules, present mainly in the first 10 - 15 km of the atmosphere, are transparent to short-wave radiation but opaque to long-wave. They thus permit the sun's radiation to penetrate to the earth surface and by partially trapping and remitting the outgoing thermal radiation (at approximately 8-14 μm) are responsible of the increase of earth's surface and lower atmosphere temperature of about 33°C (around 15ºC rather than –18ºC, *Schlesinger, 1995*).

This phenomenon is known as the GREENHOUSE EFFECT. Though clouds and water vapour are the main contributors to this process, other gases present at low or trace concentrations contribute significantly to the greenhouse effect. These so-called trace gases include carbon dioxide ($CO_2$), methane ($CH_4$), tropospheric ozone ($O_3$), nitrous oxide ($N_2O$) and some chlorofluorocarbons (CFC-11, CFC-12) (*IPCC, 1990*). While clouds and water vapour dynamics follow a natural climatic pattern (atmospheric hydrogeological cycle), human activities have significantly increased the atmospheric concentration of these trace gases during the last century. The increase in these gas are documented by a 20 year or longer record of atmospheric measurements at a range of sampling stations around the world (*Rasmussen & Khalil, 1986; Prinn et al., 1990; Boden et al., 1992; Steele et al., 1992*) and by the long term record provided through the analysis of gases trapped in air bubbles in glacial ice (*Raynaud & Barnola, 1985; Raynaud et al., 1988; Oeschger & Arquit, 1989*). The soil ecosystem, in particular converted and agricultural soils, is considered as one of the important anthropogenic source for Greenhouse gases emissions to the atmosphere. Soil microorganisms produce and consume NO, $N_2O$ and $CH_4$. The net emission of these gases from soils result from the balance of oxidative and reductive microbial metabolism.

### 1.2.1 NITROUS OXIDE

Nitrous oxide ($N_2O$) is a very important trace greenhouse gas, which, although present at a minor concentration in the atmosphere than $CO_2$, has high absorbing capacity for infrared radiations and long residence time. According to *Bouwman (1990a)* $N_2O$'s thermal absorption potential is about 150 times higher than that of $CO_2$ and about 5 times higher than that of $CH_4$. It has a global warming potential (i.e. the direct warming effect in relation to $CO_2$ at a time horizon of 100 years) of 310 (*Houghton et al., 1995*). It has a residence time against stratospheric photochemical destruction of evaluated between 166 years and 120 years (*Prinn et ai., 1990, OTA, 1991, IPCC, 1994*).

In addition to contributing to the greenhouse effect, $N_2O$ is a natural regulator of the production of ozone. Nitrous oxide is photo-oxidized in the stratosphere to NO, which consumes ozone in a catalytic reaction (*Crutzen, 1970; Cicerone, 1987; Solomon, 1999*). It has been estimated that doubling the concentration of $N_2O$ in the atmosphere would result in a 10% decrease in the stratospheric ozone layer (*Crutzen and Ehhalt, 1977*).

Ice core measurements show that the pre-industrial value of $N_2O$ was relatively stable at about 285 ppbv for most of the past 2000 years, and started to increase around the year 1700 (*Pearman et al., 1986; Khalil and Rasmussen, 1988b; Etheridge et al., 1988; Zardini et al., 1989*). Its actual atmospheric concentration is about 313 ppbv (*Rahn and Wahlen, 1999*), contributing at present approximately 5% to the observed global warming (*Myhre et al., 1998*) and it is further increasing with at a rate of 0.75% (*Robertson, 1993*), or 0.25% (*IPCC, 1994; Houghton et al., 1996; Battle et al., 1996*) per year.

The anthropogenic sources of $N_2O$ include the burning of fossil fuels, industrial processes and agriculture, but it is agriculture, particularly the use of mineral fertilizers, that is considered the most important of these sources (*FAO* and *IFA, 2001*). Agricultural activities are presently estimated to contribute from 30% to 90% of the total $N_2O$ emission (*Davidson, 1991, Iserman, 1994, Mosier, 1994*), depending on the agroecosystem and agricultral practices considered.

Overall, terrestrial ecosystems represent the main source of $N_2O$, about 57% of the global atmospheric sources of $N_2O$ are related to emissions from soils (*Mosier and Kroeze, 1998*).

The exchange of $N_2O$ between soils and the atmosphere depends specifically on the simultaneous, opposing microbially mediated processes of nitrification and denitrification (*Firestone and Davidson, 1989; Bouwman, 1990b; Wrage et al., 2001*). The concept of $N_2O$ formation in, and emission from, soil can be illustrated by the " hole in the pipe" model of *Davidson (1991)* (Fig 1.).

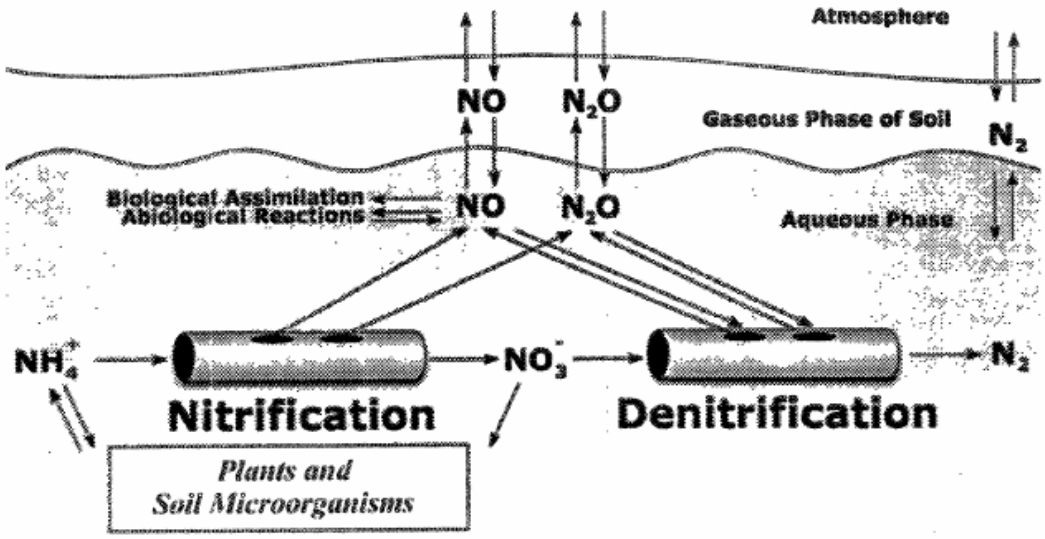


**Figure 1-10.** Diagram of the hole in the pipe conceptual model (*Firestone and Davidson, 1989*; revised *Davidson, 1991*).

The rate of flow of nitrogen through the pipes is analogous to rates of nitrification and denitrification and, more generally, to nitrogen cycling through the ecosystem. The NO and $N_2O$ trace gases "leak" out of holes in the pipe, and the sizes of the holes, through which they leak, is determined primarily by the soil water content. Soil acidity and relative abundance of electron donors (soil organic carbon) and acceptors (primarily oxygen, nitrate, and sulphate) may also affect the relative proportion of $N_2$, $N_2O$ and NO emissions from nitrification and denitrification (*Nõmmik, 1956; Firestone, 1982; Firestone and Davidson, 1989*), but soil water content appears to be the most common and the most robust controller of these ratios (*Davidson, 1993*).

$N_2O$ is a by-product of the first step of autotrophic nitrification (§ 1.1.8) and it is an obligate intermediate product of denitrification (§ 1.1.11) (*Granli & Bøckman, 1994*). Although some studies have concluded that nitrification can produce a significant portion of the total $N_2O$ measured (*Koops et al., 1997; Ambus, 1998*), other researchers have found that

most of the $N_2O$ attributed to nitrification is actually produced by nitrifier denitrification (*Castignetti and Hollocher, 1982; Poth and Focht, 1985; Bollmann & Conrad, 1998; Wrage et al., 2001*). When the supply of $O_2$ is limited by diffusional constraints the nitrifying bacteria can use nitrite produced during the oxidation of $NH_4^+$ as an electron acceptor and reduce it rather than being further oxidized to $NO_3^-$. This reduction may proceed to the formation of both NO, $N_2O$ as occurs during denitrification (**Fig. 1.11**). *Castignetti and Hollocher (1982)* recognized the production of denitrification enzymes by a heterotrophic nitrifier. However, unlike nitrification, this process occurs under low $O_2$ conditions (*Wrage et al., 2001*). *Koops et al. (1997)* found that in addition to low $O_2$, nitrifier denitrification also becomes important under low available $NO_3^-$. *Poth and Focht (1985)* rejected nitrification as a significant source of $N_2O$, and attributed $N_2O$ production to nitrifiers performing denitrification.

Nitrous oxide is also produced through the abiotic process of chemodenitrification (*Broadbent and Clark, 1965; Van Cleemput and Baert, 1984*) (**Fig. 1.11**).

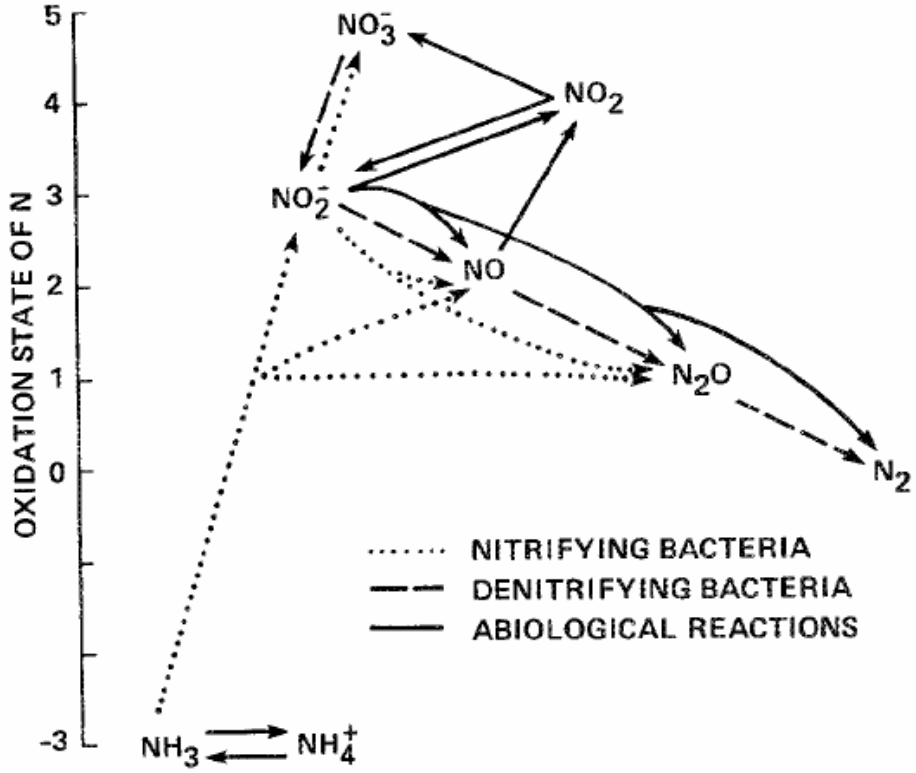


**Figure 1-11.** Biological and abiological processes of production and consumption of NO and $N_2O$ (*Davidson, 1991*).

Chemodenitrification is the generation of nitrogen gas products through reactions that are non-biologically mediated (*Knowles, 1981*). Chemodenitrification involves the chemical decomposition of nitrous acid ($HNO_2$) or reaction of $HNO_2$ with amino acids, ammonia, urea and other soil constituents such as metal ions. The process occurs primarily, but not necessarily, at low pH values (5 or less). NO, $N_2O$ and $N_2$ have been reported as products of chemodenitrification and among these NO is the most abundant (*van Cleemput et al., 1976*). Overall, chemodenitrification is not considered to be an important form of denitrification (*Tiedje, 1988; Robertson and Tiedje, 1987*), and $N_2O$ production. However, fertilizer or urine inputs in grazed areas may lower soil pH and cause significant chemodenitrification in the short term.

The importance of other soil processes in the production of $N_2O$, including any role of dissimilatory nitrate reduction to ammonium, heterotrophic nitrification by fungi and anaerobic oxidation of $NH_4^+$, remains poorly understood (*Wrage et al., 2001; Wolf and Brumme, 2002; Dalsgaard et al., 2003*).

### *1.2.2 FACTORS AFFECTING $N_2O$ EMISSIONS*

The most important controls on $N_2O$ production from soil are: availability of $O_2$, availability of N and C, and climate. Soil moisture and soil temperature are factors that act as direct controls on microbial activity, and indirectly on $O_2$ supply and C and N dynamics. Microbial activity is also influenced by pH.

Considering that $O_2$ is the preferred electron acceptor in the process of electron transport phosphorylation, the level of $O_2$ in the soil should determine how readily nitrogenous oxides are selected and reduced as electron acceptors in the process of denitrification. Studies have found that increases in soil $O_2$ result in a decline in total denitrification (*Firestone et al., 1979; Letey et al., 1980; Parkin and Tiedje, 1984; Burton and Beauchamp, 1985; Arah et al., 1991*). On the contrary, limited $O_2$ corresponds to increased $N_2O$ production and denitrification activity (*Broadbent and Stojanovic, 1951; Renault and Stengel, 1994; McKenney et al., 2001*) until scarcity of $O_2$ affects $N_2O/N_2$ ratio favouring $N_2$ versus $N_2O$, the latter being used as an electron acceptor in the extremely reduced environment (*Terry et al., 1981; Mosier et al., 1990*).

Where $O_2$ is not limiting, denitrification is suppressed and any $N_2O$ produced is related to nitrification (*Robertson and Tiedje, 1987; Parton et al., 1988*). Reaction products $N_2O/NO_3^-$ ratio increase when $O_2$ concentration decrease. Thus, in both processes, $N_2O$ formation is favoured at intermediate conditions of aeration (*Khdyer & Cho, 1983*).

The availability of $O_2$ at a particular point in the soil is determined by the rate at which $O_2$ can diffuse to that point and the rate at which $O_2$ is consumed by microbial activity (*Tiedje, 1988*) and consequently availability of oxidizable compounds (*Smith, 1990*) and diffusion of oxygen depends also on the soil texture and management. The $O_2$ status of a soil is difficult to measure in the field, so availability of $O_2$ has usually been assessed by surrogate measurements such as water filled pore space (WFPS) (*Linn and Doran, 1984; Maag and Vinther, 1999*), soil moisture content (*Skiba et al., 1993; Koops et al., 1997*), or air-filled porosity (*Letey et al., 1980*). Presence of water in pore spaces or as films on soil aggregates slows diffusion of $O_2$ (*Renault and Stengel, 1994*). Microbial activity will increase with soil water content until diffusion of $O_2$ is restricted and the environment becomes anaerobic (*Linn and Doran, 1984*).

Thus, soil water content is a control on denitrification and nitrification. *Skiba et al. (1993)* found that low soil water content (18% dry wt.) favoured production of $N_2O$ by nitrification and that an increase to a water content of 20.4% dry wt. favoured production of $N_2O$ by denitrification.

Data from seven studies by *Davidson and Verchot (2000)* show mean ratios of NO-N/$N_2O$-N well in excess of 10 at 40% WFPS or dried. The rate of nitrification, the rate of $N_2O$ production and the ratio of $N_2O$ to nitrate produced during nitrification all increase as the soil WFPS increases. *Davidson (1991)* observed that nitrification occurs up to a WFPS of 60% (**Fig.1.12**). At WFPS greater than 60% denitrification becomes dominant (*Lemke et al., 1998*). At high values of WFPS (>80%) $O_2$ diffusion may be restricted to the point where the product of denitrification is primarily $N_2$ (*Veldkamp et al., 1998*). Under these conditions, $N_2O$ is itself used as an electron acceptor and reduced to $N_2$. *Robertson and Tiedje (1987)* concluded that denitrification can act as a sink as well as a source of $N_2O$ because they also observed that when the level of $O_2$ was low, $N_2O$ was denitrified to $N_2$.

A WFPS value from 45% to 75%, although some studies indicate a higher value (*Klemedtsson et al., 1988; Hansen et al., 1993*), is generally favourable both for nitrification and denitrification and produce maximum emission of $N_2O$. This soil water content associated

with maximum $N_2O$ emission is normally close to field capacity (FC), which is defined as the soil water content after excess moisture has drained freely from the soil. At field capacity, soil micropores are water-filled, which permits microbial activity without stress, and soil macropores are air-filled, which permits relatively good aeration of the bulk of the soil, although anaerobic microsites may exist. Field capacity is the transition value of soil water content at which both oxidative and reductive processes are active in the soil.

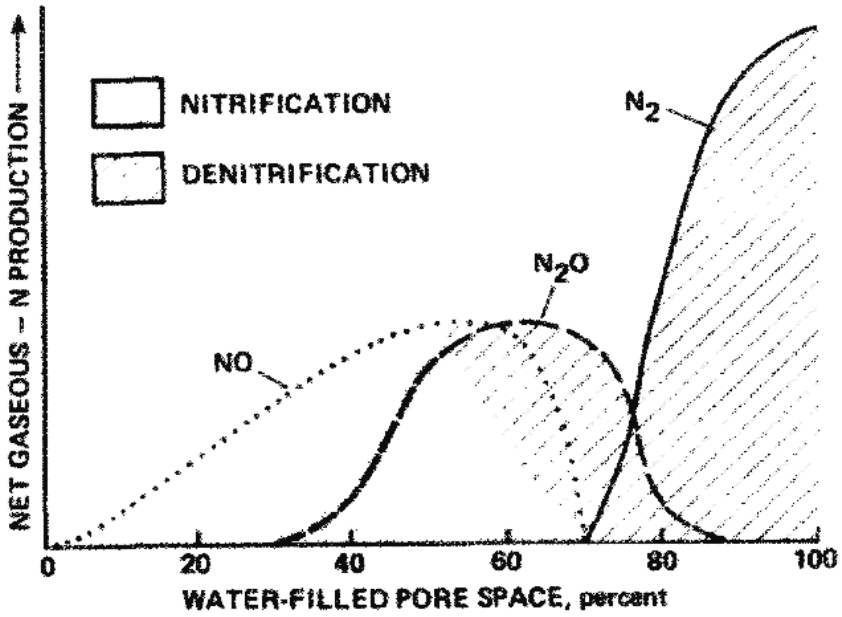


**Figure 1-12.** Relative contributions of nitrification (shaded) and denitrification (cross hatched) to emissions of NO and $N_2O$ as a function of soil water filled pore space (*Davidson, 1991*). While the figure indicates the general relationship between fluxes, the position of the maximum can vary with soil type and conditions.

A positive correlation between soil temperature and $N_2O$ evolution has been observed (*Kliewer and Gilliam, 1995; Maag and Vinther, 1999*). *Anderson and Boswell (1964)* found that nitrification was limited until the soil temperature reached 4 °C, the optimum temperature range for this process is usually between 25 and 35°C (*Bock et al., 1986; Haynes 1986*). However it seems that indigenous nitrifiers have temperature optima adapted to their climatic regions.

Soil temperature controls $N_2O$ production directly, through its control on activity of denitrifiers, and indirectly as its increase stimulates microbial activity, thus increasing $O_2$ consumption with a consequent formation of anaerobic micro-sites (*Maag and Vinther, 1999*).

Also, temperature by influencing the solubility of $O_2$ in water affect its diffusion to micro-sites of intense microbial acitivty (*Renault and Sierra, 1994*). The optimum temperature for denitrification seems to range from 30 to 67°C (*Nõmmik 1956; Bremner & Shaw, 1958; Keeney et al., 1979; Mancino et al., 1988; Malhi et al., 1990*). The reported differences reflect to some extent bacterial adaption to local conditions (*Powlson et al., 1988; Malhi et al., 1990*).

The relationship between soil temperature and $N_2O$ production in the field is not widely studied and detailed field measurements are rare. The influence of temperature is often assessed by monitoring seasonal changes in emissions and air temperature (*Corre et al., 1996; Groffman et al., 2000*). Indirectly, soil temperature can be related to an increase in $N_2O$ emissions during spring thaw from saturated soil layers (*Nyborg et al., 1997*). The saturated soil layer develops from the inability of the unfrozen soil water to infiltrate the frozen soil layer below (*Hayashi et al., 2003*).

Although C is an electron donor and nitrogenous compounds are electron acceptors in the process of denitrification, the relationship between available C and N, and $N_2O$ emission is complicated, as evidenced by the contradictory results which can be found in literature.

For example, *Bowman and Focht (1974)* found denitrification rates to be dependent upon $NO_3^-$ concentration. Denitrification rate generally increases with increasing $NO_3^-$ concentration (*Ryden, 1983; Robertson et al., 1987; Ambus and Lowrance, 1991*), but then reaches a plateau (*Mosier et al., 1983*; **Fig. 1.13**). The $N_2O/N_2$ ratio strongly increases with increasing $NO_3^-$ concentrations (**Fig. 1.13**) as high [$NO_3^-$] inhibits $N_2O$ reduction to $N_2$ (*Blackmer and Bremner, 1978; Firestone et al., 1980; Kroeze et al., 1989*). It is not clear if this is a true inhibition of $N_2O$ reduction or if it is due to the greater suitability of $NO_3^-$ as an electron acceptor as compared with $N_2O$ (*Cho and Sakdian, 1978*). In this latter case, the effect of nitrate is nullified when soils are strongly reduced, as after a flood (*Terry and Tate, 1980; Bowman, 1990*). *Firestone et al. (1980)* proposed that the inhibitory effect of $NO_3^-$ on $N_2O$ production could be actually due to $NO_2^-$, which is much more effective in such an inhibition than $NO_3^-$, and which could be produced by microbial processes when high doses of $NO_3^-$ are applied to the soil. Such an inhibitory effect of $NO_2^-$ on $N_2O$ reduction has been found also by *Van Cleemput et al. (1988)*. Low concentrations of $NO_3^-$ (5 mg N kg $soil^{-1}$) have been found to stimulate formation and activity of nitrous oxide reductase (*Blackmer and Bremner, 1979*).

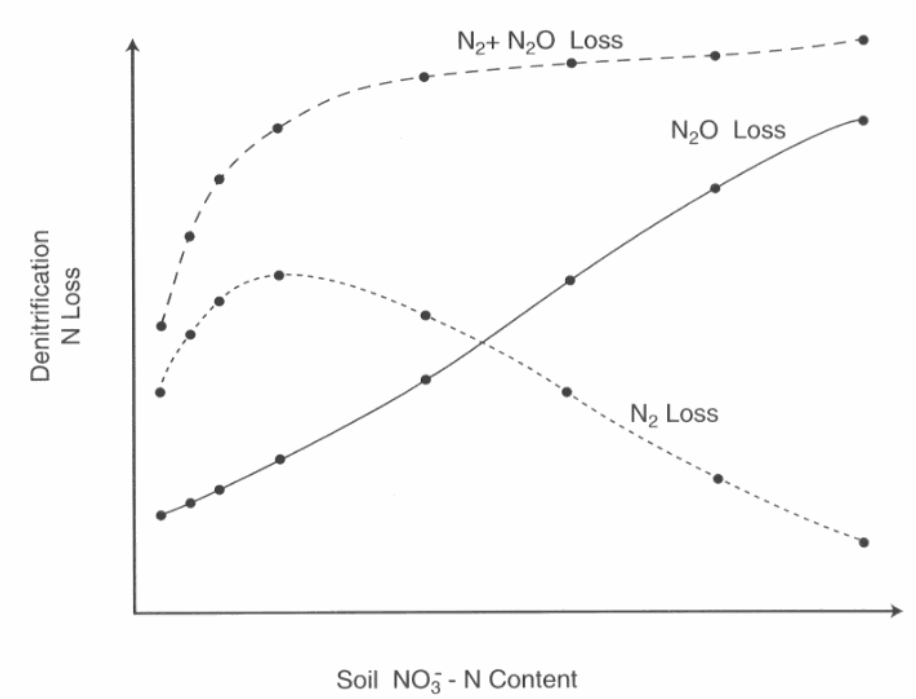


**Figure 1-13.** The idealized effect of soil $NO_3^-$ on $N_2$ and $N_2O$ losses associated with denitrification. (From *Mosier et al., 1983*).

However, *Linn and Doran (1984)* did not observe any relationship between $NO_3^-$ levels and $N_2O$ emissions, although they did find a relationship between soluble organic C and $N_2O$ emissions as did *Stanford et al. (1975). Tiedje (1988)* and *Myrold and Tiedje (1985)* suggest that N supply will have an effect on emissions in a soil that has a low concentration of $NO_3^-$. *Limmer and Steele (1982)* have found denitrification potential to be independent from $NO_3^-$ concentration for values greater than 25 mg $NO_3^-$ -N $kg^{-1}$ in a range of soils.

Thus $NO_3$ concentration exerts a control on $N_2O$ emissions where it is limiting and less or no control where $NO_3^-$ is not limiting. *Corre et al. (1996)* found that C and N availability became important to $N_2O$ emissions only when soil moisture conditions were favourable for denitrification (i.e., availability of $O_2$ was restricted), in fact, when other factors are limiting, denitrification can be rather insensitive to variation in $NO_3^-$ concentration (*Bremner, 1978; Aulakh et al., 1983, Kroeze et al., 1989*).

Availability of N is commonly assessed by measurement of soil mineral N levels (*Firestone et al., 1979; Drury et al., 1998; Del Grosso et al., 2000*). The importance of C has been assessed through a measure of bio-available C such as soluble organic carbon (*Burford and Bremner, 1975; Drury et al., 1998*), extractable (glucose equivalent) C (*Stanford et al.,*

*1975*) and total organic C (*Burford and Bremner, 1975; Stanford et al., 1975*). Measurements of available N and C typically access discrete nutrient pools and fail to account for N and C cycling. A better understanding of N and C cycling in agricultural and natural systems may improve our understanding of N and C available for $N_2O$ processes.

Soil pH has been occasionally cited as an influencing factor on $N_2O$ production or denitrification (*Firestone et al., 1980; Knowles, 1981; Stevens and Laughlin, 1998*). It is also linked to chemodenitrification, which occurs at a pH below 5 (*Tiedje, 1994*). *Firestone et al. (1980)* observed that pH did not influence $N_2O$ production until $NO_3^-$ levels were not limiting. *Stevens and Laughlin (1998)* noted that the influence of pH was small compared to WFPS, but it did have an effect on the mole fraction of $N_2O$. The mole fraction of $N_2O$ decreased with an increase in pH. *Van Cleemput and Patrick (1974)* found that the reduction of $NO_3^-$ increased with increasing pH.

### 1.2.3 METHANE

Methane is a radiatively-active gas, contributing approximately 12% to the enhanced greenhouse effect and contributing to about 15% of the potential global warming (*OTA, 1991; Abrol, 1995*). Its concentration in the atmosphere reaches 1.7 ppmv, with an increasing rate of 0.9% per year (*Blake and Rowland, 1988; Sombroek and Gommes, 1996*). The significant increase in atmospheric $CH_4$ concentration in the last two decade has been reported by many researchers (*Steele et al., 1992; Chapman et al., 1996 and Subadiyasa et al., 1997*).

Although methane residence time in the atmosphere of 12 to 15 years is an order of magnitude lower than that of carbon dioxide (120 years), its global warming potential is 32 times higher than that of $CO_2$ (which is the most significant greenhouse gas) for a 100 years time-scale (*OTA, 1991*).

The global annual methane emission is estimated at 500 Tg $y^{-1}$, with an uncertainty of 10 to 20% (*lAEA, 1992*). Wetlands, including rice paddies, contribute between 15 and 45% of global methane emissions (*Prather et al. 1995*). Projections for the year 2100 suggest that due to an increased population and waste generation, landfills will become a major source of atmospheric methane (*Kreileman and Bouwman, 1994*). The wide range of estimated values is mainly ascribed to the fluctuations of $CH_4$ emissions associated with agricultural practices

such as water management (*Sass et al., 1992; Murase et al., 1993 and Xu et al., 2003*), fertilizer application (*Lauren et al., 1994 and Martinez et al.,2003*), seasonal variation (*Sass et al.,1990; Kimura et al., 1991b and Snell et al., 2003*), diurnal variation (*Miura et al., 1992*), temperature (*Holzapfel-Pschorn and Seiler, 1986*).

Methane fluxes from or to soils result from the interaction of several biological and physical processes in the soil (*Cicerone & Oremland 1988; Conrad 1989; Bouwman 1990; Hogan 1993; Schimel et al. 1993; Wang et al. 1996*). Methane flux is the difference between $CH_4$ oxidation and methanogenesis, which may occur simultaneously even in arable terrestrial ecosystems (*Conrad, 1995*), and the three groups of organisms that may be involved are the methanotrophic bacteria, ammonia oxidizing and methanogenic bacteria (*Schimel and Gulledge, 1998*).

Methane production (methanogenesis) in soils is a microbiological process, which is predominantly controlled by the absence of oxygen and the amount of easily degradable substrate. It can occur when organic matter is degraded anaerobically (*Oremland 1988; Conrad 1989; Svensson & Sundh 1992*). Several bacteria that degrade organic material via a complex food web are needed to perform this process. The final step is performed by methanogens, methane producing bacteria which are a group of archeobacteria. Methanogenic bacteria are strictly anaerobic, they can use a limited number of substrates to produce $CH_4$ by two dominant pathways, cleaving acetate to $CO_2$ and $CH_4$, and reducing $CO_2$ with $H_2$ (*Whitman et al., 1992*). Acetate and hydrogen are formed by fermentation from hydrolysed organic matter (*Dolfing 1988*). Other substrates such as methylamines may also be used (*Yarrington & Wynn-Williams, 1985; Whitman et al., 1992*) but these are generally minor in terrestrial systems. Because methanogenesis is obligatory anaerobic it generally only occurs at high rates in systems which are continuously water saturated and are rich in organic matter; thus soil sources include natural wetlands and rice paddies. Upland soils are commonly recognized as important sinks for atmospheric $CH_4$ (*Schimel et al., 1993; Conrad, 1996*), most of the aerobic soils seem to function as $CH_4$ consumption sites (*Seiler and Conrad, 1987; Steudler et al., 1989*), $CH_4$ production can only occur in pulses after rain events that saturate the soil for an extended period.

Methane consumption ($CH_4$ oxidation) is also a microbiological process and is considered to be mainly performed by a single class of microorganisms: the methanotrophs (*Cicerone & Oremland 1988; King 1992*). Methane consumption is essential for

understanding methane emission. Methanotrophs can consume $CH_4$ produced in the soil or $CH_4$ entering the soil from the atmosphere (*Moiser et al., 1997; Schmidt et al., 2001*). Although the methods for determining in situ methane oxidation on the field scale are under debate (*Denier van derGon&Neue 1996; Frenzel&Bosse 1996; King 1996; Lombardi et al. 1997*), it is likely that a large and a varying part (1–90%) of the produced methane could be consumed again, either in the oxic top layer or in the oxic rhizosphere (*De Bont et al., 1978; Holzapfel Pschorn & Seiler 1986; Schütz et al. 1989; Sass et al. 1990; Fechner & Hemond 1992; Oremland & Culbertson 1992; Happell et al. 1993; Epp & Chanton 1993; Kelley et al. 1995; King 1996; Denier van der Gon&Neue 1996; Schipper&Reddy 1996; Lombardi et al. 1997*). Major controls are soil oxygen and soil methane concentrations. Methanotrophs are a diverse group of aerobic bacteria that oxidize $CH_4$ through methanol, formaldehyde, and formic acid to $CO_2$. The oxidation pathway is initiated by the methane monooxygenase (MMO) which requires both $O_2$ and reducing equivalents for activity. According to *Conrad [29]*, the biggest problem for the energy metabolism is the activation of the relatively inert $CH_4$ molecule. The activation is achieved in the initial step by the MMO which converts $CH_4$, $O_2$ and reducing equivalents to methanol and $H_2O$, i.e.:

$$CH_4 + O_2 + 2NAD(P) \xrightarrow{MMO} CH_3OH + H_2O + 2\,NAD(P)$$

Methanotrophs use three methods to consume $CH_4$, each beginning with the conversion of $CH_4$ to formaldehyde (*Mancinelli, 1995*). The dissimilatory method restricts bacteria to capturing energy from the oxidization of formaldehyde $CO_2$ and $H_2O$ and does not allow the retention of any C (*Paul and Clark, 1996*). The ribulose monophosphate method allows methanotrophs to assimilate the formaldehyde, converting it to different C molecules for use as biomass (*Mancinelli, 1995*). Methanotrophs using the serine method assimilate the formaldehyde and convert it to carboxylic acids and amino acids used in biomass production (*Mancinelli, 1995*). Methanotrophs require $CH_4$ for growth, being unable to use other substrates and can be distinguished in two kinds of methanotrophic activity: high affinity (low atmospheric methane concentrations) and low affinity (high methane concentrations). $CH_4$ is oxidized in soils primarily by two enzymes: a) $CH_4$ monooxygenase (MMO), found in methanotrophic bacteria, as written above; and b) $NH_3$ monooxygenase, found in nitrifying

bacteria (*Bédard & Knowles, 1989*). $NH_3$ monooxygenase is capable of oxidizing $CH_4$ because $CH_4$ and $NH_3$ are similar in shape and size (*Weast, 1976*). While nitrifiers can oxidize $CH_4$, they are unable to grow on it (*Bédard & Knowles, 1989*).

Microbial oxidation of methane plays a significant role in reducing the emission of methane to the atmosphere (*Oremland and Culbertson, 1992; Lelieveld et al., 1998*). The oxidation of $CH_4$ by methanotrophic bacteria accounts for approximately 10% of the global $CH_4$ sink (*Topp and Pattey, 1997*). Much interest has focused on the role of aerobic soils as sinks for methane and on the ecological and land use practices such as agriculture that affect its magnitude. Rates of methane uptake in soils have been determined for a wide range of natural environments including agricultural soils (*Hütsch et al., 1994; Hütsch, 1998*), forest soils (*King and Adamsen, 1992; Bender and Conrad, 1993; King and Schnell, 1998*), tundra soils (*Whalen and Reeburgh, 1990*), and peatlands (*Sundh et al., 1995; Dedysh and Panikov, 1997*).

### *1.2.4 FACTORS AFFECTING $CH_4$ FLUXES*

$CH_4$ production only occurs at substantial rates in fully saturated soils. As the water table drops below the soil surface, $CH_4$ efflux rates drop rapidly (*Harriss et al., 1982; Moore & Knowles, 1989*). This is due to combined effects of reduced $CH_4$ production as the anaerobic soil volume shrinks and increased $CH_4$ consumption in the aerobic surface soil. As methanogenesis is a strictly anaerobic process, it can only occur at low redox potentials. $CH_4$ production often begins at redox potentials below +100 mV, but usually become rapid until the redox drops below 0mV (*Yagi & Minami, 1990; Lindau et al., 1991*). Once anaerobiosis is established, organic substrate is considered as the major limiting factor for methane production; both the addition of direct methanogenic substrates, like hydrogen or acetate, and the addition of indirect substrates, like glucose and leaf leachate, enhanced methane production in anaerobically incubated soil samples (*Williams & Crawford 1984; Bachoon & Jones 1992; Valentine et al. 1994; Amaral & Knowles 1994*). Alternative electron acceptors like $NO_3^-$ , $Fe^{3+}$, $Mn^{4+}$, $SO_4^{2-}$, and possibly humic acids (*Lovley et al., 1996*) suppress methane production, because reduction of alternative electron acceptors supplies more energy than methanogenesis (*Zehnder & Stumm 1988*). The primary controls on the rate of

methanogenesis are $O_2$ and carbon availability (*Schimel et al., 1993*). Environmental factors also affect methane production and include soil texture (*Neue et al., 1994; Sass et al., 1994*), climate (*Schütz et al., 1990; Sass et al., 1991*), and agricultural practices such as water regime and management (*Sass et al., 1992; Lewis, 1996; Yagi et al., 1996*), these are distal controls on process to the progressively larger scale.

Methane oxidation is controlled by a number of environmental factors that partly explain the variability in observed methane oxidation rates. Factors such as soil water nutrient concentration and pH have previously been found to be important in the regulation of methane oxidation capacity (*Steudler et al., 1989; Mosier et al., 1991; Amaral et al., 1998; Gulledge and Schimel, 1998*). However, physical determinants such as soil water content and temperature may be of even greater importance in many areas and at certain times of the year. Changes in land use and climate may affect such physical determinants of methane oxidation capacity in ways which both increase and decrease the size of the soil methane sink (*Whalen and Reeburgh, 1990; Hutsch et al., 1994; King and Schnell, 1994*).

Several physico-chemical factors influence rates of methane oxidation in soil, including soil diffusivity; water potential; and levels of oxygen, methane, ammonium, nitrate, nitrite, and copper. Most of these factors exert their influence through interactions with methane monooxygenase (MMO), the enzyme that catalyzes the reaction converting methane to methanol, the first step in methane oxidation.

The ideal temperature range of MMO is between 20 and 40ºC, but diurnal changes in temperature do not change $CH_4$ consumption (*Topp and Pattey, 1997*).

Ammonia is known to inhibit methane oxidation in soils as a result of competitive interaction of $NH_4^+$ with methane for the active sites of the MMO enzymes (*Hanson and Hanson, 1996*). However, it has also been suggested that the inhibition of methane oxidation by $NH_4^+$ is not always the direct result of its concentration but rather its nitrification rate (*Sitaula et al., 1995*) or N turnover (*Hütsch et al., 1994*). The oxidation of $NH_4^+$ by MMO also produces toxic N compounds that inhibit the methanotrophic population (*Hütsch, 2001*) and therefore repeated $NH_4^+$ fertilisation decreases the methanotrophic microbial population and the soil consumption of $CH_4$ long after application of $NH_4^+$ ceases (*Mancinelli, 1995; Mosier et al., 1996*). Nitrate ($NO_3^-$) fertiliser has no effect on $CH_4$ consumption or can stimulate it (*Lessard et al., 1997; Hütsch, 2001*).

Soil structure is important to $CH_4$ flux because methanotrophs accumulate on the surface of coarse-textured soils and within soil aggregates (*Conrad, 1996; Mosier et al., 1997*). Tillage disturbs these structures and may reduce habitat necessary for methanotrophs (*Willison et al., 1995*). Undisturbed soils tend to consume $CH_4$ more often than they produce it (*Wang and Bettany, 1995*).

## 1.3 MEDITERRANEAN-TYPE ECOSYSTEM

Mediterranean-type ecosystem can be generally delineated according to the classification adopted, climatic (*Köppen, Wilson, 1967*), according to vegetation (*Emberger, 1930*), or a combination of both (bioclimatic map, *UNESCO, 1963*) and it surrounds the Mediterranean sea between the latitudes 40 and 30°N and it occurs in other four word regions: in the central Chile, in the Cape region of South Africa, in southwestern and southern Australia and, of course, in southern California and northern Baja California.

The Mediterranean Sea, a marginal and semi-enclosed sea, is located on the western side of a large continental area and is surrounded by Europe to the north, Africa to the south, and Asia to the east. Its area, excluding the Black Sea, is about 2.5 million $km^2$; its extent is about 3700 km in longitude, 1600 km in latitude and surrounded by African, Asian and European countries. The average depth is 1500 m. with a maximum depth of 5150 m in the Ionian Sea. The Mediterranean Sea is an almost completely closed basin, being connected to the Atlantic Ocean through the narrow Gibraltar strait (14.5 km wide, less than 300m deep at the sill). These morphologic characteristics are rather unique. In fact, most of the other marginal basins have much smaller extent and depth or they are connected through much wider openings to the open ocean. Moreover, high mountain ridges surround the Mediterranean Sea on almost every side. Furthermore, strong albedo differences exist in south-north directions (*Bolle, 2003*). These characteristics have important consequences on air masses and atmospheric circulation at the regional scale (e.g. *Xoplaki 2002*).

The Mediterranean climate is characterized by mild and rainy winters, and warm to hot dry summers, with high solar radiation and high rates of evaporation. *Leisz (1982)*, after *McCutchan (1977)* describes the Mediterranean-type ecosystem as: "it is one influenced by Mediterranean climate; that is, it exists in an area with (1) warm-to-hot summers and mild

winters; (2) a moderate marine air influence throughout the year; (3) moderate precipitation concentrated during winter months, with summers that are very dry; and (4) extended periods of sunny weather and few clouds, especially in summer".

Specific climate is one of four attributes that make the Mediterranean world indeed different and largely determine the nature of its soils, together to other three characteristics: its mountain, dust from the desert and the long term effects of man (*Yaalon, 1997*). From the point of climatic constraints on the soil, it is not the total annual amount of rainfall which counts (from 200 to over 1000 mm, in high mountains up to 2000mm) but its seasonal distribution. According to the *Köppen* definition, the Mediterranean climate is characterized by winter rainfall that is more than three times on summer rainfall. This strong seasonal winter/summer rainfall contrast results in root zone drying of the soil during the summer, often for several months, and represents the basis for defining the xeric soil moisture regime (*Soil Survey Staff, 1994*). The xeric moisture regime is defined by the length of summer dryness of the soil and characterized by winter rainfall in excess of evapotraspiration. It is not confined to the Mediterranean region, but it is present also in areas with lesser extension in America, Africa, Asia and Australia.

The natural vegetation of Mediterranean regions is a generally broad-leaved sclerophyllous forest, with dominant evergreen trees or drought deciduous shrublands. This type of vegetation is well adapted to the summer drought and the light to moderate frost in winter and it is resistant to the decomposition process. The herbaceous plants communities of Mediterranean region in the dry season act a strategy: in the summer, above ground portion of the perennial herbaceous plants dies and survive only the below ground portion, from which in winter or in spring aerial biomass develops; instead the annual herbaceous plants reproduce at the end of winter or in spring, in the summer they die leaving only the seeds (*Venturelli and Virli, 1995*).

Roughly 1% of the world's terrestrial vegetation is inside the Mediterranean type-ecosystems and about half of this occurs around the Mediterranean Sea. *Naveh & Lieberman (1984)* stated that in France this vegetation is called *maquis* or *guarrigue*, depending on the substratum, in Italy *macchia*, in Spain and Chile *mattoral*, in Greece *xerovoni*, in Israel *choresh*, in California *chaparral*, in South Africa *fynbos*, in Australia *malle*, *heath* and *shrub*. Due to the limited extent and isolation of each area of the Mediterranean ecosystem, there is frequently a high degree of endemism in the flora and fauna.

The elevated diversity of vegetal species in the basin of the Mediterranean sea is related to coexistence of areas with different successional stages, mainly the holm oak, the Mediterranean shrublands and herbaceous plants communities. Typical tree species in the regions around the Mediterranean, include *Quercus ilex* L. and *Quercus rotundifolia* L. (holm oak), which are widespread in the western Mediterranean; as well as *Quercus suber* L. (cork oak), *Quercus coccifera* L., *Olea oleaster* L. (wild olive tree), and *Ceratonia silicua* L. (carob tree). The characteristics shrub species for the region include *Pistacia lentiscus* L., *P. terebinthus* L., *Rhamnus sp*., *Cistus sp.*, and *Arbutus Unedo*. In the higher, rainier zones, the typical mediterranean vegetation gives way to deciduous tree species (*Quercus pubescens*). In contrast, steppe species take over the evergreen forest on the dry margins of the region. Coniferous trees (*Pinus halepensis*, *P. brutia*, *Cedrus libanotica*) are regionally abundant, and significantly dense in some altitudinal stages.

The sclerophyllous vegetation of California resembles much the Mediterranean vegetation (for istance, *Quercus agrifolia* is very similar to *Quercus ilex*), and some of its genera (*Quercus*, *Cupressus*, *Arbutus*) include a large number of species.

The Mediterranean regions of Chile, South Africa and Australia have rather different floristic composition. For example, *Eucalyptus* species are dominant in Southwestern Australia.

Besides flora, in the Mediterranean regions the fauna is also very rich and diverse, and has great importance from the biological diversity point of view.

Of the eleven orders of Soil Taxonomy, four (*Entisols*, *Inceptisols*, *Vertisols* and *Alfisols*) probably account for 90% of the soil-covered surface of Mediterranean regions. Another three (*Mollisols, Aridisols* and *Ultisols*) occur less frequent and can be locally significant.

## 1.4 AIM OF THE RESEARCH

The purpose of this research was to improve our knowledge on a) the nitrogen cycle in terrestrial Mediterranean ecosystems-type and b) their potentiality in the production and consumption of greenhouse gases (GHGs), in particular of nitrous oxide ($N_2O$) and methane ($CH_4$). The existing informations about these matters in natural areas of Mediterranean are very scarce in literature. Considered the economic importance of the agriculture in the basin of the Mediterranean, greater attention in the past has been turned to the improvement of the nitrogen levels in agricultural ecosystems finalized to optimize the crop and the production of livestock. Much less attention and research has been focused on N cycle and GHGs in natural Mediterranean ecosystems. Only recently, Mediterranean sites have been included in biogeochemically oriented projects (NITROEUROPE; CARBOEUROPE, etc) where these topics are dealth with, mainly in response to the growing interest on GHGs and climate change mitigation and impacts. These ecosystems can be expected to be extremely sensitive to the future predicted global changes. The Mediterranean area is a densely populated area where man modification of primary ecosystems can be dated back to the Roman ages, and where the antrophic impacts of the increased productive activities will increase in the next decades. On the other hand, climatic predictions for the area suggest a worstening of the climatic criticalities. Model simulations (HadCM2 model, Jhons *et al*., 1997) predict an average increase in temperature from 3 to 10°C (5°C for Italy) in the Mediterranean basin in the next 20-100 years, with a decrease in the total rainfall and some changes in its temporal distribution (HadCM2 model, Jhons *et al*., 1997). This would lead to a decrease of soil water content of about 10% as an annual average (HadCM2 model by Jhons et al. 1997), with more drastic reductions in the drier periods. Stronger and longer periods of aridity might induce a further increase in the occurrence of fires, also associated to the expanding urbanization. Land use change, fires, antropic disturbance, climate modifications can all impact the structure of Mediterranean plant communities, the ecosystem and associated cycles of elements. The magnitude of the effect of such disturbance of nitrogen transformation, losses and GHGs fluxes in Mediterranean ecosystem is unknown.

The present study aims to improve our understanding of N cycle and GHGs in Mediterranean ecosystems, both in terms of natural mechanisms associated to this type of environment and in terms of response of ecosystem to modifications and disturbance. This

might provide information for a correct management of Mediterranean ecosystems and might help us to improve our predictions and estimates of the budget of $N_2O$ and $CH_4$ fluxes in the area.

In the present job of doctorate thesis, four case studies were set up to answer to four questions that can help to improve the understanding of the Mediterranean ecosystems in relation to N cycle and GHGs:

-Which will be the impact of a variation of rainfall regime on N cycle and related GHG fluxes?

A rainfall manipulation experiment in Mediterranean woodland of Tolfa was carried out in order to evaluate, on the short-term, the impact of variation of rainfall regime of about 20% on the nitrogen cycle and on GHGs emissions (**chapter 3**);

-Do Mediterranean sclerophyllous plants produce allelophatic substances which control/affect N transformations and GHG fluxes?

In the same site of Tolfa, the role of allelopathic compounds produced by *Arbutus Unedo* as possible nitrification activity inhibitors was investigated by means of field observations and laboratory toxicity experiments (**chapter 4**);

-Given the high numbers of leguminous plants ($N_2$ fixers) which dominate the early stages of secondary successions following disturbance, which is the potentiality of $N_2$-fixation vs mineral N exploitation by herbaceous plants in Mediterranean grasslands and does the presence of $N_2$ fixing species increase N pools, fluxes and losses in Mediterranean grasslands?

A study was set to estimated the amount of N which enters into the ecosystem in Med. grasslands via $N_2$ fixation, considering that for these herbaceous species there is a complete lack of information in relation to this topic and N processes and pools were investigated in grassland areas covered by a different percentage of $N_2$ fixers (**chapter 5**);

-Which is the potential for NO and $N_2O$ emissions in Mediterranean natural ecosystems and which soil factors contribute to determine the $NO/N_2O$ ratio associated to a specific ecosystem/soil?

Four Mediterranean woodland ecosystems with different soil characteristics and 3 successional stages within the same soil type were compared for their potential to produced NO and $N_2O$ emissions in controlled and N excess conditions (**chapter 6**).

# 2 MATERIALS AND METHODS

## 2.1 SOIL PHYSICAL-CHEMICAL PROPERTIES

### 2.1.1 SOIL WATER CONTENT

Water content of soil is generally expressed as "gravimetric water content $\theta_g$", i.e., the mass of water per unit mass of oven-dry soil, or as "volumetric water content $\theta_v$)", i.e. the volume of water per unit volume of soil, typically $cm^3$ $H_2O$ $cm^{-3}$. For routine purposes the chosen method was the former. About 10 g of fresh soil were weighed in small cups and placed in an oven at 105 °C overnight. The next morning samples were cooled in a desiccator and reweighed. Typically results are expressed as g $g^{-1}$ or as a percentage.

### 2.1.2 SOIL BULK DENSITY AND WATER FILLED PORE SPACE (WFPS)

Soil bulk density represents the mass of oven-dry soil present in a given volume of naturally structured soil. For the measurement, a metal cylinder is placed against the soil and gently hammered into the soil. It is then excavated, the soil surfaces smoothed flush with the ends of the cylinder, using a knife, and the ends are closed with plastic lids. In the laboratory, the cylinder is then sealed in a polythene bag. The cylinder is placed in the oven at 105 °C till the weight stabilizes. The volume and the weight of the empty cylinder is required, as well as the fresh and the dry weight of the bulk soil sample.

The bulk density is then given by:

**Soil bulk density** = mass dry soil / bulk volume of soil

The water-filled pore space (WFPS), often expressed as a percentage, is the ratio of volumetric soil water content ($\theta_v$) to total porosity of the soil ($\varepsilon$), i.e. [100 x $\theta_v$]/ $\varepsilon$, where $\varepsilon$ = $cm^3$ pore space/$cm^3$ soil. The volumetric water content can be easily derived from the gravimetric water content as it is equal to:

$$\theta_v = \theta_g \text{ x bulk density/ density of water}$$

The total porosity of soil can be deduced from the following relationship:

$\varepsilon$ = 1 - [bulk density/particle density]

Some typical values of particle density and porosity of soil are reported in Table 2.1.

**Table 2-1.** Reproduced from *D. L. Rowell (1993).*

| | *Particle density* ($g\ cm^{-3}$) | *Bulk density* ($g\ cm^{-3}$) | *Porosity* ($cm^3\ cm^{-3}$) |
|---|---|---|---|
| Cultivated mineral soils, plough horizons | 2.60 | 0.8 - 1.4 | 0.69- 0.46 |
| medium-heavy textured, light texture | 2.60 | 1.1 - 1.7 | 0.46 - 0.35 |
| Subsoils and parent materials | 2.65 | 1.2 - 1.8 | 0.47 - 0.32 |
| Grassland and woodland, A horizons | 2.4 | 0.8 - 1.2 | 0.48 - 0.50 |
| Peats | 1.4 | 0.1 - 0.3 | 0.93 - 0.79 |

Although $\theta_v$ and $\theta_g$ parameters cannot be easily compared among soils with different textures, the WFPS can be used, as it takes into account the total porosity of the soil and the compaction, and is consequently directly related to gas diffusivity.

### 2.1.3 SOIL PH

Soil pH measurements by potentiometric method were determined on suspensions of the soil in water ( 1:2,5; soil:$H_2O$ ratio).

10 g of air-dried soil, sieved with a 2 mm mesh sieve, are shaken with 25 ml of deionised water for half an hour on a shaking machine. The solution is then stirred, and the pH is measured inserting a glass electrode in the solution; pH is recorded after about 30 seconds. The pH meter is calibrated before the measurement with buffer solution at pH 4.0 and 7.0. Calibration for routine purposes is generally carried out at ambient temperature.

### 2.1.4 AMMONIUM AND NITRATE CONCENTRATION

$NH4^+$ and $NO_3^-$ concentration in the soil was determined by extracting soil with a solution 0.5 M of $K_2SO_4$ (1:5 soil extract v/v) by potentiometric analysis using ion-selective electrodes.

Soil extracts was obtained adding 10 g of fresh soil and 50 millilitres of potassium sulphate, then after 1 hour of agitation on a shaker orbital, the extracts (on triplicate) have been filtered with Whatman 42 and the content of ammonium and nitrate in solution has been determined. The measures have been carried out using specific potentiometric electrodes for the reading of the ammonia (ORION, Model 95-12) and for the reading of the nitrate (ORION, Model 97-07), connected to a portable pH/ISE-meter (ORION, Model 290A).

The detection limit for the two electrodes, when a low level measurement procedure is used for the calibration curve, is 0.01 ppm of $N\text{-}NH_4^+$ and 0.1 ppm of $N\text{-}NO_3^-$. The calibration is performed with a series of standards, obtained using progressive dilutions (0.01, 0.1, 1, 10, 100 ppm for $N\text{-}NH_4^+$, and 0.1, 1, 10, 100 ppm for $N\text{-}NO_3^-$). When the concentration of mineral N in the samples is expected to be low, the concentration of standards used is below 10 ppm and the number of low concentration standards is increased (always 4 points).

$K_2SO_4$ is used to extract soil mineral N instead of 1M KCl, as chlorine highly interferes with the nitrate electrode. On the contrary the ion sulphate represents the least interfering

anion. As a general procedure a Nitrate Interference Suppression Solution (Orion cat. No. 930710) is added to the sample extract (1:1) for the removal of interferences caused by the different anions which might be present in the soil extract. Suppressor is added both to samples and standards. The optimal concentration of extracting solution has been determined on an experimental base.

The concentration of the samples is determined by comparison to the standards, at the same temperature and after addition of Ionic Strength Adjustor (Orion cat. No. 951211 and 930711) to all solutions to ensure that samples and standards have a similar ionic strength and proper pH.

### 2.1.5 SOIL EXTRACTABLE α-AMINO N

The amino acids, peptides and proteins available in soil in a free form are generally only a minor organic component of the soil, as in the free form they represent a source of N and C for the microorganisms and are immediately degraded. This component can be extracted by shaking the soil within an aqueous solution and can be measured with methods based on the ninhydrin reaction.

When an aqueous solution of an α-amino acid is treated with ninhydrin (triketohydrindene hydrate), a violet colour is produced. In the first stage of the reaction, the amino acid is oxidized to give an α-imino acid. This is further hydrolyzed to an α -keto acid and ammonia. The ammonia reacts further to give the violet pigment and the α -keto acid decarboxylates to give an aldehyde. The violet solution shows a significant absorption at 570 nm, and the intensity of absorption is proportional to the α -amino acid present. This specific reaction does not occur with proline as it is a secondary amino acid; the product obtained in this case is different and absorbs at another wavelength. Alternatively $CO_2$ or $NH_3$ formed in the course of the reactions can be measured.

Spectrophotometric ninhydrin method, developed by *Moore and Stein (1948, modified 1954)*, essentially includes slowly addition of 1ml of the ninhydrin color reagent to 2 ml of standards or samples and test tubes are shaken to mix. Test tubes were then heated for 25 min in a vigorously boiling water bath. After cooling at room temperature, 20 ml of ethanol (50%

v/v) was added to the tubes, mix and the absorbance was measured in 1 cm a path length cuvette at 570nm on a spectrophotometer. The concentration in solution was calculated from a calibration curve prepared with the same procedure, using L-leucine as a standard.

### 2.1.6 SOIL TOTAL CARBON AND NITROGEN

Soil total carbon and nitrogen content were determined by Elemental Analyzer (*USDA, 2004*). According Dumas method, soil organic and inorganic compounds were converted into gaseous products by extremely rapid and complete "flash combustion", successively they were separated by a gas chromatograph (GC) and measured with thermal conductivity detector (TCD).

Approximately 20 mg of dried, homogenized soil sample were weighed into a tin cup, properly closed and inserted into autosamples for measurements by flash combustion-gas chromatography on a NCS-soil Thermo FlashEA 1112. Into this instrument, samples were transferred to combustion column (Oxidation Column, 950°C), here by elevated temperature, they were oxidised. In order to complete oxidation process, gaseous products were transported by a He carrier through a specific catalyser (silver cobalt oxide and chromium oxide). Combustion products, then, are transported through a reduction furnace (reduction column, 840°C) to remove excess oxygen and to convert all nitrous oxides into $N_2$, and through a drying tube containing anhydrous magnesium perchlorate ($Mg(ClO_4)_2$) to remove water produced during combustion. The gas-phase products, mainly $CO_2$ and $N_2$, were separated by a gas chromatograph (GC) with thermal conductivity detector.

Soil total carbon and nitrogen content, as percentage of dry soil, were directly obtained by a calibration curve obtained using a series of standard samples with known concentrations (aspartic acid: C=36.09%; N:10.52%; H=5.20%; $O_2$=48.08%) using a software of management of Elemental Analyzer (EAGLE2000).

### 2.1.7 NITROGEN ISOTOPE RATIOS

Isotopic analysis were performed using an Elemental Analyzer coupled online via a ConFlo interface with an Isotope Ratio Mass Spectrometer (EA-IRMS; Delta C, Thermo Electron).

Stable isotope composition is reported in the conventional delta (δ) per mil notation (‰). This notation is used since the absolute abundance of the heavier isotopes is usually less then 1% of the total for a given element.

$$\delta\ (‰) = \frac{R_{sample} - R_{standard}}{R_{standard}} \times 1000$$

where $R_{sample}$ and $R_{standard}$ are the heavy to light isotope ratios of the sample and standard, respectively.

The isotopic standard for nitrogen is atmospheric air. $^{15}N$ represent the less abundant (0.366%) stable isotope of N, the more abundant being the $^{14}N$ (99.634%). For nitrogen $R_{standard} = [^{15}N]/[^{14}N] = 0.00367$. Atmospheric $N_2$ is considered for convention to have a $\delta^{15}N = 0‰$.

Stable isotope ratios of nitrogen ($^{15}N/^{14}N$) were mostly measured using mass spectrometry that is an instrument which separates charged atoms or molecules on the bases of their mass in the presence of a strong magnetic field. An IRMS is constituted of 4 main components: 1) inlet system; 2) ion source; 3) mass analyzer (with a magnetic and flight-tube assembly) and 4) ion detector system. The samples were prepared for analysis by weighing dry soil into tin cups. Following flash combustion, the $N_2$ gas released from the sample was separated chromatographically. Using helium as a carrier gas, the $N_2$ from each sample was transferred to the mass spectrometer via inlet system as a viscous gas flow to avoid fractionation during the path. It was ionized on an electrically heated filament in the ion source and accelerated with high voltage (ca. 3.5 kV). In the magnetic field (mass analyzer) the positively charge ions were deflected, and forced in a curve according to their masses of 28, 29 and 30. The charged ions were counted in an ion detector and added up to an electrical signal, proportional to the isotopic abundance, which was measured with the help of a defined standard, so that the electric current is proportional to the isotope ratio.

### 2.1.8 QUANTIFICATION OF SYMBIONTIC $N_2$ FIXATION BY ANNUAL LEGUMES

Any field estimate of $N_2$ fixation input requires an assessment of (a) the percentage of the legume N derived from the atmosphere (%Ndfa) and (b) the total amount of N in legume biomass during the interval of the study. These two components are then compounded to give an amount of N fixed and, by subtraction from the total N in the crop, the amount of legume N derived from the soil.

It is often stated that plant growth, or dry matter (DM) yield, is the driving factor behind $N_2$ fixation. While this may be true from the perspective that growth creates the demand for N, and photosynthesis the raw materials to sustain $N_2$ fixation (*Pate and Layzell, 1990*), it does not hold in low N environments where the growth of legumes is limited by poorly effective bacteria or inadequate nodulation. Thus plant DM yield will determine the amount of $N_2$ fixed only when the symbiotic machinery is operating effectively.

The %Ndfa for a legume is not a trait determined by a legume genotype and rhizobia alone, but rather is a product of the interaction between the soil N environment and total legume growth. It is well known for example that mineral N in the soil will generally depress both nodulation and $N_2$ fixation (*Streeter, 1988; Waterer and Vessey, 1993*) and thereby push the legume towards dependence on soil mineral N. The reverse will apply under low mineral N availability.

Different methods are available to determine the %Ndfa which include the N difference technique, the acetylene reduction assays, $^{15}N$ isotope dilution, relative ureide abundance technique, $^{15}N$ natural abundance (NA) technique (*Unkovich and Pate, 2000*).

#### *2.1.8.1. THE N DIFFERENCE TECHNIQUE*

In this approach the total N accumulated by a non legume 'reference' plant, or the legume in non-Nr fixing mode, is used as proxy for uptake of soil N by an adjacent

symbiotically active legume. The amount of N in the reference plant is subtracted from the total N of the legume under study and the difference ascribed to $N_2$ fixation.

### *2.1.8.2. $^{15}N$ NATURAL ABUNDANCE (NA) TECHNIQUE*

This technique relies on the slight natural enrichment of $^{15}N(\delta)$ that is observed in many soils, relative to atmospheric $N_2$ ($\delta = 0‰$) (*Shearer et al., 1978; Ledgard et al., 1984*).

Plant sampling is conducted near peak biomass to capture total seasonal $N_2$ fixation, but judiciously timed before the commencement of plant senescence which can influence both ($\delta$ and total N values, and give rise to less accurate estimates of $N_2$ fixation (see *Unkovich et al., 1994*). A selection of non-$N_2$-fixing weeds are sampled alongside the legumes to provide discrete $\delta^{15}N$ values of soil available N at each sampling point. These values were then used for paired estimates of $N_2$ fixation for the legumes (see *Unkovich et al., 1994*).

Despite considerable concerns about the heterogeneity of natural $^{15}N$ abundance in grazed ecosystems (*Kerley and Jarvis, 1996; Eriksen and Hogh-Jensen, 1998*), several studies have successfully used the NA technique in grazed pastures to estimate $N_2$ fixation (e.g. *Sanford et aI., 1994, 1995; Bolger et aI., 1995; Peoples et al., 1995c; Riffkin et al., 1999*). *Unkovich et al. (1998)* used the NA technique to study the influence of grazing intensity by sheep on $N_2$ fixation by subterranean clover (*Trifolium subterraneum*) in a mixed annual pasture. Although the NA technique is by no means applicable to all situations, guidelines applying to its judicious use have been well established (*Unkovich et al., 1994; Peoples et al., 1997*). One important potential problem with the methodology is the requirement for a 'B' value. This is defined as the $\delta^{15}N$ value for a legume when completely dependent on $N_2$ fixation for growth. Under fully symbiotic conditions legume shoot N is typically depleted in $^{15}N$ relative to atmospheric $N_2$ and thus estimations of $N_2$ fixation based on the $\delta^{15}N$ of legume above-ground biomass relative to a non-legume reference plant $^{15}N$ will overestimate %Ndfa unless the 'B' value of the legume is taken into account (*Shearer and Kohl, 1986*). Since the B value varies with species, plant age (*Unkovich et al., 1994*), micro symbiont (*Unkovich and Pate, 1998*), and growing conditions (*Ledgard, 1989*), a single B value is not adequate for all legumes and environments. For some species, like *Lupinus*, we have found

the B value to be conserved across species and growing conditions. By comparison values for chickpea are much more variable, introducing some uncertainty where %Ndfa is high. Both *Unkovich et al. (1994)* and *Peoples et al. (1997)* have indicated the likely magnitude of errors associated with the determination and application of B values for estimates of $N_2$ fixation using the NA technique. As a general rule where %Ndfa estimates are less than 85% the errors associated with an inaccurate B value are likely to be small (*Unkovich et al., 1994*). Despite clear evidence that shoots of symbiotically N-dependent legumes are depleted in $^{15}N$ relative to atmospheric $N_2$, the $\delta^{15}N$ values for $N_2$-dependent annual legumes are not significantly different from their N source on a whole-plant basis (*Unkovich et al., 1994*) thus it can be concluded that there is little evidence of significant N isotope fractionation associated with $N_2$ fixation per se for annual legumes. However, internal cycling of N within legumes most often results in shoots being depleted in $^{15}N$ relative to atmospheric $N_2$. Clearly further work is required to validate our observations, and to elucidate the mechanisms causing some fully symbiotic legumes to vary considerably in their $^{15}N$ distribution.

The %Ndfa using $\delta^{15}N$ (‰) was estimated according to *Ledgard(1989)*:

$$\%\text{Ndfa} = 100 \times \frac{\delta^{15}\text{N (reference plant)} - \delta^{15}\text{N (legume)}}{\delta^{15}\text{N (reference plant)} - \text{B}}$$

Where: $\delta^{15}N = \frac{R(spl) - R(std)}{R(std)} \times 1000$ $R = \frac{^{15}N}{^{14}N}$ or $R = \frac{^{15}N}{^{15}N + ^{14}N}$

$^{15}N$ represent the less abundant (0.366%) stable isotope of N, the more abundant being the $^{14}N$ (99.634%). The natural abundance of $^{15}N$ at natural level is expresses as (‰) excess (‰ $^{15}N$).

Atmospheric $N_2$ is considered for convention to have a $\delta^{15}N$ = 0‰. In ecosystems compartments $\delta^{15}N$ varies between –10 and +15 ‰.

For the present study the fractionation factor B was = 0‰. Thus no fractionation was assumed. The factor B is usually determined growing the leguminous plant in a hydrophonic medium without mineral N, where $N_2$ is the sole N source for the plant. Wild leguminous of Mediterranean grasslands, and in particular the specie investigated, are quite difficult to

germinate in laboratory, requiring scarification, temperature conditioning, and having anyway a quite low success of growth.

## 2.2 SOIL BIOLOGICAL PROCESSES

### 2.2.1 NET AEROBIC N MINERALIZATION ACTIVITY

N mineralization was determined by incubating fresh soil samples (3 replicates) aerobically (60% of soil water holding capacity) in the dark at 25°C and extracting inorganic nitrogen ($NH_4^+$ and $NO_3^-$) at t0 and after 14 and 28 days in order to calculate the mineralization rates using a time vs. concentration curve *(Kandeler 1995a).*

### 2.2.2 POTENTIAL NITRIFICATION ACTIVITY

Potential nitrification was measured as described by *Kandeler (1995b)* on 10 grams of fresh soil (on triplicate) amended with ammonium sulphate (100 µg N $g^{-1}$ dry soil) and incubated at 60% of water holding capacity at 25°C for 28 days.

### 2.2.3 DENITRIFICATION ENZYME ACTIVITY (DEA)

Denitrification enzyme activity (DEA) was used as a stable measure of denitrification activity was modified from *Smith and Tiedje (1979).* 5g of fresh soil were incubated anaerobically in helium atmosphere in 50 ml air tight flasks after addition of 2 ml of solution containing nitrate and glucose (C/N, 4/1; *Castaldi and Smith 1998a*). 10% (v/v) acetylene was

added to each vial to inhibit nitrous oxide reductase activity (*Yoshinary and Knowles, 1976*). Samples were then incubated at 30°C for three hours. This incubation time was chosen after preliminary tests in order to get a measure of denitrification enzyme activity already present in soil, without further enzyme synthesis. This allows to avoid the use of chloramphenicol, a protein synthesis inhibitor (*Dendoveen et al., 1994*), as many problems may occur when antibiotics are added to soil because they can act as substrates, can interfere with activity of on-target organisms, can alter in a different way the activity of different microorganisms, especially the ones involved in the nitrogen cycle (*Badalucco et al., 1994; Castaldi and Smith, 1998b)*. After 3 hours, 1 ml gas sample was withdrawn with an air tight syringe and injected into the gas chromatograph for $N_2O$ analysis.

## 2.3 GAS FLUX MEASUREMENTS

### 2.3.1 $N_2O$ AND $CH_4$ FLUXES

Gas fluxes from soil were measured, by closed static chambers (*Hutchinson and Mosier 1981; Smith et al. 1995*) made of high-density polyvinyl chloride (15 cm high x 15 cm in diameter). Chambers were placed on collars (5 cm high) one hour after these had been inserted at random into the soil. Each chamber was provided with a sampling port fitted with a three-way tap. Three gas samples were taken from each chamber (time zero and at about 30 and 60 minutes) and stored, in 20 ml air-tight evacuated vials. Concentrations of $N_2O$ and $CH_4$ were determined, within a week, using a gas chromatograph (Series 800 Fisons, Milan, Italy). A modified system from *Loftfield et al. (1997),* was set up to analyse both gases on 1 ml gas sample. Gas was loaded on a 1 ml loop connected to a 10-ports valve (Valco Europe, Switzerland). A precolumn of 1 m (O.D. 1/8", 0.08" I.D.), filled with Porapak 80-100 Q and maintained at 60°C, was connected to the 10-port valve in order to operate frontflush and backflush. From the pre-column, the gas passed into the main column (T Porapak 80-100 Q, O.D. 1/8", 0.08" I.D., 2 m length), also held at 60°C. Then it was directed, via a 4-ports valve

(Valco Europe, Switzerland) firstly to a flame ionization detector (FID) and, after 86 seconds, to an electron capture detector (ECD), held at 280°C. Pure nitrogen was used as carrier gas at a flow rate of 40 $cm^3$ $min^{-1}$. Calibrated standards (Air Liquide Italia; 0.8 ppm, 2.00 ppm, 3.10 ppm of $CH_4$; 0.350 ppm, 2.6 ppm, 5.2 ppm of $N_2O$) were used for $N_2O$ and $CH_4$ concentration determination, and were injected on duplicate every 20 samples.

The flux $f$ for the two gases was calculated as:

$$f = \frac{\frac{dC}{dt} \cdot V}{A}$$

where $V$ is the volume of the chamber, $A$ its basal area and $dC/dt$, the rate of concentration change. This was calculated from the linear regression of $N_2O$ production over time. The same calculation was applied for $CH_4$ uptake, as depletion over time followed a zero order kinetic model (*Striegl et al. 1992; Borken et al. 2000; Castro et al. 1994; Castaldi and Fierro 2005*). A negative sign is used to indicate $CH_4$ consumption.

### 2.3.2 NO FLUX

Nitric oxide (NO) fluxes were measured from the soil columns using a gas flow-through system (*Dick et al., 2001, Sanchez et al. 2008*). The inlet air of the column was previously filtered through charcoal and aluminium/$KMnO_4$, to remove $O_3$ and NOx. The flow rate over the headspace of the column was about 40 ml $min^{-1}$. NO was analysed by chemiluminescence (42C model, Thermo-Environmental Instrument) and $O_3$ by UV absorption (427 model, Thermo-Environmental Instrument). Both instruments require a flow rate of around 1L $min^{-1}$ each, so additional filtered air free of $O_3$ and NOx was supplied to the analysers. All data: flow rates, gas concentrations were recorded every 10 seconds with a datalogger (21X model, Campbell Scientific).The fluxes were estimated with the steady state of the NO concentration occurring approximately 10 min after the column closing and were interspersed with measurement from an empty column, in order to take into account reactions with the chamber walls and lids. They were calculated as the product of the flow rate of the column headspace

with the difference of the steady state concentrations between the soil columns and an empty column and with a dilution factor (ratio between the total and the column flow rate) divided by the weight of the dry soil (100g).

## 2.4 STATISTICAL ANALYSIS

For each parameter, for each sampling date, a mean value as arithmetic mean was calculated and bars in the graphs represent one standard error of the mean. To compare the effect of treatment on measured parameters, one way analysis or two way analysis of variance was applied to the data for each sampling event. When the difference was significant ($P<0.05$) an "all pairwise" comparison was carried out using the "Student-Newman-Keuls test". Multilinear and non-linear regression analysis was performed to evidence the relationship bteween depend and independent variables. Statistical analyses and graphics were done using SIGMA STAT 3.11 and SIGMA PLOT 9.0 (Jandel Scientific).

# 3 SOIL NITROGEN CYCLE AND FLUXES OF GREENHOUSE GASES ($N_2O$ AND $CH_4$) FROM AN ITALIAN MEDITERRANEAN WOODLAND UNDER CHANGING PRECIPITATION REGIME

## 3.1 INTRODUCTION

Variations in the distribution, frequency and total amount of precipitation is one of the predicted effect of the occurring climate change (*IPCC, 2007*). In the Mediterranean region the observed climatic trend indicates a constant increase of temperature (*Peñuelas et al., 2002, 2005; Peñuelas & Boada, 2003*) and a decrease of precipitation, in particular during the summer period (dry season) (*Esteban-Parra et al., 1998*). The reduced amount of precipitation together with the rise in potential evapotranspiration, led by increased temperature, has resulted in increased aridity (*Piñol et al., 1998; Peñuelas et al., 2005*). Changes in intensity and frequency of precipitation has resulted in increases of drought durations in many semi-arid regions (*Feddema, 1999; Lelieveld et al.; 2002; Moonen et al., 2002; Ragab and Prudhomme, 2002; Ventura et al., 2002*), in shifts in the "wet-dry cycles" of soils, or increases in extreme events. Future predictions for the Mediterranean basin indicate a further increase in warming and drought in the coming decades (*Sabate et al., 2002; Peñuelas et al., 2005*). These environmental variations might have significant impacts on biogeochemical cycles in natural ecosystems (*Ryan et al., 1998*).

Rainfall represents a key ecological factor for terrestrial ecosystems which directly controls net primary production (NPP) (*Churkina & Running, 1998; Knapp & Smith, 2001*), decomposition (*Swift et al., 1979; Villela and Proctor, 2002*) N mineralization (*Emmett et al., 2004*), soil respiration (*Orchard & Cook, 1983; Savage & Davidson, 2001; Davidson & Janssens, 2006*). Excess and scarcity of water affect negatively ecosystem processes. Where excessive rain leads to soil water saturation and flooding, anoxic conditions can develop inhibiting oxidative processes, thus resulting in partial and slow decomposition of soil organic

matter (*Freeman et al., 2001*). On the other hand, reduction of precipitation can lead to soil drought, which affect organic matter input to the soil, by reducing NPP, but also reduces soil organic matter decomposition and mineralization by limiting diffusion of enzymes and substrates, mobility and surviving of soil micro-organisms. However, the modified frequency of rain events also leads to repeated cycles of drying-rewetting which are known to cause the disruption of soil aggregates, exposing physically protected organic matter (*Adu and Oades, 1978; Lundquist et al., 1999*) and to induce peaks of mineralization (*Fisher et al., 1987; Jackson et al., 1988; Davidson et al., 1993; Appel, 1998*) and N and C gaseous emissions (*Ryan et al., 1998; Austin et al., 2004*). The exact mechanism of this phenomenon and the relevance of these events on annual budget of N and C losses in seasonally-dry ecosystems is still an open debate. However, strong trend of soil organic matter decomposition and C losses, recently evidenced in Europe (*Bellamy et al. 2005, Ciais et al. 2005*), have been explained by a combination of increased drought and temperatures (*Ciais et al. 2005; Schulze & Freibauer 2005*).

In a variety of ecosystems, rates of net N mineralization and the total quantity of soil N are indicators of soil fertility (*Nadelhoffer et al. 1983; Pastor et al. 1984; Vitousek and Matson 1985*). Given the high sensitivity of decomposition and mineralization processes to soil water content, N availability and its dynamics and stocks in ecosystems are expected to be significantly affect by climate changes. In particular where reduced water can lead to reduced N mineralization, significant impacts both on ecosystem performance and on long-term C sequestration in terrestrial ecosystems could be expected (*Hungate et al., 2003; Luo et al., 2004)*, in particular in Mediterranean regions where nutrients are often a limiting factor for NPP (*Hanley & Fenner, 2001; Sardans et al., 2004, 2005a*).

The objective of the present work is to evaluate the impact of changes in the precipitation regime on the N cycle in a Mediterranean ecosystem by using a manipulation experiment to artificially increase and reduce the water input to the system by a 20%.

Up to now most studies on the effect of climate change on ecosystem processes have focused on the effect of elevated atmospheric $CO_2$ (*Körner et al., 2005; Luo et al., 2006*) and increased soil temperature (*Giardina & Ryan, 2000; Davidson & Janssens, 2006; Parmesan & Yohe, 2003; Badeck et al., 2004; Luo, 2007*) and most of the manipulation studies on climate have been performed in cold and moist temperate and Arctic ecosystems (*Chapin et al., 1995; Harte & Shaw, 1995; Parsons et al., 1995; Henry & Molau, 1997; Luekewille &*

*Wright, 1997; Wright, 1998; Buckland et al., 2001; Valpine & Harte, 2001; Rasmussen et al., 2002*).

Less information exist on the effect of changes in the rain regime on C and N cycles for Mediterranean, arid and semi-arid environments (*Weltzin & McPherson, 2000; Shaw & Harte, 2001, Knapp et al., 2002; Austin A.T., 2004; Aranibar, 2004*). The present study aims to contribute to fill this gap of knowledge together with other published studies (*Papatheodorou E.M., 2004*), relatively to Mediterranean ecosystems. Short and long term effect could be observed in C and N cycle in response to climatic change. In this study we present the response of soil N cycle after two years of variation in the precipitation regime. This it represent the response on the short term. The study is still in progress so that we will be possible to compared these data with long term effects.

## 3.2 MATERIALS AND METHODS

### 3.2.1 DESCRIPTION OF THE SITE OF STUDY

The experimental site, located on the Tolfa hills in Central Italy (42°11' N 11°56' E, 180 m a.s.l..), is a coppiced woodland dominated by *Arbutus unedo* L. (65%-90% plant cover) of about 6ha. Other species are also present with a lower cover density, often as isolated trees: *Erica arborea* L. (13%), *Fraxinus ornus* L. (8%), *Quercus pubescen, Willd*. (5%), *Quercus cerris* L. (4%). Climate is typically Mediterranean, with a mean annual precipitation of 650 mm and a mean annual temperature of 15°C. The geology of the site is characterized by the presence of emerging eruptive deposits that, by the action of hydrothermal processes, gave origin to alum mineral beds.

The soil is classified, according to the USDA system, as an *Andisol*, a sandy loam soil, with a mean bulk density of 0.25 $gcm^{-3}$ in the top 10 cm, and 0.91 $gcm^{-3}$ from 10 to 30 cm, which is the maximum average depth of the A horizon. Soil pH is 4.3 in the organic layer and 4.0 in the mineral layer. Soil C and N concentrations are 18.9% and 1.2%, respectively, with a

C/N ratio of 15.7 in the organic layer, and 2.6% and 0.4%, respectively, in the mineral with a C/N ratio of 6.5.

### 3.2.2 EXPERIMENTAL DESIGN AND SAMPLING

The present experiment is part of a wider study on large scale plots of about 1 ha each, where towers for Eddy covariance measurements have been set. In order to avoid disturbance on these main plots smaller areas have been organized where physiological, chemical-physical and soil analyses could be made. These plots are about 10 x 10 metres wide. The manipulation regime includes the following treatments: DRY, WET and CONTROL. On the DRY plot, throughfall was intercepted by a number of belowcanopy metal drains that were placed right at about 1.5 meters height, connected to pipes to allow the displacement of the intercepted water outside the plot. In this way, approximately 20% of the ground area was covered by drains in order to reduce by about a 20% natural rainfall input. The irrigation treatment (WET) was obtained by an irrigation system (sprinkler irrigators uniformly distributed within the plot) able to simulate rain event and to add about 20% of water to precipitation regime. Irrigations were carried out during summer period, in order to maintain soil moisture above the drought stress threshold (*Alberti et al. 2007*) (**Fig. 3.1**). A third main experimental plot without treatment (CONTROL) was created to take in account an unaltered precipitation regime. Each treatment was replicated 3 times using three blocks located within an hectare (**Fig. 3.2**) and distributed along a transect South-Nord, at about 200 meters distance one from the other.

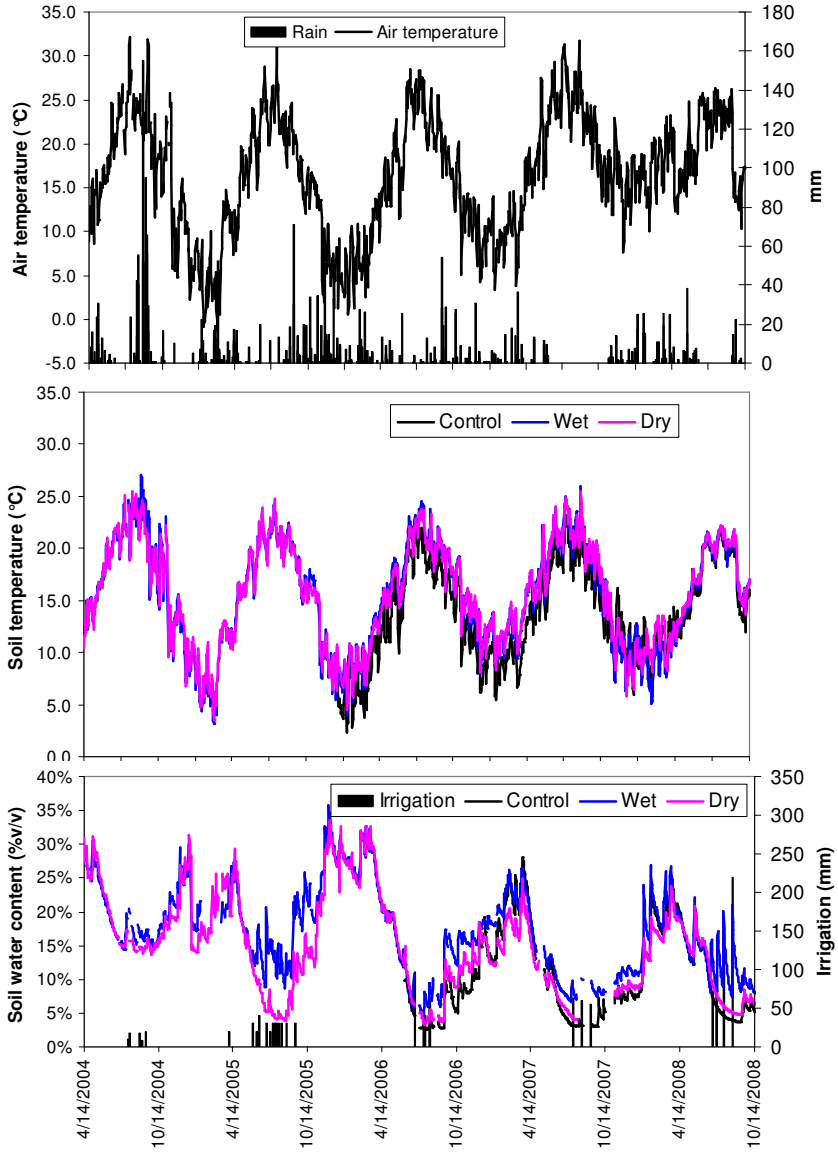


**Figure 3-1.** Meteorology data from Tolfa, plus soil temperature and volumetric water content.

Seasonal soil sampling were carried out at the site from spring 2005 to autumn 2006. In each occasion soil cores (0-10 cm depth, 5 cm diameter) were collected from three sampling points uniformly chosen within each subplot (100 $m^2$ each), for a total of nine replicates per treatment. Immediately after sampling, soil was sieved (2 mm mesh) and stored at 4°C until subsequent analysis in laboratory. Gas sampling were carried out by static closed chamber method in five seasonal sampling dates in each subplot area using again 3 replicates per each subplots for a total of nine replicates per treatment.

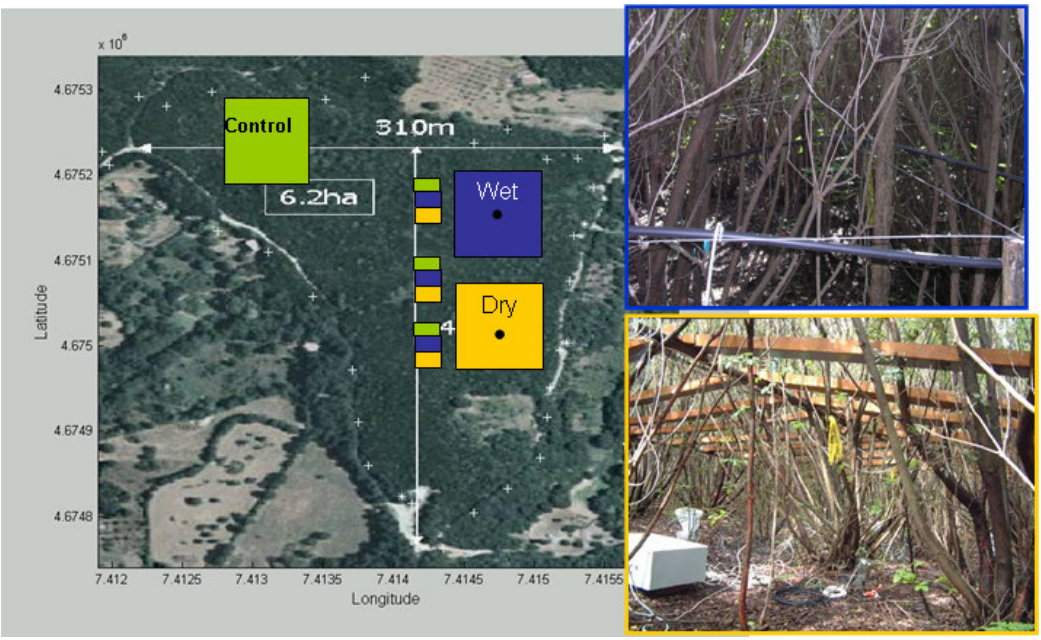


**Figure 3-2** Tolfa experimental design. The big squares represent the Eddy towers locations, the small rectangles represent the small replicated plots for destructive measurements utilized in the experiment. On the left details of irrigation system and drains.

### 3.2.3 SOIL AND GAS ANALYSIS

Soil physical-chemical properties and soil biological processes related nitrogen cycle were measured in each sampling event. Methods for analysis and measurements of soil water content, pH, organic carbon, total nitrogen and carbon, mineral nitrogen, organic α-$NH_2$, mineralization rate, nitrification rate, denitrification enzyme activity and gas fluxes are described in detail in the chapter 2.

### 3.2.4 STATISTICAL ANALYSIS

For each parameter, for each sampling date, a mean value as arithmetic mean was calculated and bars in the graphs represent one standard error of the mean. To compare the effect of manipulation on measured parameters, taking into account the block design (3

replicates on 3 blocks) a two way analysis of variance was applied to the data for each sampling event. When the difference was significant ($P<0.05$) an "all pairwise" comparison was carried out using the "Student-Newman-Keuls test". Multilinear and non-linear regression analysis was performed to evidence the relationship bteween depend and independent variables. Statistical analyses and graphics were done using SIGMA STAT 3.11 and Sigma plot (Jandel Scientific).

## 3.3 RESULTS

### 3.3.1 SOIL ANALYSIS

### *3.3.2 GRAVIMETRIC SOIL WATER CONTENT*

The pattern of soil water content exhibits clear seasonal fluctuations (**Fig. 3.3**) with lower values in summer, a maximum during winter and intermediate values during spring and autumn. The difference in treatment were more evident in the first year of study and in most cases resulted significant only when comparing DRY vs. WET. The control fluctuated among the two treatments often assuming intermediate values without having, however a statistical difference. In general, the gravimetric water content in DRY treatment was always lower than CONTROL and WET treatments, then latter being on average always above the other two treatments. Corresponding volumetric water contents can be derived by multiplying the gravimetric water content for the Bulk density at the same depth (0.47 g/cm$^3$). Overall we can say that the volumetric water content varied between 9 and 23 % in the DRY treatments and between 14.1 and 49.3 in the WET treatment. The strongest differences between WET and the others was observed in summer 2005 thanks to irrigation. This was less intense (**Fig. 3.1**) in summer 2006 with consequence less difference in soil water content. In winter, instead it was possible to observe the higher difference between DRY treatment and the others.

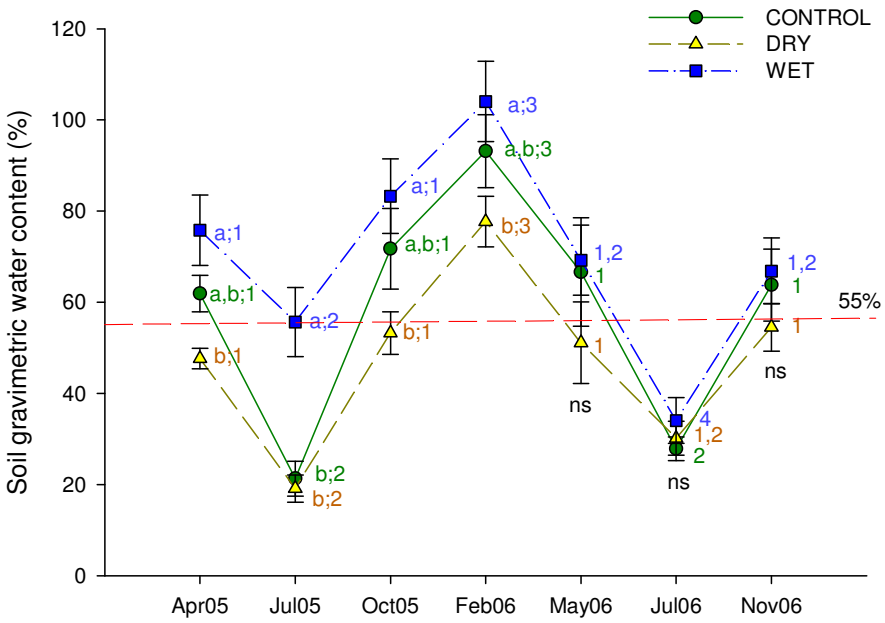


**Figure 3-3.** Pattern of gravimetric soil water content during the study period. Different letter indicate significant (P<0.05) differences among treatments, different numbers indicate significant differences among seasons.

### *3.3.3 NET NITROGEN MINERALIZATION RATE*

Net nitrogen mineralization rate was measured over 28 days of laboratory incubation, in each sampling event and the sole product of organic N mineralization was $NH_4^+$, which increased linearly during the time of incubation. No $NO_3^-$ production was observed. Net nitrogen mineralization rate (**Fig. 3.4**) was significantly influenced by the sampling date, showing a strong seasonal trend with higher rates in autumn and winter, intermediate values in spring 05 and lower rates in summer and spring 06. No statistical difference was instead observed among treatments. In most cases, only as a general trend the mineralization in the DRY treatment was the lowest, whereas CONTROL and WET treatment were basically very similar. The spatial variability among blocks was higher than the variability between treatments so that only strong variations in seasonal driving parameters could overtake this spatial variability.

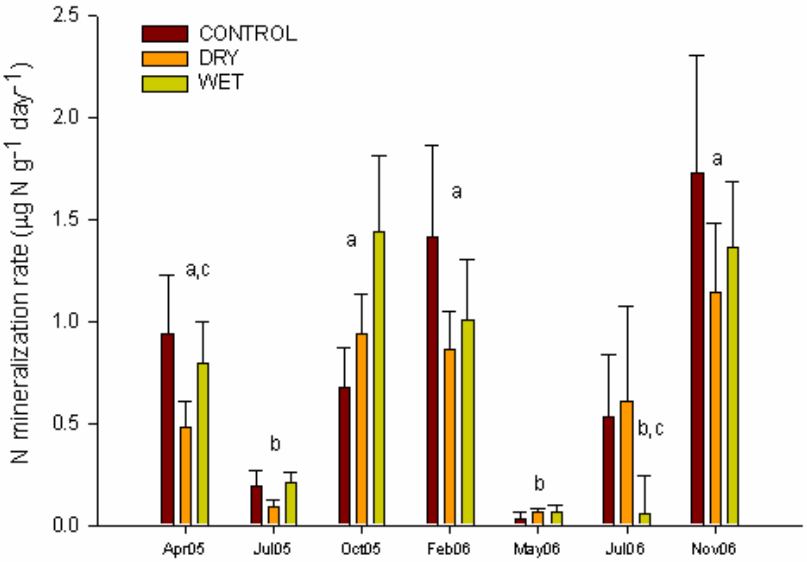


**Figure 3-4** Net nitrogen mineralization rate (average ± SD). Different letter indicate significant differences between sampling date (two way anova, P<0.05).

Soil gravimetric water content was indeed a factor which seem to influence the net mineralization trend at Tolfa, as in fact the latter increased exponentially with increasing water content (**Fig. 3.5**). The steepest rise started for values above the 25% of volumetric water content, which at Tolfa was reached only in short winter periods (**Fig. 3.1**).

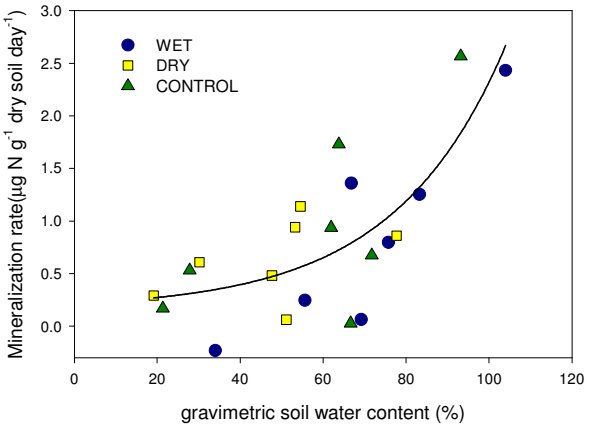


**Figure 3-5** Variation of net N mineralization rate in function of soil water content. (y = 0.16+0.05*(1.04)x R2=0.81).

This indicates that only above this threshold a difference of volumetric soil water content of about 10%, such as that in the treatments, might have a visible influence of mineralization activity. Mineralization was also correlated positively with alpha amino N content in the soil **(Fig. 3.6)**.

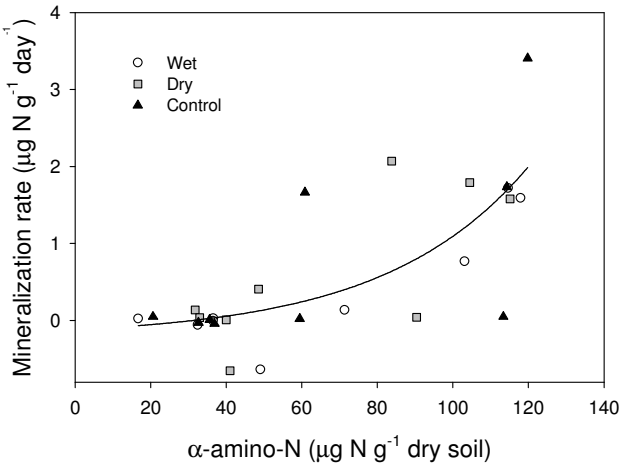


**Figure 3-6** Variation of net N mineralization rate in function of alfa amino N content in the soil.

Higher mineralization activity seemed associated to those situation presenting higher values of both soil water content and alpha amino N (**Fig. 3.7**).

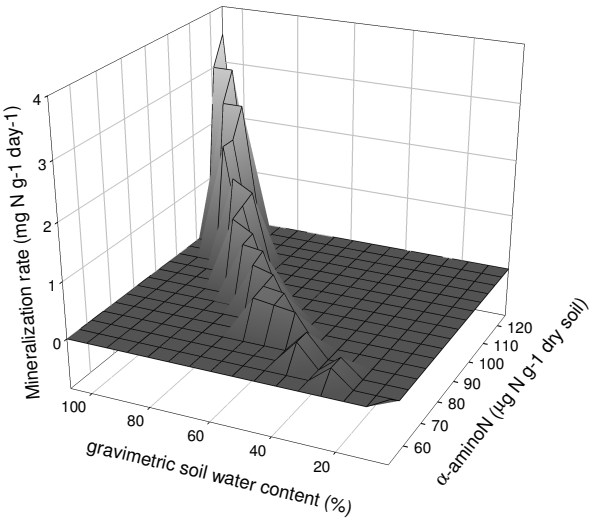


**Figure 3-7** Variation of net N mineralization rate in function of soil water and soil alpha amino N content.

### *3.3.4 POTENTIAL NITRIFICATION RATE*

In the test of potential nitrification activity no $NO_3^-$ was produced, despite the addition of 100 µg N $g^{-1}$ dry soil as $(NH4)_2SO4$, which was still found in the soil after 28 days of incubation at the same concentration measured at time zero (**Fig 3.8**) plus the amount mineralized from soil organic matter during the 28 days of incubation.

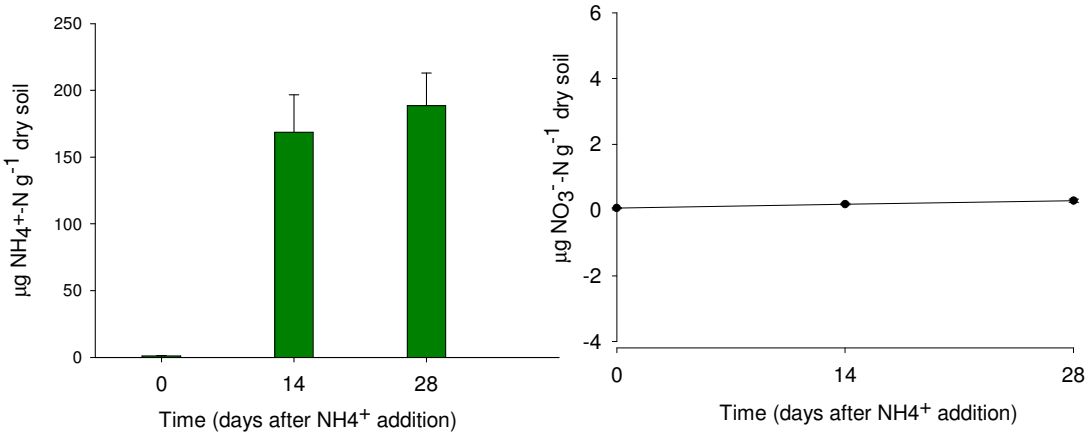


**Figure 3-8** Soil ammonium (a) and nitrate (b) concentration after addition of 100 µg NH4-N/g dry weight in potential nitrification test on CONTROL plot.

Thus, apparently no net nitrification was occurring in this soil. Possible reasons of absence of net $NO_3$ production were studied and were described in detail in the chapter 4. No field trend can be hence described for nitrification activity.

### *3.3.5 AMMONIUM AND NITRATE SOIL CONTENT*

The **Fig 3.9** shows soil ammonium content in the seven sampling events. Again a good and significant seasonality was evidenced, with highest values were recorded in winter. Concerning the treatments the only differences were evidenced in October 2005 and February 2006, when the ammonium content in the WET treatment was significantly higher than those in DRY and CONTROL treatments, while in other sampling dates there wasn't any significant differences in soil ammonium content among treatments.

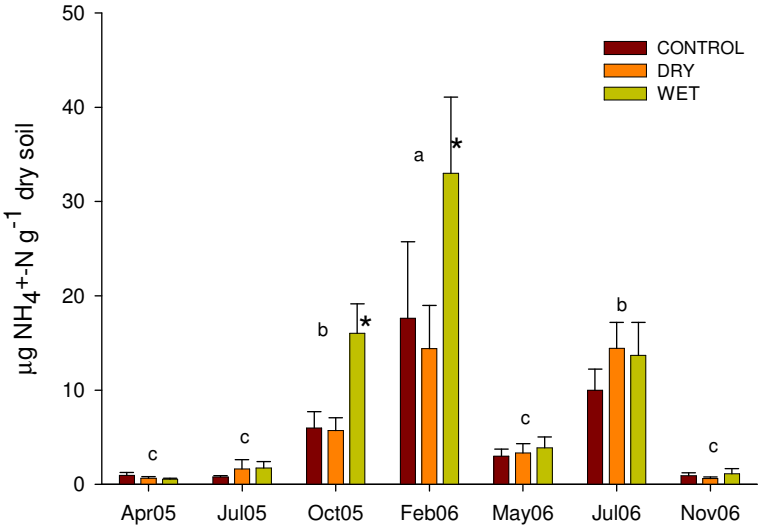


**Figure 3-9** Soil Ammonium content in seven sampling events. Asterisks show significant differences between treatments for each sampling date. Different letters indicate significant differences between sampling dates.

No soil $NO_3^-$ content was detected in Tolfa site over 1 and ½ years of sampling, so $NH_4^+$ was the dominant form of mineral N at all sampling occasions. $NO_3^-$ measured values in field samples are always below the limit of analytical detection for the electrodes (see chapter 2).

### *3.3.6 ORGANIC α-NH₂*

Organic α-$NH_2$ represents a soluble fraction of chemical compounds which have the amino group in alpha position, such as in aminoacids. Thus this fractions can include aminoacids, peptides, proteins, enzymes. This analysis did not start immediately so that only 3 sampling dates are available. From **Figure 3.10a** quite high content of this fraction is noted in all occasions, with higher values observed in autumn, respect to summer and spring. In each sampling event there wasn't significant differences among treatments.

The N inorganic/organic ratio (**Fig. 3.10b**) was strongly dependent on the season, with a clear dominance of organic forms in the spring/summer particularly evident in the WET treatment in summer. In autumn the ratio was around 1, then with an equal distribution of available N between the organic and the mineral form.

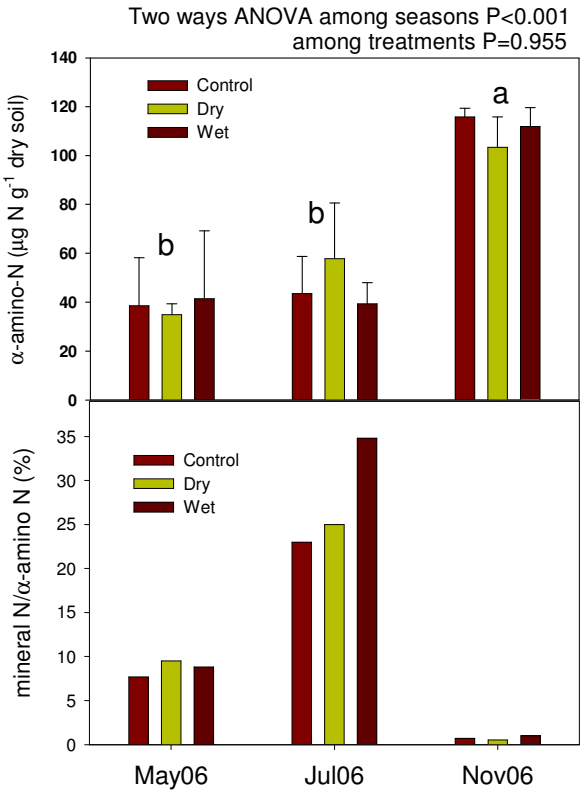


**Figure 3-10** (a) Soil organic α-$NH_2$ content (average ± SD) and ratio of (b) inorganic N/organic-N as α-amino N.

### *3.3.7 DENITRIFICATION ENZYME ACTIVITY*

Denitrification enzyme activity (**Fig 3.11**) measured at Tolfa was always very low, with maximal values never exceeding 15 ng $N_2O$-N $g^{-1}$ $h^{-1}$.Values were slightly lower in autumn-winter months and, although the difference was not significant, the DRY treatment showed always lower DEA than the WET and control treatments. Part of the lack in statistical difference among treatments can be attributed again to the spatial variability, as in fact it can be seen from **Fig 3.12** that the fluxes in the different blocks vary quite differently with water content. The highest fluxes are reached in block 1. In this case and in block 3 there is an exponential trend of increasing DEA with increasing water content.

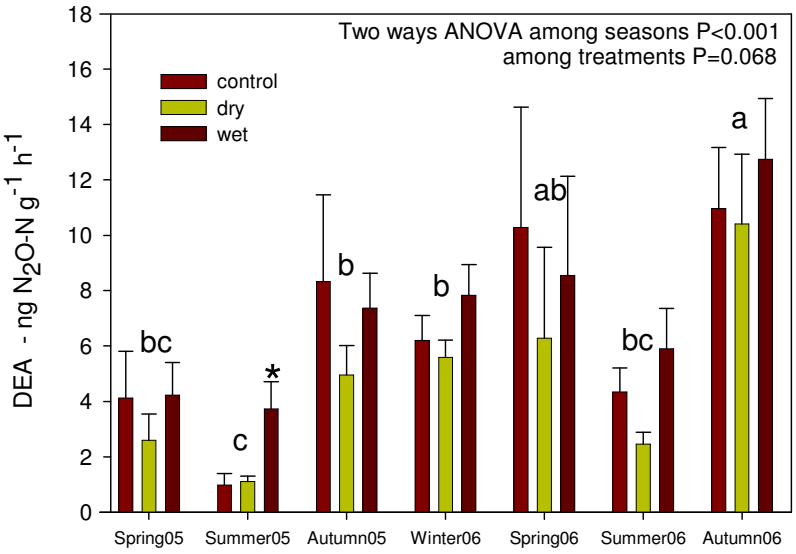


**Figure 3-11** Denitrification enzyme activity (average ± SD).

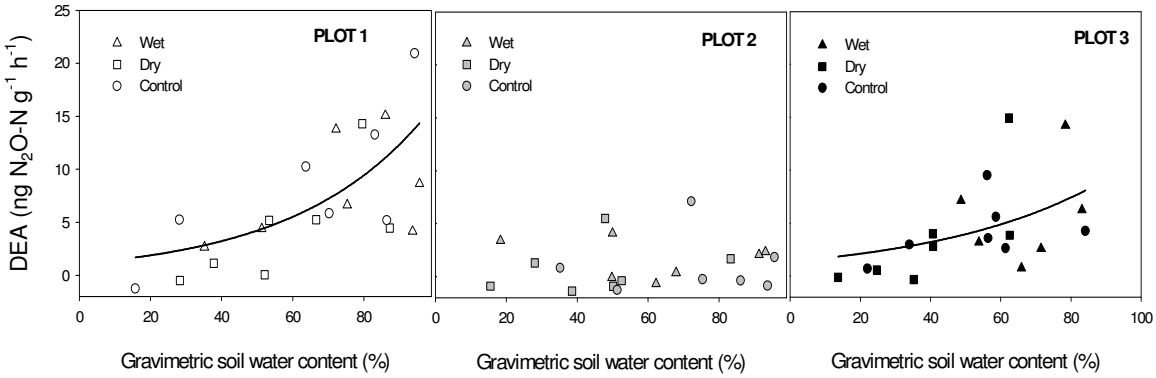


**Figure 3-12** DEA plotted versus soil water content, keeping separated the three blocks.

### 3.3.8 GAS ANALYSIS

### *3.3.9 METHANE FLUXES*

The sites acted overall as a methane sink (**Fig 3.13**). The only exception was observed on February 2006 when soil water content exceed the saturation value. In this case the $CH_4$ flux became positive. i.e. the soil became a source of $CH_4$.

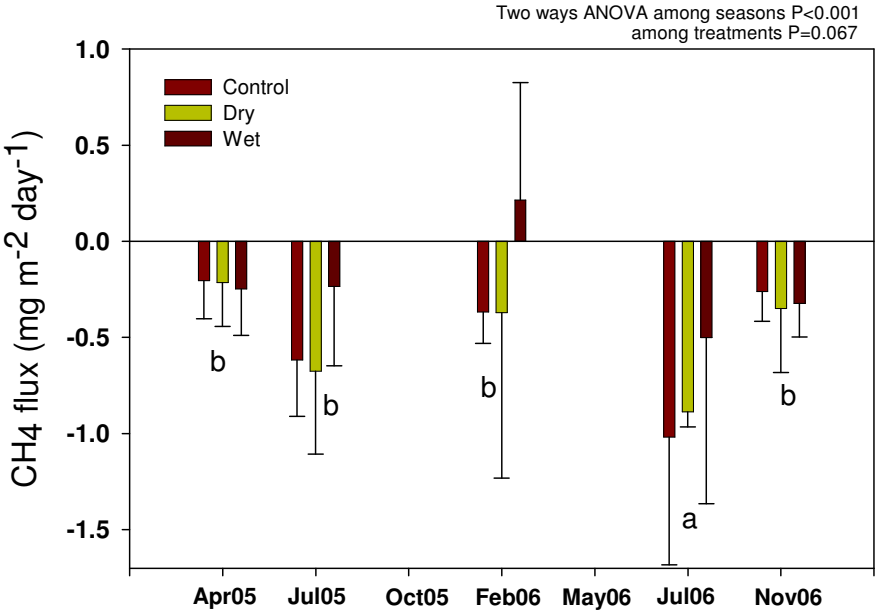


**Figure 3-13** Methane flux rate (mean ± SD, mg $CH_4$ $m^{-2}$ $day^{-1}$) of CONTROL, DRY and WET treatments averaged across three plots in 2005-2006 years.

The main driving factor for the observed temporal variability is the soil water content (**Fig. 3.14**). The relationship between $CH_4$ uptake and volumetric soil content is exponential ($R^2$=0.90), having higher consumption rates at lower soil content, and a soil uptake that get towards zero when soil water content rise up (**Fig. 3.14**). At saturation there is the shift from sink to source. No significant differences (P=0.067) in $CH_4$ consumption between treatments

(DRY, WET and CONTROL) were observed, at each sampling event, as the water content which controls the flux was not enough different, taken into account the spatial variability within plots and among plots (**Fig. 3.1** and **3.3**), as also evidence by the high standard deviation associated to the mean value (Fig. **3.13**). However, also in this case, a tendency of the WET treatment to present lower $CH_4$ uptake and even $CH_4$ production can be evidenced as a result of the rain manipulation.

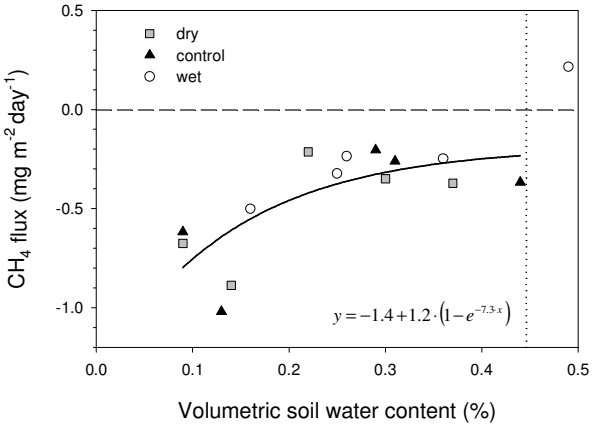


**Figure 3-14** $CH_4$ flux plotted versus volumetric soil water content for CONTROL, DRY and WET treatments.

Using the relationship reported in **Fig 3.14**, the annual $CH_4$ flux was modelled for the whole experimental period using the water content data available at the site from Tetra probes. These were set into the control plots only from the 9$^{th}$ of June 2006, so to compare all the three treatments only data from 2007 can be used. **Fig. 3.15** shows the modelled trend of $CH_4$ uptake from 2004 to 16$^{th}$ October 2008.

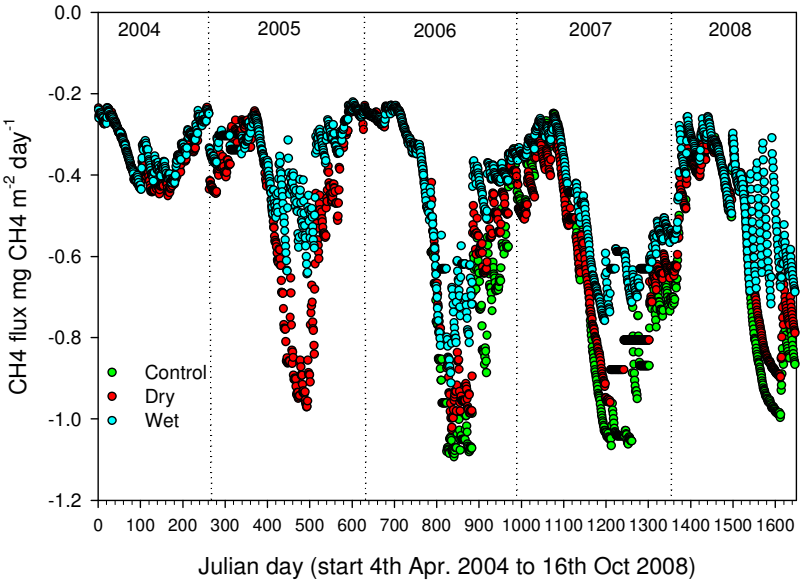


**Figure 3-15** Modelled $CH_4$ flux at Tolfa soil, obtained using the relationship derived in Fig. 3.14 and the water content data.

These modelled data were used to calculate annual values of $CH_4$ uptake. In **Table 3.1** these are expressed as mg $CH_4$ $m^{-2}$ $year^{-1}$ or g $CO_2$ equivalents $m^{-2}$ $year^{-1}$ for the WET and DRY in 2006 and also CONTROL for 2007. On a yearly base the difference among treatments was significant with the WET treatment showing lower uptake than the DRY in 2006 (-14.2%) and 2007. In the 2007 the lowest values were measured in the control, which showed also the lowest values of soil water content.

**Table 3-1** Annual values of $CH_4$ uptake expressed as mg $CH_4$ $m^{-2}$ $year^{-1}$ or g $CO_2$ equivalents $m^{-2}$ $year^{-1}$

| | **CH4 uptake** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **mg $CH_4$ $m^{-2}$ $year^{-1}$** | | | | **g $CO_2$ equivalente $m^{-2}$ $year^{-1}$** | | | | |
| | C | D | W | %diff D-W | C | D | W | | |
| **2006** | | 184.4 | 158.2 | 14.2 | | 3.8 | 3.3 | D > W P<0.0001 | Paired t-test |
| **2007** | 243.1 | 230.1 | 183.8 | 20.13 | 5.1 | 4.8 | 3.8 | C>d>w | one way anova |

### 3.3.10 NITROUS OXIDE EMISSIONS

In all sampling events, no nitrous oxide flux was measured. $N_2O$ fluxes at Tolfa were absent or below the detection limit.

To double check this lack of $N_2O$ production soil was sampled and within a day was incubated, fresh, in laboratory in glass jars of 1 litre, adding 2 ml of water per gram of soil with and without (untreated) nitrate ($KNO_3$, 100 μg N $g^{-1}$ dry soil). We also amended half of the samples with 10% (of headspace volume) of acetylene which allows to block nitrification and $N_2O$ reduction to $N_2$ during denitrification, so that we can be distinguished the source of $N_2O$. Gas was measured after 2, 24, 44 and 180 hours by gas chromatographic analysis. Results from **Table 3.2** show that in absence of $NO_3$ addition no $N_2O$ is produced. When $NO_3$ is added after 24 hours a significant activity of $N_2O$ production develops, and it increases in presence of acetylene. This means that the source of $N_2O$ is denitrification activity.

**Table 3-2** $N_2O$ production from Tolfa soil incubated with or without $KNO_3$, (100 μg N $g^{-1}$ dry soil) and with or without acetylene ($C_2H_2$).

| Incubation | $N_2O$ production (ng $N_2O$ $g^{-1}$ $h^{-1}$) | | | | |
|---|---|---|---|---|---|
| Time (h) | Untreated | Untr. + 10% $C_2H_2$ | Time (h) | $+NO_3$ | $+NO_3$ + 10% $C_2H_2$ |
| 2 | -0.26 ± 0.11 | -0.27 ± 0.21 | 2 | -0.20 ± 0.23 | -0.09 ± 0.08 |
| 24 | 0.09 ± 0.09 | 0.05 ± 0.04 | 24 | 79.22 ± 8.30 | 82.10 ± 10.41 |
| 44 | 0.10 ± 0.11 | -0.04 ± 0.03 | 172 | 95.71 ± 8.60 | 106.75 ± 35.54 |
| 180 | 0.01 ± 0.01 | 0.01 ± 0.01 | | | |

## 3.4 DISCUSSION

The Tolfa site has demonstrate to have a quite unusual N cycle for Mediterranean woodlands (*Rovira and Vallejo 1997, Gallardo and Merino 1998, Castaldi and Aragosa 2002)*, in particular concerning the total absence of net nitrification and $NO_3^-$ production, as well as its very high organic N content in the extractable fraction of N.

This site is characterized by typical net rates of N mineralization for Mediterranean woodlands, which lower values during the dry periods and higher values up to 3 μg N g-1

$day^{-1}$ when conditions of soil water are particularly favourable, mostly during the autumn winter months. The increase of activity for soil water content is quite slow between 10% and 30%, and above 30% it start to increase more rapidly. This condition is hardly met in Tolfa where the soil volumetric water content very rarely reaches or overtake the 30% (Fig. 3.1). From Fig. 3.3 it can be noticed that the difference of gravimetric soil water content between WET and DRY treatments never exceeds 30% and it is even less (15%) between control and one of the two treatments. A 30% increase of soil water content from 20% to 50% of soil water content induces and increase of mineralization rate from 0.25 to 0.50 μg N g-1 $day^{-1}$ (Fig. 3.4). Looking at Fig. 3.4 this variation of soil mineralization is within the standard deviation associated to the mean value of mineralization, in almost all cases. This means that the spatial variability associated to this parameter obscure the effect of water manipulation resulting in no significant difference among treatments. Thus to see a real change in mineralization rates the rain regime should change in such a manner to lead to a variation of gravimetric soil water content higher than 30% (about 15% of soil volumetric water content). This is true both considering the variability within each single plots or all the 3 plots together.

Although N mineralization gives as end product the $NH_4^+$, this latter did not follow the same temporal trend of mineralization. However, while mineralization represents an activity rate, soil mineral N is the net balance between production and uptake from plants and microbes, or losses. The highest values of $NH_4^+$ in soil correspond to winter months, when plant uptake might be to a minimum, and in this case it can be seen that WET treatments have more available soil $NH_4^+$ than DRY and control. Thus pools seems more sensitive than processes to manipulation. Data indicate that this $NH_4^+$ is not transformed into $NO_3^-$. The measure of potential nitrification carried out with the addition of ammonium sulphate at optimal conditions (aerobic incubation to 25°C) has shown an accumulation of ammonium in the 28 days of incubation and absence of production of $NO_3^-$. This result in accord with field data, which show absence of soil $NO_3^-$. This soil, hence is able to mineralize but not to nitrify. *Northup et al. (1995)* hypothesised that plant communities adapted to strong acidic and/or infertile soils can sustain productivity despite the low nitrogen availability by a mechanism associated to the high polyphenols concentration of decomposing litter which apparently controls and slows down soil mineralization and even more nitrification, so that N is "short circuited and can be directly used by the plant in form or organic N or $NH_4^+$. Not all plant are rich in polyphenols. Mediterranean plants are indeed reach in substances and in this is true

also in the case of *Arbutus*, as it will be shown in details in the following chapter. *Northup et al. (1995)* also demonstrated that in these ecosystems DON (dissolved organic N) is found to be the dominant form of available N although fractions reveal that rather than amino acids or proteins, this component might be represented by protein-tannin or humic substances complexes. Indeed, in Tolfa soil a very high concentration of N compounds, sensitive to the nynhidrine reaction ($NH_2$-groups exposed as in aminoacids and proteins), were found at concentrations from 2 to 10 times higher than those found in other maquis ecosystems (Castaldi and Carfora, pers. Comm). In fact, at Tolfa this form of available N was found to strongly prevail over mineral N ($NH_4^+$) in spring and summer, and being in the same order of magnitude in winter. If this fraction was indeed associated to a higher release of phenols in the soil which slows down decomposition of available fractions and complex organic N, then indeed we should expect the highest values in spring and summer when plants are most active. *De Luca et al. (2006)* demonstrated that in forests rich in tannins and polyphenols (*Pinus ponderosa* Lows) nitrification is completely inhibited but fire can unblock this inhibition by charcoal production. This in fact was hypothesised to act as a absorbent of these aromatic substances, thus reducing their concentration in soil and hence their negative effect on nitrification.

Clearly the distinction between litter N made avauilable as DON, $NH_4^+$ or $NO_3^-$ is crucial because the form in which N is mobilized determines which organisms can utilize it and also the potential for its loss leaching or gas losses. Plants very often may prefer $NO_3^-$ over $NH_4^+$ or amino acid N (*Persson et al., 2003*) as a result of the great mobility of $NO_3^-$ in the soil ecosystem compared with the latter forms of N, as the possibility of roots to access $NO_3^-$ by mass flow rather than root interception or diffusion is less energy costing. However the plants present at Tolfa are mostly *Arbutus unedo* and *Erica arborea*, both presenting two special kinds of mycorrhizae associations, arbutois and ericoid, respectively which play a fundamental role in their ability to extract N and P from organic sources, allowing these plants to colonize nutrient-poor sites where most of the nutrients are bound up in litter and dead organic matter. The fungi that form arbutoid mycorrhizae are both ascomycetes and basidiomycetes that form ectomycorrhizae on other species (such as conifers). Also the Ericoides fungal partners. *H. ericae* were demonstrated to have the ability to break down a wide variety of organic materials (including peptides, chitins and polyphenols) to mobilize nitrogen, phosphorus and other nutrients (*Read 1996; Smith and Read 1997; Read et al 2000*).

The ability of mycorrizae to uptake N in form of organic soluble N (aminoacids, peptides, proteins) does not seem affected by the increased availability of inorganic N, having these organisms a high affinity transport system for aminoacids. In addition starting from recalcitrant forms, N can be made available by plants and fungal exudates or ectoenzymes (*Wallenda et al. 2000*). In several cases mycorrhizae are also able to promote root uptake of N in form of $NH_4^+$, which being less mobile than $NO_3^-$ can be less easily uptaken by mass flow. The use of $^{15}N$ labelled ammonium has established that arbuscular mycorrhizae can derive N from ammonium salts (*Johansen et al., 1991*). This systems has been demonstrated to become more and more predominant with increasing drought, which reduces salt mobility (*Tobar et al., 1994*).

Thus the following mechanisms might be assumed to occur at Tolfa. N arrives to the soil with litter, which contains a high level of phenols (see chapter 4), which first of all slows down the decomposition of soluble compounds containing organic N (DON). A part of the organic N is mineralized to $NH_4^+$. This same substances do not allow for nitrification to occur. Thus no $NO_3^-$ is produced, or it is produced at very low rates. This does not represent a problem for the plants they are all in symbiosis with mycorrhizae which can easily exploit both $NH_4^+$ and organic N as sources of N. The lack of nitrification and $NO_3^-$ as substrate denitrification might hence explain the absence of $N_2O$ production recorded at the site. In fact, when $NO_3^-$ was added to the soil (Table 3.2) a significant $N_2O$ production occurred after 1 day from treatment, while the control soil (no $NO_3^-$ addition) did not show any $N_2O$ emissions. The acetylene test confirmed the heterotrophic nature of this $N_2O$ production. Denitrification enzyme activity was indeed found in Tolfa soil at each sampling event. Give the absence of $NO_3^-$and $N_2O$ emissions it is not clear if this DEA can fall in the classical definition of denitrification activity. Nitrous oxide has been reported to be produced by a number of $NO_2^-$ and $NO_3^-$ reducing bacteria common in soil (species of *Bacillus, Enterobacter, Klebsiella, Citrobacter, Escherchia, Erwina*), which do not fit the classical definition of denitrifiers (*Tiedje, 1981; Smith and Zimmerman, 1981; Anderson and Levine, 1986*). Also, many other organisms have been found to have the same ability to denitrify aerobically, often associated with heterotrophic nitrification activity (for a review see *Robertson and Kuenen, 1990b*) (see chapter 1 § 1.1.12).

In any case for all the measured activities the high spatial variability associated to the measure was not allowing for clear treatment effect, although in general processes proceeded lower in the DRY treatment compared with WET.

Concerning the other greenhouse gas non $CO_2$, $CH_4$, the site acts as a sink for this gas. The treatment effect is visible only when seasonal extreme are reached and even in this case the spatial variability index any significant difference among treatments. However, modelling the emissions over the whole year allow to have a denser dataset on which to build an annual budget. The modelled sink is always stronger in the DRY treatment compared to the WET in both considered years. In the second year however, where the control is available, the $CH_4$ sink is DRY<CONTROL, because the water content is lower in the CONTROL in summer. This anomaly might be attribute to the different quantity of biomass, higher in the CONTROL compared to the DRY which might induce a higher evapotranspiration during the hottest months.

## 3.5 CONCLUSIONS

The particular N cycle presented here makes this woodland less vulnerable to N losses, in the case of excess rain (WET) or to N limitation (DRY) because: 1) nitrous oxide production and $NO_3^-$ leaching are the two forms of losses which might be more interested by increased soil water content, but in our case both are absent; 2) increasing drought might reduce mineralization and N availability to plants; however the mycorrhizal system seems particularly efficient in exploiting N sources, especially in dry conditions when mass transport becomes less efficient. So a change in water regime in this ecosystems might have little influence on N limitation of NPP and N losses. Long term variations of $CH_4$ uptake potential, being correlated to the water balance in the system, might depend on the overall ecosystem functioning in the two treatments.

# 4 INFLUENCE OF ALLELOCHEMICALS PRODUCED BY SCHLEROPHYLLOUS MEDITERRANEAN ON NITROGEN CYCLE

## 4.1 INTRODUCTION

Nitrification represents one of the main biological processes involved in the nitrogen cycle, it contributes to regulate $NO_3^-$ availability to plants and microbes and net nitrification rates can reflect the potential for N losses, either through $NO_3^-$ leaching or by gaseous emission ($N_2O$ and $NO_x$). (*Likens et al. 1969; Vitousek and Melillo 1979; Krause 1982; Vitousek and Matson 1985*). Several environmental factors are known to control nitrification, such as substrate availability, soil water content, temperature, soil pH, aeration and texture (*Granli and Bøckman 1994, Castaldi and Aragosa 2002*). Forests on strongly acidic soils show little conversion of ammonium to nitrate, and no correlation between net mineralization and net nitrification (*Aber et al. 1985*). Although low soil pH *per se* was once believed to account for this inhibition of nitrification, strains of nitrifying bacteria that can sustain activity at very low pH have been isolated from acidic forest soils (*Hankinson & Schmidt 1988*). Soil nitrification rates have been found to be controlled more by the presence or absence of particular tree species than by soil pH (*Ellis & Pennington 1989*). In some cases, nitrification has also been reported to be controlled by allelophatic compounds (*Killham 1990*). These are secondary metabolites, waste products that no serve an obvious purpose in primary metabolism, and they are synthesized by plants and microorganisms, which can be found in different tissues including leaves, stems, flowers, fruits, seeds and roots, and which are released into the environment by means of volatilization, leaching, decomposition of residues, and root exudations, in large quantities. They can have an important ecological adaptative role, as chemical-defence products, on competition with other organisms and can profoundly

alter soil properties and nutrient cycling dynamics to advantage producer plant's phenology on soil environment.

The most common allelophatic compounds include phenolics, alkaloids and terpenoids (*Putnam 1988, Bertin et al. 2003*). Polyphenols (as purified tannins or polyphenol-rich litter extract) have been demonstrated to inhibit nitrification in incubation studies (*Basaraba 1964; Rice and Pancholy 1973; Lodhi, 1977; Lodhi & Killingbeck 1980; Thibault et al., 1982; Olson & Reines, 1983; Baldwin et al. 1983, Howard and Howard 1991, Erickson et al. 2000*) but some evidence is given that also monoterpenes might be involved in the observed suppression of nitrification (*White 1991, 1994; Langheim 1994; Paavolainen et al. 1998; Smolander et al., 2006*). Though hydrolysable and condensed tannins and tannin derivates are important inhibitors of nitrification, flavonols and other phenolic compounds also inhibit the oxidation of $NH_4^+$ to $NO_3^-$ through toxicity towards bacteria nitrifiers, *Nitrobacter sp.* and *Nitrosomonas sp*. (*Rice & Pancholy, 1974*).

These results have been much discussed by *Bremner & McCarty (1993)* who found no inhibitory effects using pure phenolic compounds on soil and reported that phenolics and terpenoids enhanced the immobilisation of $NH_4^+$ by soil organisms (*Schimel et., 1996; Castells et al., 2003*) rather than the inhibition of nitrifying bacteria. Then, phenolic monomers and phenolic acids can form complexes with nutrients, with formation of polyphenol-N complexes (*Northup et al., 1995; Hattenschwiler and Vitousek, 2000*) and thereby influence the nutrient availability and nutrients turnover in soil (*Kuiters 1991; Apple 1993*).

Direct consequence of minimizing the formation of nitrate can be to act as a N conservation mechanism in N-limited ecosystems (*Jordan et al. 1979*). This would provide a feedback to soil conditions that involves an adaptation to N limitation, enabling the plant to maximize N recovery, by minimizing potential N losses by leaching or denitrification, and by maintaining litter N in a form that the plant's associated mycorrhizal fungi can utilize, this result in the shift of the dominant pathway of nitrogen cycling from mineral to organic forms (*Northup et al., 1995b; 1998*). Previous study of *Llinares (1994)* reported the allelopatic inhibitory effect of *Elaeagnus angustifolia* leaf litter , after an year of incubation, on density and activity of nitrifying microorganisms present in degraded soils and the possible role in nitrogen economy in the soil supporting these plants and their possible use for restoring process of degraded soil.

Such allelopathic substances have been often found to be produced as secondary metabolites by Mediterranean sclerophyllous plants. For example, arbutin and tannins have been found as characteristic metabolites for the Ericaceae (Akhtardzhiev, 1966), while the main constituents of the extract of *Laurus nobilis* are terpenoid guaianolides and a p-menthane hydroperoxide, (*Nahoko et al. 2002*). Cyclic diterpene alcohol and quinonic acid have been purified from *Quercus ilex*, volatile terpenes and fatty acids from *Pistacia lentiscus* and pentacyclic terpenoid saponins from *Hedera elix* (*Rogosic et al. 2006*). As, in general, Mediterranean shrublands are a mosaic of different species, the input of chemicals and secondary metabolites to the soil via litter decomposition, exudates, leaching, deposition of VOCs, can be expected to be quite heterogeneous. This should avoid the high concentration of plant species-specific chemical compounds in the soil. However, a different situation could occur in monospecific Mediterranean woodlands or shrublands, where the continuous input of metabolites to the soil, assured by perennial/evergreen nature of the donor plant, might result in high concentrations of a specific plant-produced compound, with potentially toxic effects on soil microorganisms. Some of these situations in Mediterranean ecosystems could be represented by shrublands dominated by *Cistus sp*. after fire events, woodlands of *Quercus ilex* or *Quercus robor* in undisturbed conditions, or woodlands dominated by *Arbutus unedo L.*. This latter woodland type can be found in several areas of Central and Southern Italy, Greece, Turkey, Spain and even in Ireland. Moreover, *Arbutus unedo L.* can be found with significant cover density (20-40%) in many mixed evergreen woodlands of the Mediterranean basin.

In general, reported data on N cycle in Mediterranean shrublands ecosystems show well developed nitrification activity in soil, mainly limited by mineral N availability (*Rovira and Vallejo 1997, Gallardo and Merino 1998, Castaldi and Aragosa 2002*) and summer soil aridity (*Castaldi and Aragosa 2002*). However, investigating N gaseous emissions in a woodland characterized by a high cover density of *Arbutus unedo L*, in central Italy, we evidenced a complete absence of $N_2O$ emissions and $NO_3^-$ production (see chapter 3), both in the field and in the laboratory. In order to correctly simulate and quantify the N cycle processes, N gaseous emissions and greenhouse budget for this ecosystem type, it seemed necessary to further investigate the reasons for the observed results. Thus, in the present work we aimed at: a) verifying if the lack of $NO_3^-$ production was occurring in all seasons and if this was due to the inhibition of potential rates of mineralization and/or nitrification; b) to

investigate possible reasons for absence of $NO_3^-$ production, focusing the attention on potential toxic effects of chemicals produced by *Arbutus unedo* L. on soil nitrification.

To meet the first objective, concentrations of $NH_4^+$ and $NO_3^-$, and potential rates of mineralization and nitrification were measured on soil sampled form the site at each season over one year and half. To test the hypothesis of a role of plant produced chemicals on nitrification inhibition, the following steps where followed: a) to characterize the chemicals present in leaves and roots of *Arbutus unedo L.*; b) to verify their presence in the soil and quantify their concentration; c) to test leaf extracts toxicity on nitrification and produce a rough dose-response relationship to be compared with calculated concentrations of metabolites input and concentration in the soil of the studied site.

Although several studies dealing with allelophatic effects on the N cycle have been published, most of them refer to coniferous and North deciduous temperate forests and results are sometime controversial (*Baldwin et al. 1983, Schimel et al., 1996, Castells et al. 2003, Gundale and DeLuca 2006*). This makes difficult to transpose available results to other ecosystems just on the base of observations of specific chemicals produced by the plants. On the other hand, a correct evaluation of the role of allelophatic chemicals on soil N processes is particularly interesting in the Mediterranean environment, where these substances are frequently produced by plants, in order to correctly model and predict N losses from the Mediterranean ecosystems.

## 4.2 MATERIALS AND METHODS

### 4.2.1 SITE OF STUDY DESCRIPTION

The experimental site is described in detail in § 3.2.1. Soil sampling was carried out at the site in areas where *A. unedo* had a cover density of about 85%-90%.

### 4.2.2 LABORATORY TESTS

As the field sampling gave evidence of absence of nitrification activity and $NO_3^-$ production, a preliminary set of experiments was organized to investigate the possible reason for such results. Two possible causes could be advocated to explain field results. A low pH which might be unfavourable for the nitrifiers population or the presence of potentially toxic compounds in different fractions of the Tolfa environment, which might affect the nitrifying population.

In first, a short term assay was done on the Tolfa soil where soil was limed with a solution of $CaCO_3$ which raised soil pH from 4.3 to 6.3. Control and limed soil were then incubated (on triplicate) at 25°C and at optimal water content conditions (60% water holding capacity) for a month and the rate of potential nitrification (§ 2.2.2), after addition of 100 µgN/g soil as $(NH_4)_2SO_4$ was determined.

The second set of experiments was done to investigate potentially toxic effect of water extracts of different components of Tolfa ecosystem (soil, litter and fresh leaves extracts) on test soil population of nitrifying microorganisms. For this purpose a test soil from a Mediterranean oak woodland was chosen where the nitrification activity had been previously measured at significant rates during the whole year (*Castaldi and Aragosa, 2002*). This test soil was sampled in a coastal area of a protected natural reserve under *Quercus ilex* canopy. Soil is classified as an *Calcaric Arenosol* (FAO, 1998) with neutral pH 7.0 and 2% of soil C and 0.5% of N, in the top 10 cm.

In a first screening experiment the test soil was incubated with different extracts taken from the Tolfa site:

1) fresh leaves of *Arbutus unedo L.*;

2) partially decomposed litter of *Arbutus unedo L.*;

3) Tolfa soil;

4) fresh leaves of *Quercus ilex* from the test soil site.

The latter extract was used as a control to verify that the nitrification activity of the test soil was not sensitive to extracts of the dominant plant species (*Quercus ilex*) present in the site. Soil and litter extracts were obtained by shaking 100 grams of soil (top 10 cm, sieved at 2 mm mesh sieve), 35 grams of fresh leaves, reduced by hand in little pieces, with 500 ml of

distilled water for 2 hours. The extract was than filtered with Whatman 42 paper and 30 ml of each of three extract were added to 100g of the test soil (on 4 replicates). A control was used with sole distilled water (30 ml $H_2O$ 100 $g^{-1}$ dry soil). Soil was then incubated to determine the rate of potential nitrification as described in § 2.2.2.

Then a second test was performed. Fresh mature leaves (100 g), sampled at the beginning of July 2005 from tree branches, were broken in little pieces by hand and infused for 18 hours (on a rotary shaker) in 1 L of three different types of extractant (fresh leaves: extractant ratio 1:10):

1)an acid solution of $H_2SO_4$ 0.0005 M with pH 3 in distilled water;

2) a basic solution of NaOH 0.0001 M with pH 10 in distilled water;

3) a hydroalcoholic solution of $H_2O$ – EtOH (1:1).

After the extraction, the solutions were filtered on Whatman 42 paper, neutralized and then lyophilized by using a HetoDry Winner lyophilzator. The ethanolic extract was first evaporated by Rotavapor Sistem to let all the ethanol to distillate and then lyophilized. A toxicity test was then organized using the lyophilized extracts. Test soil (10 g each sample on 4 replicates) was treated with solutions of different crude extracts (acid, basic and ethanolic) to have a final concentration of 0, 4, 40, 400, and 4000 μg of extract $g^{-1}$ dry soil. Soil samples were incubated to determine the rate of potential nitrification.

A third toxicity essay was performed to test the inhibiting effect of the aqueous and the organic fractions of the hydroalcoholic extract, which were expected to contain chemicals with different $K_{ow}$ and solubility, having hence, different affinity for organic membranes and cellular uptake. The crude hydroalcoholic extract (6.4 g in 100 ml of water) was chromatographed on Amberlite XAD-4, eluting with water first, and then with methanol. Both the solutions were dried, thus obtaining a crude aqueous of 3.35 g and a crude organic of 1.13 g fraction. To perform the test, the aqueous fraction was dissolved both in distilled water (solution **A/A**) and in methanol: water (1:1) (solution **A/M**), whereas the organic fraction was dissolved only in methanol: water (1:1) (solution **M/M**). Solution A/A and A/M of the aqueous fraction were prepared to take into account possible toxic effects of methanol on nitrifiers. The final concentration of extract in soil was again 0, 4, 40, 400, 4000 μg of extract $g^{-1}$ dry soil for the organic fraction, and 0, 40, 400, 4000 μg of extract $g^{-1}$ dry soil for the aqueous fraction of the hydroalcoholic extract. Soil was then incubated (on 3 replicates) to measure the rate of $NO_3^-$ production.

### 4.2.3 CHARACTERIZATION OF CHEMICAL COMPOUND IN PLANTS, ROOTS AND SOIL

The organic and aqueous fractions obtained from the hydroalcoholic extract of fresh leaves were analyzed to determine the most abundant natural compounds present. Raw extracts were purified using different chromatographic techniques (CC, FCC, HPLC, TLC) and their structure was defined by using spectroscopic (UV-Vis, NMR 1D e 2D) and spectrometric (ES/MS) methods as described in more details by *Fiorentino et al.* (2007). Chemical analysis were carried out at the department of Science of Life (Second University of Naples, Italy).

The organic fraction was chromatographed on column using Sephadex LH-20 as stationary phase and water and methanol solutions as eluents to collect fractions of 20 mL of volumes. Fractions from 22 to 29, eluted with $H_2O$, contained pure arbutin **(2)** (62.7 mg); fractions from 183 to 193, eluted with $H_2O$ – MeOH (3:1), consisted of ethyl gallate **(1)** (44,0 mg). Fractions from 287 to 304, eluted with $H_2O$ – MeOH (1:1), was rechromatographed on preparative TLC eluting with $CHCl_3$ – MeOH (4:1), to obtain pure p-hydroxybenzoylarbutin **(3)** (2.5 mg) and galloylarbutin **(4)** (11.4 mg). Fraction 317 to 340, eluted with $H_2O$ – MeOH (1:1), was purified by TLC eluting with $CHCl_3$ – MeOH (4:1), to obtain gallocatechin **(5)** (35.5 mg) and catechin **(6)** (54.6 mg). Fractions 406-430, eluted with $H_2O$ – MeOH (1:1), was purified by TLC eluting with $CHCl_3$ – MeOH – $H_2O$ (13:9:3), to obtain kaempferol 3-O-α-L-ramnopyranoside **(7)** (11.0 mg), quercetin 3-O-α-L-ramnopyranoside **(8)** (33.0 mg), myricetin 3-O-α-L-ramnopyranoside **(9)** (18.0 mg). Finally, fractions 445 to 458, eluted with $H_2O$ – MeOH (1:3), was purified by TLC eluting with $CHCl_3$ – MeOH – $H_2O$ (13:9:3), to obtain kaempferol 3-O-β-D-arabinofuranoside **(10)** (1.3 mg), quercetin 3-O-β-D-arabinofuranoside **(11)** (3.1 mg), myricetin 3-O-β-D-arabinofuranoside **(12)** (2.8 mg).

In order to identify the compounds present in extracts of roots of *A. unedo* and in extracts of Tolfa soil, samples (3 replicates) of 5 g of dry roots of *A. unedo* and 5 g of Tolfa soil were extracted by Soxhlet apparatus (4 h in 250 ml of methanol) (Alonso et al., 1998). The crude extracts were dried to obtain a residue (140 mg for the roots and 70.0 mg for the soil) which was redissolved in methanol to have a final concentration of 0.5 mg/ml. An

aliquot (50 µl) of the solution was analysed by $C_{18}$ HPLC with detection at 258 nm. The metabolites in the mixture were identified by comparing their retention times with those of the pure standard isolated and characterized from *A. unedo* (Fiorentino et al. 2007). The HPLC apparatus consisted of a pump (Beckman System Gold 127), a UV-Vis detector (Beckaman 166) and a Shimadzu Chromatopac C-R6A recorder. Analytical HPLC was performed using RP-18 (Gemini 50µm, 250 x 4.6 mm i.d., Phenomenex) column using the following gradient: solution A ($H_2O$ – AcOH 99:1 v/v) and solution B (MeCN-MeOH, 4:1 v/v); A:B (49:1) in isocratic mode for 5 min; gradient A:B from 49:1 to 7:3 in 20 min, then isocratic mode for 10 min, finally 100% B in 10 min. The flow rate was 0.7 mL min-1 with detection at 258 nm. All the root and soil analyses were performed in triplicate and the results are reported as the average of the values ± standard deviation.

### 4.2.4 STATISTICAL ANALYSIS

A one way analysis of variance was used to evidence significant differences among different season. When the difference was significant ($P<0.05$) an "all pair wise" comparison was carried on using the "Student-Newman-Keuls test". Non-linear regression analysis was performed to find the relationship between the decrease of nitrification activity and the increase in extract concentration (Sigma Stat 3.11, Jandel Scientific).

## 4.3 RESULTS

### 4.3.1 N TRANSFORMATION PROCESSES AND MINERAL N IN SOIL AT THE TOLFA SITE

In the Tolfa soil, $NH_4^+$ was the dominant form of mineral N at all sampling occasions (**Table 4.1**). $NO_3^-$ was completely absent, with the sole exception of the 5th of April 2005 when, however, its concentration was to the limit of analytical detection (0.1 ppm of $N\text{-}NO_3^-$). Significant N mineralization activity was measured at each sampling occasion and was generally lower in spring (**Table 4.1**).

**Table 4-1.** Values (± 1stdev) of mineral N extracted from fresh soil, mineralization and nitrification rates measured in laboratory incubations on soil sampled in year 2005 and 2006 at the Tolfa site.

| Date of field sampling | **Soil ammonium** μg $NH_4^+$-N $g^{-1}$ dry soil | **Soil nitrate** μg $NO_3^-$-N $g^{-1}$ dry soil | **N mineralization rate** μg N $g^{-1}$ $day^{-1}$ | **Nitrification rate** μg N $g^{-1}$ $day^{-1}$ |
|---|---|---|---|---|
| 5th April 2005 | 0.93 ± 0.26 | 0.05 ± 0.01 | 0.92 ± 0.79 | 0.01 ± 0.00 |
| 20th July 2005 | 1.69 ± 0.07 | 0.00 ± 0.00 | 0.33 ± 0.51 | 0.00 ± 0.00 |
| 19th October 2005 | 5.02 ± 1.30 | 0.00 ± 0.00 | 0.54 ± 0.31 | 0.00 ± 0.00 |
| 2nd February 2006 | 17.92 ± 24.18 | 0.00 ± 0.00 | 1.44 ± 1.3 | 0.00 ± 0.00 |
| 12th May 2006 | 0.79 ± 0.21 | 0.00 ± 0.00 | 0.06 ± 0.06 | 0.00 ± 0.00 |
| 11th July 2006 | 10.00 ± 6.65 | 0.00 ± 0.00 | 0.34 ± 0.23 | 0.00 ± 0.00 |

As evidenced in the field samples, also in the laboratory incubations no $NO_3^-$ was produced over 28 days, and the sole product of organic N mineralization was $NH_4^+$, which increased linearly over the 28 days of incubation. Similarly, in the test of potential nitrification activity no $NO_3^-$ was produced, despite the addition of $(NH_4)_2SO_4$, which was still found in the soil after 24 days of incubation at the same concentration measured at time zero (see chapter 3, § 3.3.4, Fig. 3.8). The same result, i.e. complete absence of $NO_3^-$ production, was obtained when the soil was limed with $CaCO_3$ (**Table. 4.2**).

**Table 4-2.** Values (± 1stdev) of nitrification rates measured in laboratory incubations on Tolfa soil with or without $CaCO_3$.

| Sample | **Nitrification rate** μg N $g^{-1}$ $day^{-1}$ |
|---|---|
| Control soil | 0.007 ± 0.006 |
| Limed soil | -0.006 ± 0.008 |

### 4.3.2 TOXICITY TESTS

First toxicity test in **Fig. 4.1**, carrying out on a "test" soil, have showed nitrification rates obtained adding aqueous extracts of different components of Tolfa ecosystems. Tolfa soil extract doesn't reduced nitrification rate, while aqueous extract of fresh leaves and partially decomposer litter on nitrification rate was resulted in a significant reduction. Nitrification rate no resulted decrease on soil with addition of aqueous extract of *Quercus ilex*. The highest

nitrification inhibitory activity on test soil, with 37% of reduction respect to control, was associated to fresh leaf extract of *Arbutus Unedo*.

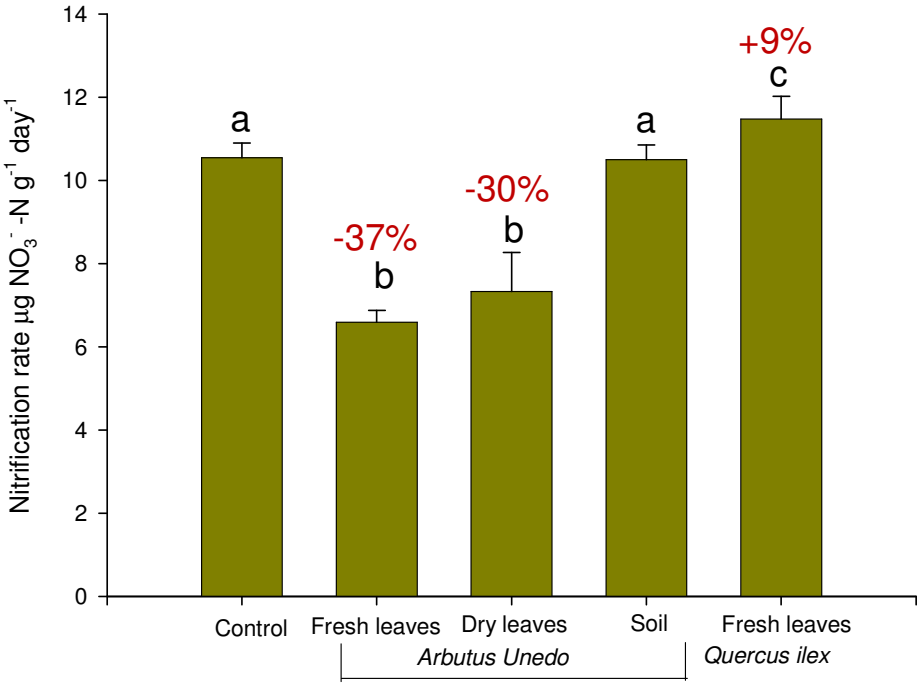


**Figure 4-1.** Nitrification rate of Castel Volturno soil with addition of $H_2O$, aqueous extract of fresh leaves, litter and soil underneath *Arbutus Unedo* from the Tolfa site and aqueous extract of *Quercus ilex*. (Bars represent one standard deviation. Different letters indicate significant differences among treatments (one-way ANOVA, $P<0.05$).

The inhibitory effect of extracts of fresh leaves of *Arbutus Unedo* on nitrification was cleared in **Fig 4.2**. Here, compounds present in the fresh leaves was extracted using three solutions with different pH, then neutralized and lyophilized and ridissolved in water to obtain four concentrations. A dose-response curve was constructed for each type of extract, all of three extracts was resulted in inhibitory effect on soil nitrification, but the hydroalcoholic extract showed inhibitory effect at lower concentration.

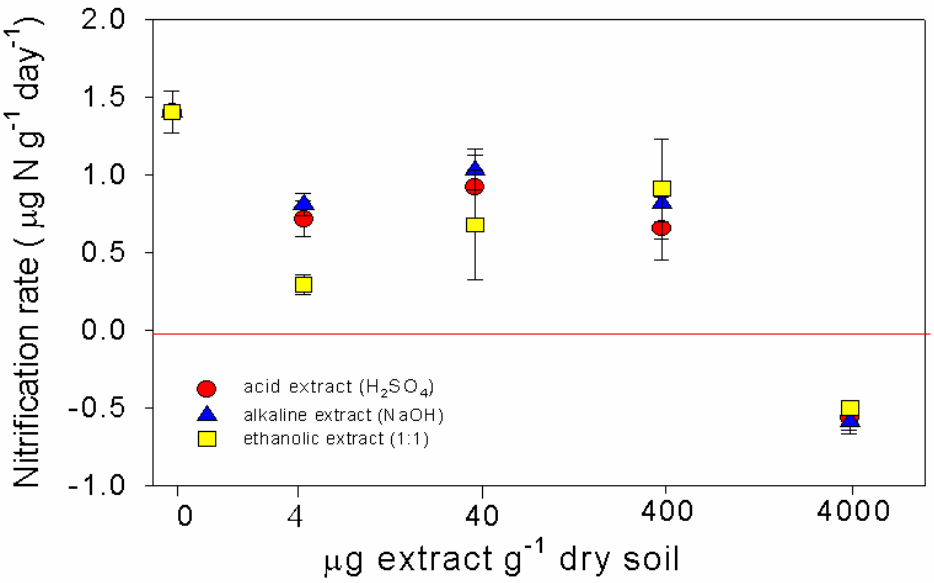


**Figure 4-2.** Nitrification rates measured in the test soil amended with fresh leaf of *Arbutus unedo* extracting in acid (red), basic (blue) and hydroalcholic (yellow) solutions. (Bars represent one standard deviation. Different letters indicate significant differences among treatments (one-way ANOVA, $P<0.05$).

In Figure **4.2** net nitrification rate of hydroalcholic extract (yellow) are plotted against the amount of leaf extract added to the soil. The "zero" leaf extract concentration represents the control treatment (treated with slightly acid water), where 163.3 µg $NH_4^+$-N and 23 µg $NO_3^-$-N were extracted per gram of dry soil at $T_0$. After 28 days of incubation 85 µg $NH_4^+$-N g-1 and 67 µg $NO_3^-$-N g-1 were recovered in the control. Thus, over 28 days the microorganisms nitrified 44 µg $NO_3^-$-N and immobilized about 39 µg $NH_4^+$-N. In the soil treated with the highest concentration of leaf extract, at $T_0$ no significant difference of mineral N was measured compared with the control treatment. After 28 days, 178 µg $NH_4^+$-N g-1 were still recovered in the soil treated with the highest concentration of leaf extract, indicating that less $NH_4^+$ was immobilized and/or transformed into $NO_3^-$-N, and only 14 µg $NO_3^-$-N $g^{-1}$ were recovered, showing not only no net $NO_3^-$ production but also that some $NO_3^-$ was immobilized or lost as gaseous N, which however, was not monitored. Indeed the high input of organic extract might have stimulated some immobilization as well as some gaseous lost

via denitrification. However, immobilization might not be the sole explanation for the absence of net nitrification, as the observation that plenty of $NH_4^+$ was still present in the soil after 28 days indicate a slowdown in the process of nitrification and/or immobilization of $NH_4^+$. On the other hand, it would be difficult to expect a preferential immobilization of $NO_3^-$, which would bring to no net $NO_3^-$accumulation, when in the soil there is still plenty of $NH_4^+$. A combination of inhibition of nitrification and immobilization/loss might probably explain the observed results in the test soil. At lower extract concentrations (**Fig. 4.2**), a partial reduction of $NO_3^-$ production was also observed, which was already quite effective at 4 µg of extract $g^{-1}$ dry soil. The slight increase of $NO_3^-$ production between 40 and 400 µg of extract $g^{-1}$ dry soil probably depended on the stimulation of mineralization and nitrification due to the addition of the organic extract, which represents also a substrate for microbial activity, not balance by a sufficiently strong toxic effect or immobilization.

Testing the methanol and aqueous fractions of the hydroalcoholic extract gave good dose-response relationships described by sigmoid curves reported in **figure 4.3**, however, due to the high variability of results, only at the highest concentration of extract the effect resulted statistically significant (**Fig. 4.3**). Almost 60% of inhibition of $NO_3^-$ production was attained adding the aqueous extract (treatment A/A), indicating that the compounds responsible for nitrification inhibition are quite soluble. In fact, only a maximal reduction of activity of 20% was associated to the methanol fraction (M/M), which was significantly lower than the reduction observed for the treatments A/A and A/M (**Fig. 4.3**).

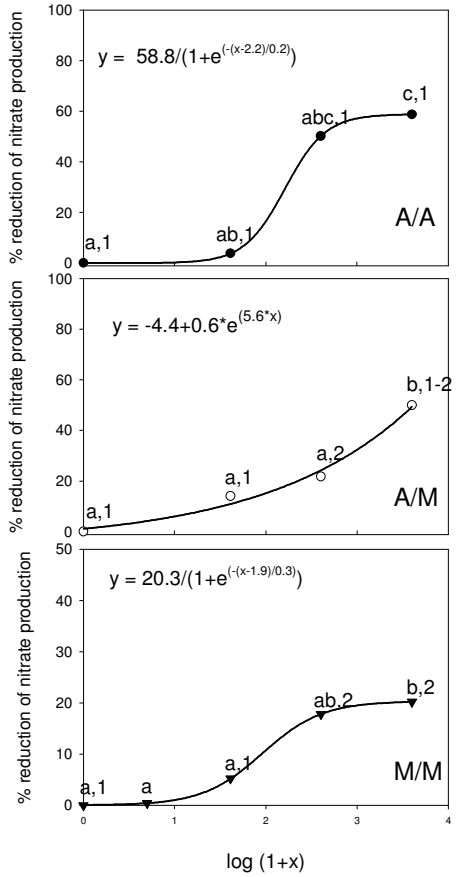


**Figure 4-3.** Dose-response graphs representing the percentage of inhibition of $NO_3^-$ production in function of the concentration of extract of fresh leaves added to the soil (0, 4, 40, 400, 4000 µg $g^{-1}$ dry soil) reported on log scale. (A/A aqueous fraction of the hydroalcoholic extract in distilled water; A/M aqueous fraction of the hydroalcoholic extract dissolved in methanol: water (1:1); M/M methanol fraction of the hydroalcoholic extract dissolved in methanol: water (1:1)). Equations represent the best fitting curve describing the relationship between the two variables. Different letters indicate a significant difference (two-way ANOVA) among concentration extracts within each treatment (A/A, A/M, M/M); different numbers indicate a significant difference (two-way ANOVA) comparing the same extract concentration among different treatments.

### 4.3.3 CHARACTERIZATION OF THE MAIN SUBSTANCES PRESENT IN LEAVES, ROOTS AND SOIL

The pure metabolites purified from the hydroalcoholic extract by chromatographic techniques, have been identified on the basis of their spectroscopic features as ethyl gallate **(1)**, arbutin **(2)** and its p-hydroxybenzoyl **(3)** and galloyl **(4)** derivatives, (+)-gallocatechin **(5)** and catechin **(6)**, six flavonoid glycosides **7**-**12** characterized by the kaempferol, quercitin and

myricetin as aglycons, and by a rhamnopyranose or arabinofuranose as glycone units (**Fig 4.4**) (*Fiorentino et al. 2007*).

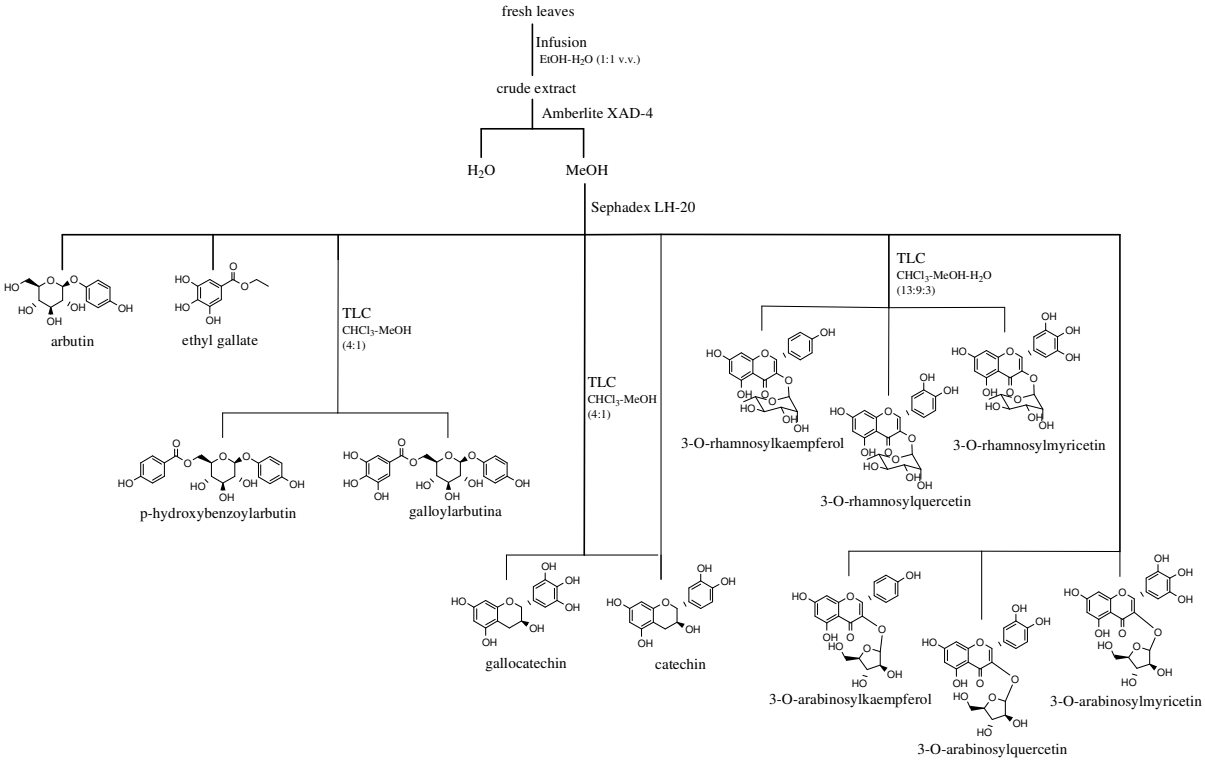


**Figure 4-4.** Main compounds purified from hydroalcholic extract.

The same compounds were found in the methanol and in the aqueous fraction, although in different quantities (**Fig. 4.5**). In particular, gallocatechin was the most abundant compound in the aqueous fraction (AHF), corresponding to 74% of the total gallocatechin of the initial hydroalcoholic extract (HLE). Root extracts (R/E) (**Fig. 4.5**) showed the same constituents previously described, described, with the exception of the less abundant flavones **10**-**12**, and again gallocatechin was one of the most abundant compounds.

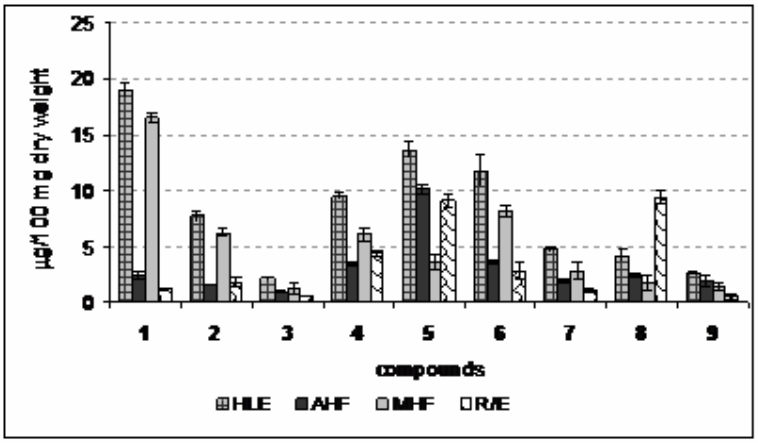


**Figure 4-5.** Quantitative analysis of secondary metabolites from *A. unedo* (µg/100 mg of dry weight ± standard deviation) determined by RP-HPLC analyses of the total hydroalcoholic leaf extract (HLE), its aqueous (AHF) and methanolic (MHF) fractions and root extract (R/E). Ethyl gallate (1), arbutin (2) and its p-hydroxybenzoyl (3) and galloyl (4) derivatives, (+)-gallocatechin (5) and catechin (6), 3-O-rhamnosylkaempferol (7) 3-O-rhamnosylquercetin (8), 3-O-rhamnosylmyricetin (9). Bars of standard deviation represent laboratory replications (3 samples).

When soil was also analysed to determine the chemical compounds present, a distinction was made between the bulk soil and the rhizosphere soil, i.e. the soil adherent to the roots. The latter soil was obtained by shaking the hand cleaned roots of *A. unedo* on a white paper sheet. This soil in contact with roots could be supposed to be richer in root exudates than the bulk soil sampled in the top 10 cm among plants. The **figure 4.6** shows the chemicals identified in the bulk soil (S1) and in the rhizosphere soil (S2). Gallocathechin, galloylarbutin and catechin were the most represented metabolites identified in S1. The root soil (S2) showed much lower concentrations of the same compounds found in S1.

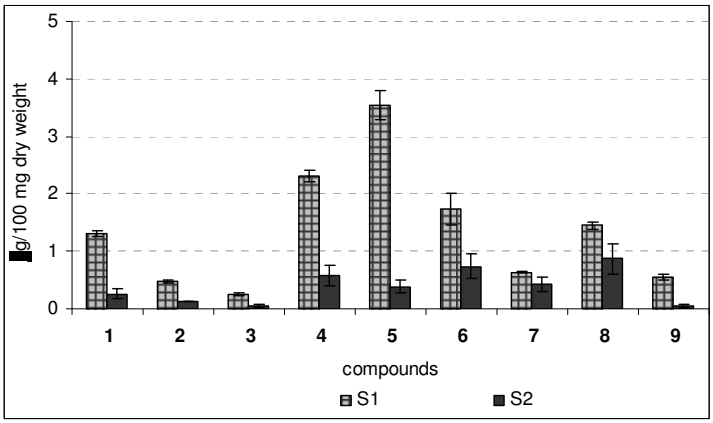


**Figure 4-6.** Quantitative analysis of secondary metabolites from *A. unedo* (µg/100 g of dry weight ± standard deviation) determined by RP-HPLC analyses of the bulk soil (S1) and the rhizosphere soil (S2) extracts. Ethyl gallate (1), arbutin (2) and its p-hydroxybenzoyl (3) and galloyl (4) derivatives, (+)-gallocatechin (5) and catechin (6), 3-O-rhamnosylkaempferol (7) 3-O-rhamnosylquercetin (8), 3-O-rhamnosylmyricetin (9). Bars of standard deviation represent laboratory replications (3 samples).

## 4.4 DISCUSSION

Field data showed that $NO_3^-$ production in the top 10 centimetres of the Tolfa soil was either absent or so low to be below the detection limit of the used technique, which would mean in any case a concentration below 0.05 µg of $NO_3^-$-N $g^{-1}$ dry soil, over the whole year. Two causes could be advocated for such result, lack of substrate or inhibition of nitrification. Lack of substrate might derive from inhibited mineralization, which provides $NH_4^+$ from organic matter decomposition, or immobilization due to competition of plants and heterotrophic microbes with autotrophic nitrifiers, known to be weak competitors for $NH_4^+$. Data show that the mineralization process occurred at significant rates (Table 4.1) over the whole year, producing, as also evidenced in the laboratory over 28 days of incubation, significant amounts of $NH_4^+$. In the laboratory, $NH_4^+$ was neither transformed to $NO_3^-$ nor immobilized, as in fact, even when the Tolfa soil was treated with additional N (150 µg $NH_4^+$-N $g^{-1}$ dry soil, potential nitrification test), after 28 days we still found the same amount of added N (191.3 ± 17.5 µg $NH_4^+$-N $g^{-1}$ at T0 and 182.5 ± 54.8 µg $NH_4^+$-N $g^{-1}$ at T28) whereas no $NO_3^-$ was detected. In the field no $NO_3^-$ was detected even when $NH_4^+$ production was exceeding immobilization and uptake (net $NH_4^+$ accumulation in the soil, Table 1). Other

studies in forests and woodlands reported $NH_4^+$ as the dominant form of mineral N in acidic soils, especially in the mature phase of the succession (*Schimel et al. 1996; Ste Marie and Paré 1999; DeLuca et al. 2002*), where, however, some $NO_3^-$ production was still observed. In fact, adapted populations of autotrophic nitrifiers and heterotrophic nitrifiers are still able to produce $NO_3^-$ at low pHs (3-5) (*Walker and Wickramasinghe 1979; Robertson 1982 a,b; Troelstra et al. 1990; De Boer et al. 1992; Pennington and Ellis 1993*), although at lower rates than neutrophilus autotrophic nitrifiers (*De Boer and Kowalchuk 2001*). On the other hand, *Northup et al (1995)* evidenced that in acidic poor soils under *Pinus muricata*, nitrification was reduced because the production of phenol rich litter slowed down the mineralization process. This was proposed as a mechanism to facilitate N uptake in organic forms by plants via mycorrhiza, "short-circuiting" the nitrogen cycle when mineralization rate cannot support plants N requirements. In the case of Tolfa soil, however, the availability of $NH_4^+$ in the field (Table 1) and the mineralization activity ($NH_4^+$ production only) measured in the laboratory over the whole study period, indicate that the limiting step for nitrification was not $NH_4^+$ production.

The second hypothesis is that inhibition of nitrification, rather than lack of substrate or competition, might be a possible cause for the lack of $NO_3^-$ production observed in this soil. In this respect, the toxicity tests gave some indication that leaf extracts might have indeed some toxic potential activity on nitrification, as in fact, at the highest leaf extract concentration no net $NO_3^-$ production was observed and more $NH_4^+$ was recovered in the soil after 28 days compared with the control. It cannot be excluded that some $NO_3^-$ might have been immobilized or reduced during processes of assimilatory or dissimilatory reduction, as in fact, in the soil treated with the highest concentration of leaf extract, some net $NO_3^-$ loss or immobilization was observed. However, the fact that this immobilization was evident in the treated samples and not in the control, plus the observation that added $NH_4^+$ was recovered in the treated soil at much higher concentration compared with the control, seems an indication that some inhibition of nitrification occurred. In fact, it could be difficult to imagine preferential uptake or immobilization of $NO_3^-$ as the sole explanation for the observed absence of $NO_3^-$ in the soil after 28 days, when there was still plenty of extractable $NH_4^+$ available, as $NH_4^+$ is known to inhibit assimilatory nitrate reduction in many plants and microorganisms (*Revilla et al. 1986; Martínez-Espinosa et al. 2007*).

When these observations are combined with field measurements and the laboratory incubation data, discussed in the first part of this paragraph, the inhibition mechanism might seem a possible explanation for the observed absence of net $NO_3^-$ production at Tolfa.

This soil receives a quite high annual input of litter (400-500 g $m^{-2}$ $year^{-1}$), which, being *A. unedo* an evergreen plant, is almost continuous along the year, with a peak in early summer and one in late autumn (Cotrufo pers. Comm.). Thus, a significant input of allelophatic compounds, produced by the plants, might occur by means of leaf fall. The chemical analysis of leaves of *A. unedo* have shown the presence of many compounds which are known to have antioxidant activity and potential allelophatic effect (Fig. 4.3 and 4.4). Several of these chemicals are present in quite high concentrations compared with previous findings (*Fiorentino et al. 2007*). In particular, (+)-catechin **(6)**, isolated with 35% enantiomeric excess, was previously identified as a phytotoxin and antibacterial agent, against root-infesting pathogens, (*Bais et al. 2002; Bais et al. 2003*). The enantiomerically pure (+)-gallocatechin **(5)**, the most representative metabolite after ethylgallate (Fig. 4.3), known for its antioxidant activity, was previously reported from Mediterranean shrubs as *Cistus sp* (*Pomponio et al. 2003*) and *Salix triandra* (*Niemi et al. 2005*). Extracted and purified from the roots of *Leucana leucocephala*, it gave direct evidence of nitrification inhibition on pure cultures of *Nitrosomonas europaea* at concentration of 50 µg/ml with significant inhibiting activity already at 12 µg/ml (*Erickson et al. 2000*). Although, *Nitrospira sp.* is the most common autotrophic nitrifier in acid soils (*De Boer and Kowalchuk, 2001*), *Nitrosomonas*-like 16S rDNA (*Carnol et al. 1998*) and *Nitrosomonas*-like PCR amplification products (*Hastings et al. 2000*) have also been obtained from acid soil and litter, respectively.

In recent studies *De Luca et al. (2006)* showed that the addition of charcoal to soil in a *Pinus ponderosa*/*Psuedotsuga menziesii* forest significantly increased the nitrification potential, net nitrification, gross nitrification, and decreased the solution concentrations of catechin (±), an allelochemical (phenolics) produced by the invasive species *Centaurea maculosa* (*De Luca et al. 2006; Gundale and De Luca 2006*), suggesting that the immobilization of phenols by charcoal might reduce the inhibiting effect of phenols on the nitrifying activity of the microbial community in the forest soil. Similar results were also shown by *Mckenzie and De Luca (2006)* who comparing the effect of charcoal addition on soil N processes under graminoid or ericoid litter, demonstrated that charcoal addition significantly stimulated nitrification under the ericoid litter by removing over 80% of phenolic

compounds produced, which were 20 times higher in ericoid leaf leachates compared with the graminoid ones, again supporting the idea that charcoal deposition after fire may modify a nitrification interference mechanism by absorbing plant phenolics secondary metabolites.

Then, if we assume that also at the Tolfa woodland site allelophatic compounds enter the soil mainly via litter decomposition (leaves, fine roots) we might roughly estimate the input of allelochemical compounds in the Tolfa soil, using the measured value of above-ground litter fall (400-500 g $m^{-2}$ $year^{-1}$) and an estimated value of below ground input (300 g $m^{-2}$ $year^{-1}$). Focusing, in particular, on gallocatechin and catechin, which were the most abundant chemicals in leaves, roots and in particular in soil extracts, the following allelochemical inputs can be calculated based on the values presented in Fig. 4.5, 70 mg $m^{-2}$ $yr^{-1}$ of gallocathechin and 60 mg $m^{-2}$ $yr^{-1}$ of catechin from leaves, 27 mg $m^{-2}$ $yr^{-1}$ of gallocathechin and 10 mg $m^{-2}$ $yr^{-1}$ of catechin from roots, thus a total input of 97 mg $m^{-2}$ $yr^{-1}$ of gallocathechin and 70 mg $m^{-2}$ $yr^{-1}$ of cathechin. Using the soil concentration of gallocathechin and cathechin reported in Fig. 4.6 and a soil bulk density of 0.47 g $cm^{-3}$, it can be estimated that 1645 mg $m^{-2}$ of gallocathechin and 846 mg $m^{-2}$ of cathechin were present in the top 10 centimetres of soil at the time of sampling (July 2005). These amounts are significantly higher than the annual calculated input via litter, indicating a quite high residence time for these compounds. A consequence of such result is that a significant amount of these allelochemicals would be present in the Tolfa soil during the whole year. Thus, the toxic action on nitrifiers might be exerted by these substances also in periods when litter inputs are lower. This would be in accord with absence of $NO_3^-$ production in the field, as well as in the laboratory incubation, observed in all sampling occasion, even when soil was limed.

Results from the toxicity tests (Fig.4.4) indicate that more than 400 µg of extract $g^{-1}$ dry soil were necessary to have a drastic inhibition in the test soil, corresponding to about 12.6 µg g of gallocatechin $g^{-1}$ dry soil and 19.3 µg of catechin $g^{-1}$ dry soil. If these same concentrations could be hypothesized to be so effective also in the Tolfa soil, it would mean that a soil concentration of 0.59 g of gallocatechin $m^{-2}$ and 0.91 g catechin $m^{-2}$ would be already sufficient to have a 50% reduction of nitrification activity. These concentrations are lower or in the same order of magnitude of those found for these two chemicals in the Tolfa soil (1.64 g $m^{-2}$ and 0.85 g $m^{-2}$, respectively). Thus, it could be plausible that the observed concentration of gallocatechin in the Tolfa soil might be sufficient to block $NO_3^-$ production.

It cannot be excluded that the compounds characterized in the *Arbutus unedo* leaf extracts might have additive or even synergistic effects on microbial activity when added together (whole extract), so that the effective toxic concentration of each compound might be even lower than what calculated on the base of their concentrations in the extract.

## 4.5 CONCLUSIONS

Field and laboratory data seem to suggest that the lack of $NO_3^-$ production observed in the Tolfa soil might depend on chemical inhibition of nitrification by phenolic compounds produced as secondary metabolites by the *Arbutus unedo* plants.

The calculated residence time of these compounds in the soil indicate that the toxic activity of these molecules might be exerted on microbes during the whole year, also far from peaks of litter input. These results might explain the lack of nitrous oxide emissions previously observed in the field (see chapter 3). In fact, inhibition of nitrification would block $N_2O$ emissions from this source, and the absence or extremely low rates of $NO_3^-$ production would also limit $N_2O$ losses from denitrification activity. Such result is relevant not only for widening the knowledge on N cycle in Mediterranean environment, but also for scaling up processes linked to greenhouse gas emissions. In fact, if $N_2O$ fluxes were modelled for this ecosystem just on the base of soil and environmental characteristics, a significant annual budget of $N_2O$ might be reached, having the soil good total C and N content, mineralization activity and water retention potential. This indicate that for some ecosystem types it could be necessary to incorporate allelophatic processes in biogeochemical modelling.

# 5 NITROGEN INPUT AND LOSSES ASSOCIATED TO $N_2$-FIXERS HERBACEOUS COVER IN DISTURBED OPEAN AREAS OF MEDITERRANEAN SHRUBLANDS

## 5.1 INTRODUCTION

In temperate region, and in particular in Mediterranean ecosystems, herbaceous legumes have been found to increase significantly in the early stages of succession (*Boring et al. 1988; Rundel 1989; Bordeleau & Prévost 1994*), in particular following different types of disturbance events such as fire (*Naven 1967, Bell and Kock 1980, Woodmansee et al. 1981, Crews 1999*) cut, logging, and blow down (*Greller 1988*). The persistence of the disturbance maintains the percentage of leguminous cover high, often higher than the cover of non fixing herbaceous species (*Esposito pers. Comm*), whereas during the course of succession, the numbers of leguminous species and the number of individuals per each specie descrease with time (*Esposito pers. Comm, Rundel 1989; Arianoutsou and Thanos, 1996*).

Several hypothesis have been suggested to explain leguminous plants disappearance during the succession (*Vitousek & Howarth 1991*): the energetic constraints associated to the activity of nitrogen fixers; the limitation of nitrogen fixers or fixation by another nutrients (in particular *P and Mo, Robson & Bottomley 1991; Smith 1992; Crews 1993*); other physical and ecological mechanisms such as high acidity, alkalinity or aridity (*Alexander 1984; Bordeleau & Prévost 1994*), preferential grazing (*Hulme 1994, 1996*), fire (*Bahre 1995*). When sufficient N is available in the soil, the non fixers should be in the condition to invest more of their NPP in aboveground than in the in belowground N-acquiring structures (*Vitousek & Howarth 1991; Vitousek & Field, 1999; Gutschick 1981, 1987*).

Moreover, in condition of light limitation, such as during plant canopy closure models suggest that it is energetically advantageous (i.e., requires less of a plant's photosynthate) to

grow roots and take up soil N than to fix N when soil N is available (*Gutschick 1981; Vitousek & Field, this vol, Vitousek & Howarth 1991*). Leguminous plants (Fabaceae) maintain high levels of N in leaf tissue in order to maximize photosynthetic rates per unit leaf area (*Crews, 1999*). Thus, their N rich tissue are assumed to enrich the soil with N during the early stages of succession, considering that part of the N required by the leguminous plant is not recycled from the soil, but is directly fixed from the atmosphere, hence with low nutrient cost for the ecosystem. However, how much of the N present in the leguminous tissue really goes to enrich the ecosystem is not clear, as part of this nitrogen could be lost as leacheate or different gaseous forms. In grasslands and herbaceous communities, an increase of N input might result in an increase of N losses as gaseous products, mineral and organic N, during the winter periods, when N release via decomposition processes is uncoupled with plant N uptake. This might be of particular interest to evaluate the effect of disturbance events on N cycle and N losses from macchia ecosystems. In fact, as these environments are mostly N limited and subjected to aridity or semi-aridity for part of the year, they are expected to be poor sources of greenhouse and trace N gases, as well as, poor sources of nitrates for ground waters, in contrast with what often observed in N saturated forests of central and northern Europe. However, disturbance might accelerate microbial processes involved in N transformation, might impair N uptake versus release, might favor, at least in a first stage of recolonization, the presence of N reach plants such as leguminous.

At present no works are available where N input and losses associated to $N_2$-fixers herbaceous cover is investigate in disturbed natural ecosystems of Mediterranean area.

In the present study the role of leguminous plants in terms of enrichment and losses of N in the ecosystems were investigated in a disturbed Mediterranean shrubland ecosystem. In this site, opean areas covered by herbaceous plants are maintained by fire and cutting management. Depending on the years and on the areas, plant cover is dominated by leguminous or by non-fixing erbaceous plants. The leguminous dominance generally persists for two or three years, after which nonfixing plants take over. To meet our aim the work was organized after the following objectives: a) to quantify the $N_2$ fixation capacity of the most frequent leguminous species to estimate the N input in the ecosystem via $N_2$ fixation; b) to quantify the gain and losses of N under $N_2$ fixing and non fixing plant covers.

## 5.2 MATERIALS *AND METHODS*

### 5.2.1 SITES OF STUDY DESCRIPTION

The field site was part of the "Castel Volturno" Nature Reserve, a flat coastal area located about 30 km North of Naples, in Southern Italy (40° 57'N; 1° 33'E). The climate is typically Mediterranean, characterised by summer drought, moderate precipitation (mean rain 760.3 mm/year during the year 2000) and relatively warm temperatures (mean annual temperature of 15.8°C during the year 2000). Soil is classified as a *Calcaric Arenosol* (FAO, 1998), and presents a pH of 7.8.

The area is interested by different plant communities which include Quercus mature stands, Pine woodlands, high Macchia, low Macchia, herbaceous cover, higrofilic plants. The area of study was located in a part of the park where the herbaceous cover constitutes a frequent community intermingled with macchia stands (**Fig 5.1**). This mosaic of plant patches is mainly related to the frequently occurring fire histories (*Esposito et al., 1998*).


**Figure 5-1.** CastelVolturno Reserve, area of study, with a detailed photo of a sampling area.

Two field campaign were organized to carry on the work:

1) One preliminary field campaign to sample the herbaceous plants needed to characterize the $^{15}N$ and N content to determine the amount of $N_2$ fixed, which occurred in spring 2005

2) A second field campaign from spring 2006 to autumn 2006 to follow the variation in pools and activities of N cycle associated to different plant cover % of leguminous plants.

### 5.2.2 FIELD CAMPAIGN 1: MEASUREMENTS OF PLANT $^{15}N$ AND TOTAL N FOR $N_2$-FIXATION DETERMINATION

$N_2$ fixation rates were determined by using two methods, the "N difference method" and the $^{15}N$ natural abundance (see chapter 2). Both methods require to measure the $^{15}N$, or the total N in a leguminous specie and in a non fixing herbaceous species (reference plant) which can resemble the fixer for shape, phenology, size. The principle is that if the two plants are contemporary present in the field in the same moment and are similar they can potentially exploit the mineral N present in the soil in the same manner. In the case of N difference technique, the assumption is that being more convening energetically to uptake mineral N than to fix it, only the extra N per unit of biomass which I find in the fixer, compared with the non-fixer, will be N fixed from atmospheric N. $^{15}N$ natural abundance techniques assumes that the mineral N has a characteristic $^{15}N$ signal which I can find in the plant tissue of plant which use it. If the plant fix atmospheric $N_2$ which as $^{15}N=0$ the signal will be diluted and from this dilution I can calculate the amount of $N_2$ fixes as explained in chapter 2. In both cases hence it is required that we analyse a $N_2$ fixer and a non $N_2$ fixer. As there is a high number of leguminous species we chose the two more abundant, which hence might be considered representative for the community of herbaceous $N_2$ fixers, from data of previous studies of abundance/dominance of species in the herbaceous community in the area (**Fig. 5.2**).

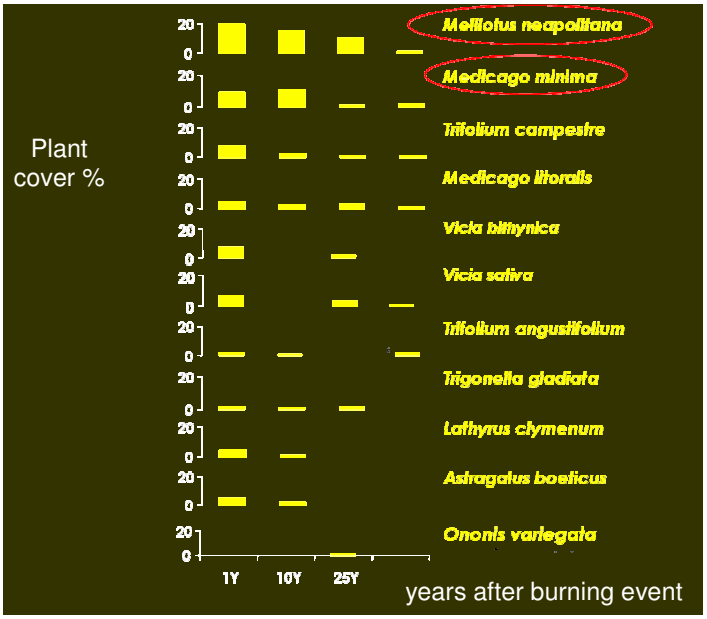


**Figure 5-2.** % plant cover of herbaceous leguminous plants determined in open areas along a chronosequence (Esposito Assunta, data unpublished).

For the present study we chose the *Melilotus neapolitana* Ten. and *Medicago minima* L. which clearly showed the highest cover. As reference plant on the base of the above mentioned required characteristics we chose *Phleum subulatum* and *Petroragia velutina* (**Fig. 5.3**).


**Figure 5-3.** Images of the leguminous and reference plants chosen for the experiment.

In spring 2005, in June, at the peak of plant biomass, 10 plots of 1 $m^2$ were selected in areas dominated by leguminous plants and 10 in areas dominated by reference plants, where 5 entire individuals (above and below ground parts) for each species (for a total of 50) were sampled per each species. However, for each plots samples were mixes to determine C and N content on 10 average samples. Plants were bought back to laboratory, dried and above, below ground biomass and seeds were first weighted and then ball-milled for subsequent analysis of total C and N by C,N,S analyser and $^{15}N$ by isotope ratio mass spectrometer (Chapt.2) (**Fig.5.4**).

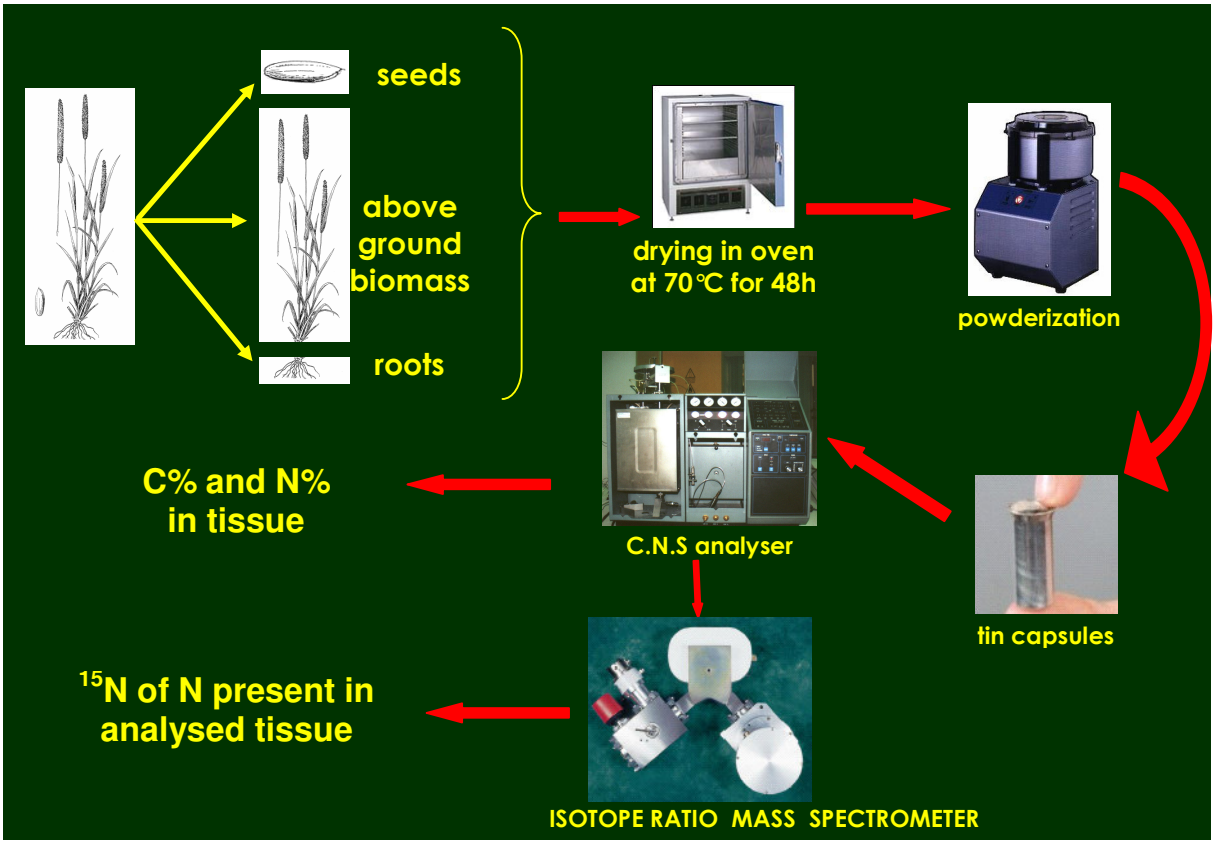


**Figure 5-4.** Procedure of samples preparation after field sampling for $N_2$ fixation determination.

### 5.2.3 Field campaign 2: VARIATION IN POOLS AND ACTIVITIES OF N CYCLE ASSOCIATED TO DIFFERENT PLANT COVER % OF LEGUMINOUS PLANTS

For this campaign, 3 open areas, covered by herbaceous plants, were chosen and within each open area 3 plots (2 $m^2$) dominated by leguminous plants and 3 by non leguminous plant where chosen for a total of 9 plots per each main cover type (Fig. **5.5**). At biomass peak, 2-3 weeks before plant senescence, closed chambers bases were set into the ground, one in each plot and a destructive sample of plants was taken to determine the amount of biomass of each species. Plant were dried in the oven at 45°C and the above and below ground biomass weight determined.

**Figure 5-5.** Closed static chambers set in 6 plots chosen in the third open area.

Soil and gas were sampled on each occasion , monthly from May 2006 to November 2006 to determine the amount of mineral N, organic soluble α-amino N, the potential N mineralization nitrification and denitrification in the different plots, starting from the assumption that plots were leguminous plants were more abundant might have had higher level of mineral N, and higher potential rates of N processes stimulated by N reach exudates when plant were alive and then by N reached tissue in the phase of decomposition. Soil samples, one per each plot were taken back to laboratory, sieved (2 mm mesh sieve) and mineral N and biological analyses were started the next day on fresh soil. For analyses details see chapter 2. Statistical analysis were carried out as descript in chapter 2.

## 5.3 RESULTS

### 5.3.1 FIELD CAMPAIGN 2005: $N_2$-FIXATION RATES

The average biomass of the different parts of the leguminous and reference plants sampled in the field from the 10 plots during the first sampling campaign is reported in **Table 5.1**. The seeds have been counted for each individual, being and weighted in group of 10 given the very low weight of each seed. The total weight of seeds per plant has then been calculated.

**Table 5-1.** Average biomass of the different part of the leguminous and reference plants sampled in the field from the 10 plots during the first sampling campaign.

| Species | Above ground Biomass (g dry weight) | Below ground Biomass (g dry weight) | n seeds per plant | g dry w.t 10 seeds | g dry w.t seeds x plant |
|---|---|---|---|---|---|
| *Medicago* | 1.81 ± 1.6 | 0.17 ± 0.08 | 200 | 0.008 | 0.165 |
| *Melilotus* | 1.94 ± 1.31 | 0.13 ± 0.10 | 600 | 0.027 | 1.602 |
| *Petrorhagia* | 0.31 ± 0.22 | 0.04 ± 0.03 | 200 | 0.001 | 0.026 |
| *Phleum* | 0.24 ± 0.08 | 0.03 ± 0.02 | 500 | 0.001 | 0.054 |

In **Figure 5.6** are reported the percentage of total N found in the different part of the sampled plants.

The seeds, as can be expected, are the most N reach tissue in the plant as they will provide N to the new individuals at the moment of germination for the following 2-3 weeks till the roots will be sufficiently big to be able to uptake sufficient mineral N or to nodulate. Both leguminous plants have a higher % of N in both above and below ground biomass compared with the reference plants, which instead present very similar values.

From the percentage values of N in each tissue and from the weight of each part of the plant the amount of N fixed by the leguminous plant has been determined using the following equation:

$$N_{dfa} = \left( \frac{\left[ N_{leg}^{a} + N_{leg}^{b} + N_{leg}^{s} \right]}{\left( w_a + w_b + w_s \right)_{leg}} - \frac{\left[ N_{ref}^{a} + N_{ref}^{b} + N_{ref}^{s} \right]}{\left( w_a + w_b + w_s \right)} \right) * 100$$

Where $N_a$, $N_b$ and $N_s$ is the total amount of N present in the plant components (aboveground, belowground and seeds) (**Fig. 5.6**), $w_a$, $w_b$, $w_s$ is the dry weight of the different components (**Table 5.1**).

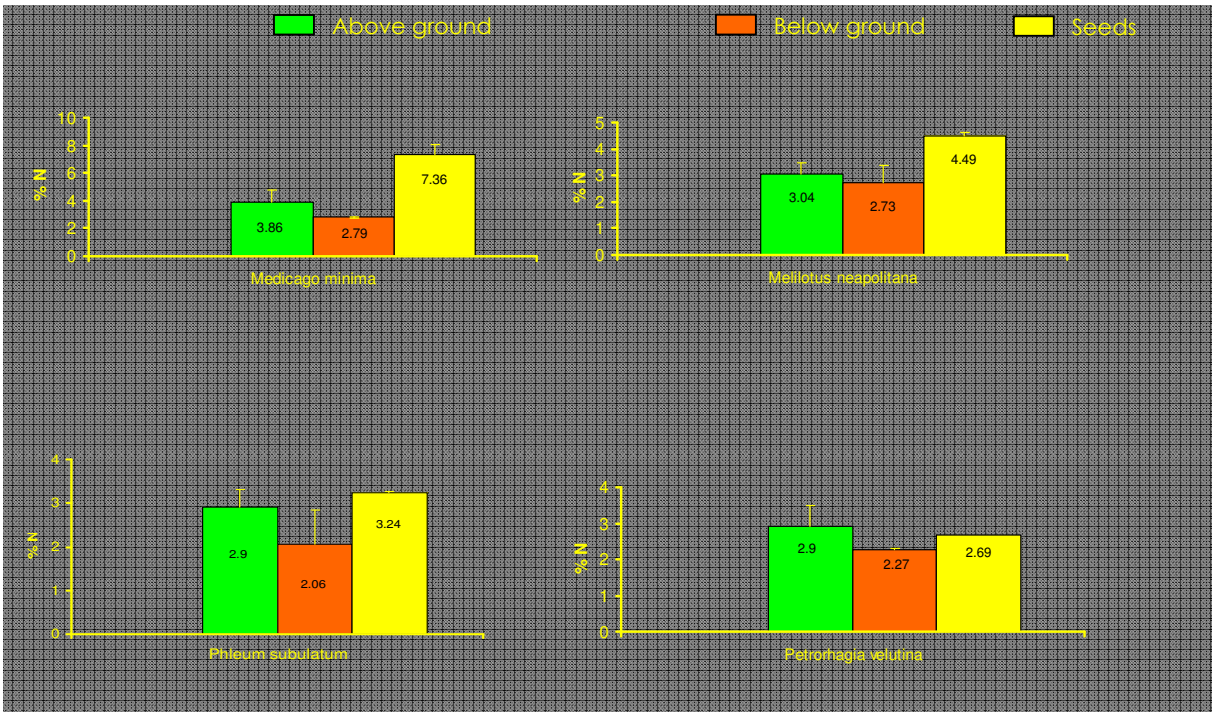


**Figure 5-6.** Percentage and amount of total N in the plant tissues of the leguminous and reference plants.

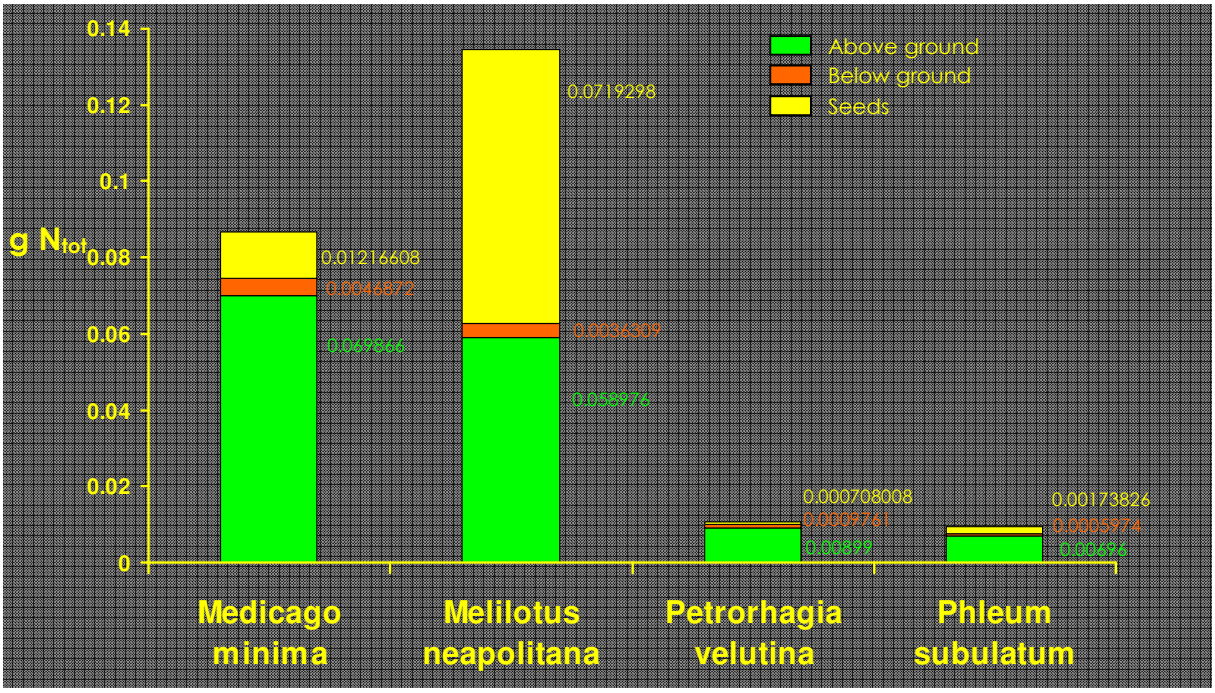


**Figure 5-7.** Total amount of N calculated in three plant tissues of the leguminous and reference plants reported as fractions of the total amount of N present in the plant.

The total amount of N normalized by the total weight of the plant comes out exactly the same for the two reference plants (0.028 g N/ g dry weight), thus we obtained just one value of $N_2$ fixation for the two leguminous plants (**Fig. 5.8**):

%Ndfa Medicago minima: 30.8%

%Ndfa Melilotus neapolitana 23.5%

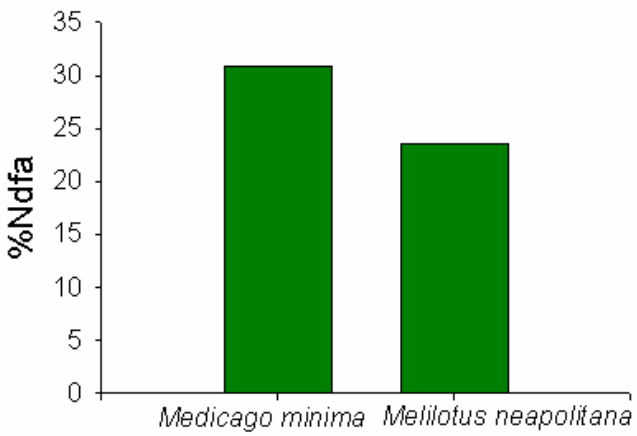


**Figure 5-8.** % of $N_2$ fixation for the two leguminous plants using "N-difference" technique.

For the determination of %Ndfa using the $^{15}N$ natural abundance the sole above ground part is generally utilized (*Unkovich et al. 1993, Pate et al. 1994*). In **Table 5.2** are reported the values of $\delta^{15}N$ (‰) determined on the above ground biomass of the four considered species.

**Tabella 5-2.** $\delta^{15}N$ (‰) values determined on the above ground biomass for *Melilotus*, *Medicago*, *Phleum* and *Petrorhagia.*

| | *Melilotus* | *Medicago* | *Phleum* | *Petrorhagia* |
|---|---|---|---|---|
| $\delta^{15}N$ (‰) | -0.75 ± 0.01 | -0.79 ± 0.19 | -1.53 ± 1.04 | -1.29 ± 0.39 |

The values are very close to each other so that the application of the technique is less precise. This might depend on the fact that wild leguminous plants often occurs in areas where the previous year leguminous plants were already present so that the signal of the decomposing organic matter N is closer to atmospheric values than would be in areas never interested by leguminous growth. The two fixers in any case have quite similar values and the same is true for the non-fixers, suggesting that they might have the a similar way to exploit soil mineral N.

In **Fig. 5.9** are reported the values of %Ndfa determined using the second approach and using either phleum or petrorhagia as reference plant. The %Ndfa varies from 37 to 50% depending on the plant and reference used. Using *Petrorhagia* as reference values are closer to those found with the "N difference", though higher. In any case, considering the very different approach the two techniques are quite in good agreement, in particular for *Medicago*.

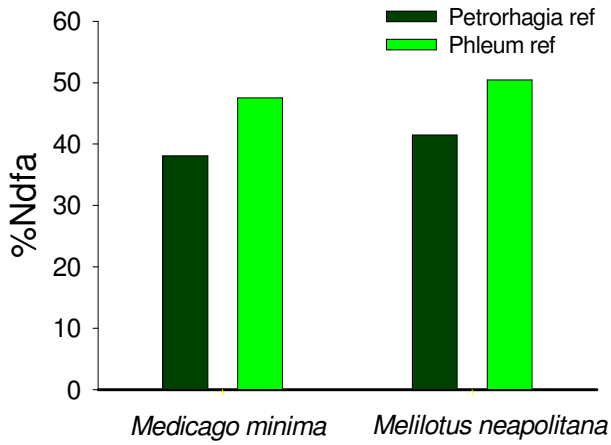


**Figure 5-9.** Value of %Ndfa, nitrogen derived from $N_2$ fixation expressed as percentage, calculated using $^{15}N$ natural abundance and the two reference plants separately.

Overall without giving any preference to any of the two techniques, the leguminous plant we studied seem to fix a percentage of N which goes from 24 to 50% in *Melilotus neapolitana* and from 30% to 47 % in *Medicago minima*. Thus a significant part of the plant N derives from mineral N in soil. In **Fig 5.10** is reported an image of *Medicago minima* and a detail of the nodules. These are very small, 1-2 mm and mostly concentrated on the first 3-4 cm of the main root. Much smaller than nodules typically occurring in crop leguminous plants or in other plants we found in macchia with much lower cover density, ex. *Lathyrus clymenum* (**Fig 5.11**).

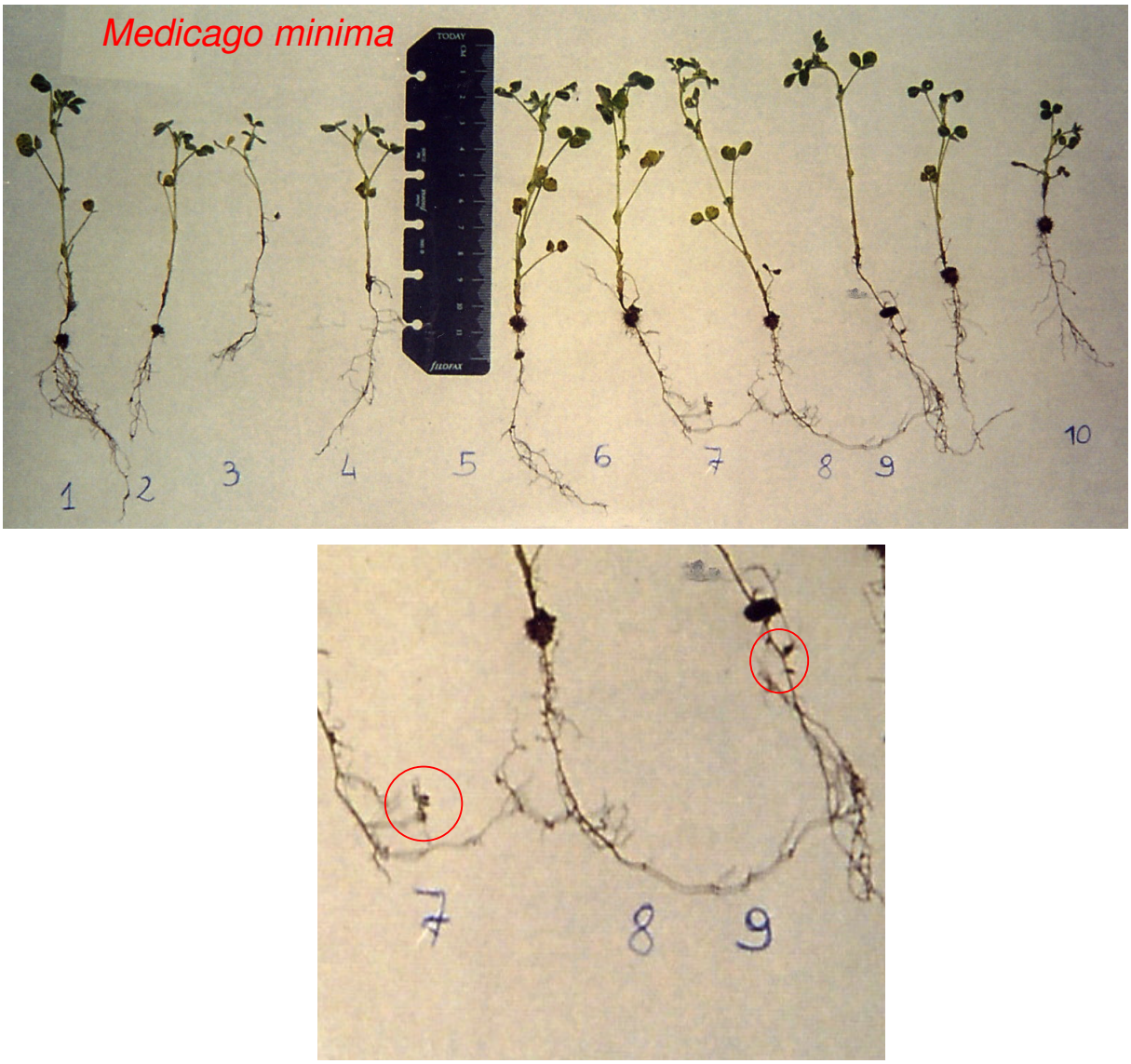


**Figure 5-10.** Plants of *Medicago minima* with nodulated roots (detail in red circles) on which analyses have been done.


**Figure 5-11.** Plants of *Lathyrus clymenum* with nodulated roots (detail on the right).

### 5.3.2 FIELD CAMPAIGN 2006: N POOLS AND MICROBIAL PROCESSES

#### *5.3.2.1. PLANT BIOMASS*

As indicated in the paragraph 5.2.3, 18 plots were identified, how which 9 plots were distributed in areas which appeared dominated by leguminous plants and 9 plots by non $N_2$-

fixing herbaceous species. In May 2006, at biomass peak, a destructive sample of plants was taken, one from each plot to determine the amount of biomass of each species present in the plot, in collaboration with the Department of Life Sciences, SUN (Esposito A. and Caporaso S.). An area corresponding to a circle of 15 cm diameter was sampled, at a depth corresponding to about 30-50 cm depending on the sample, so to collect the whole plant biomass including the below ground part. Plant were gently separated from the soil in the field, an washed in the lab. They were then one dried (45°C) and the above and below ground biomass weight was determined. For the purpose of the present study results are represented as total biomass of leguminous or non fixing herbaceous plant or mosses, present in each plot sample (**Fig. 5.12**).

The first clear result is that each plot resulted a mosaic of species, always including a certain amount of each of the tree plant cover types. The dominance resulted different in the different plots. One exception were plots 3 and 18 where a complete absence of $N_2$-fixing was reported. In some plots, the moss component was bigger than the other two components.

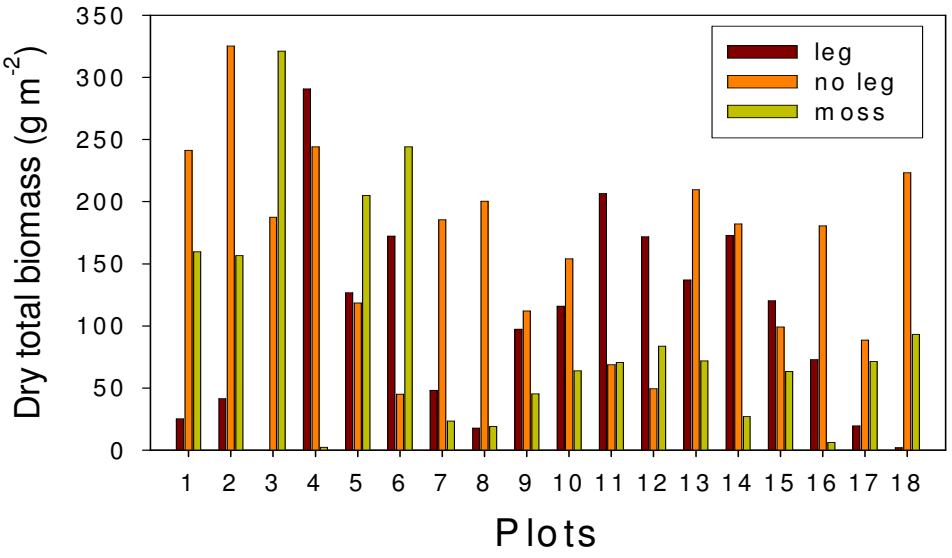


**Figure 5-12.** Dry total biomass (g $m^{-2}$) for three components collected in each plot: 1) leguminous $N_2$-fixing; 2) non $N_2$-fixing herbaceous species; and 3) moss-

Given the high variability of the plant cover in the different plots, in order to compare the effect of leguminous presence, it was decided to assemble different plots together on the base on plant cover dominance or leguminous biomass distribution. These are represented in **Fig. 5.13**. In the first case plots were grouped on the base to their percentage of leguminous plant cover <20% or 20%<x<40% or >40%; in the second case, plots were grouped on the base of biomass distribution of leguminous plants (>3g, 1.5g<x<3g, <1.5g), in the sampled rings.

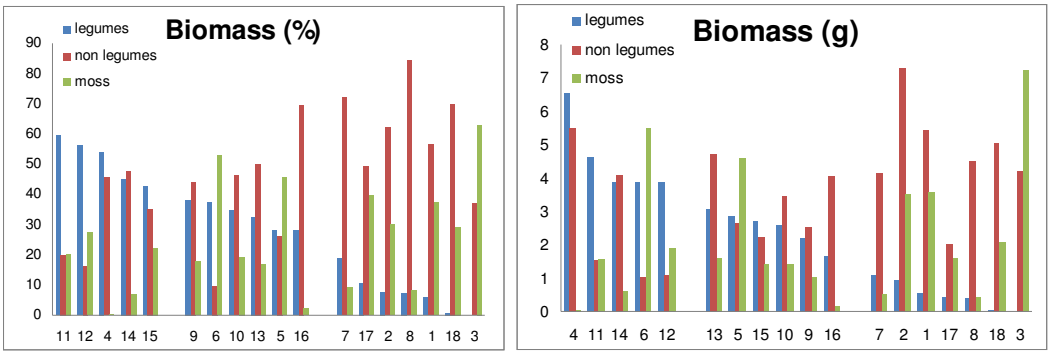


**Figure 5-13.** Redistribution of 18 plots in three main groups. In the left, plots were grouped on the base to their percentage of leguminous plant cover <20% or 20%<x<40% or >40%, on the right, plots are grouped on the base of biomass distribution of leguminous plants (>3g, 1.5g<x<3g, <1.5g).

Total biomass of herbaceous plants varied from about 200 to 300 g $m^{-2}$ (**Fig. 5.12**). Leguminous biomass varied from about 300 to 0 g $m^{-2}$. The % cover of fixing and not fixing plant showed opposite trend (**Fig 5.13**), mosses were instead randomly associated to the two cover types.

### *5.3.2.2. SOIL PARAMETERS*

Using the same approach described for the plant biomass, all the soil measured parameters, as well as gas fluxes, were grouped in 3 main groups.

Overall, soil ammonium content (**Fig. 5.14**), under herbaceous plant cover, resulted higher (up to 6.6 µg $NH_4^+$-N $g^{-1}$ dry soil) in sampling events of October and September, when decomposition of dead plant is proceeding faster, stimulated by late summer, autumn rains. No significant differences among the three groups, using both approaches, was evidence by two-ways ANOVA.

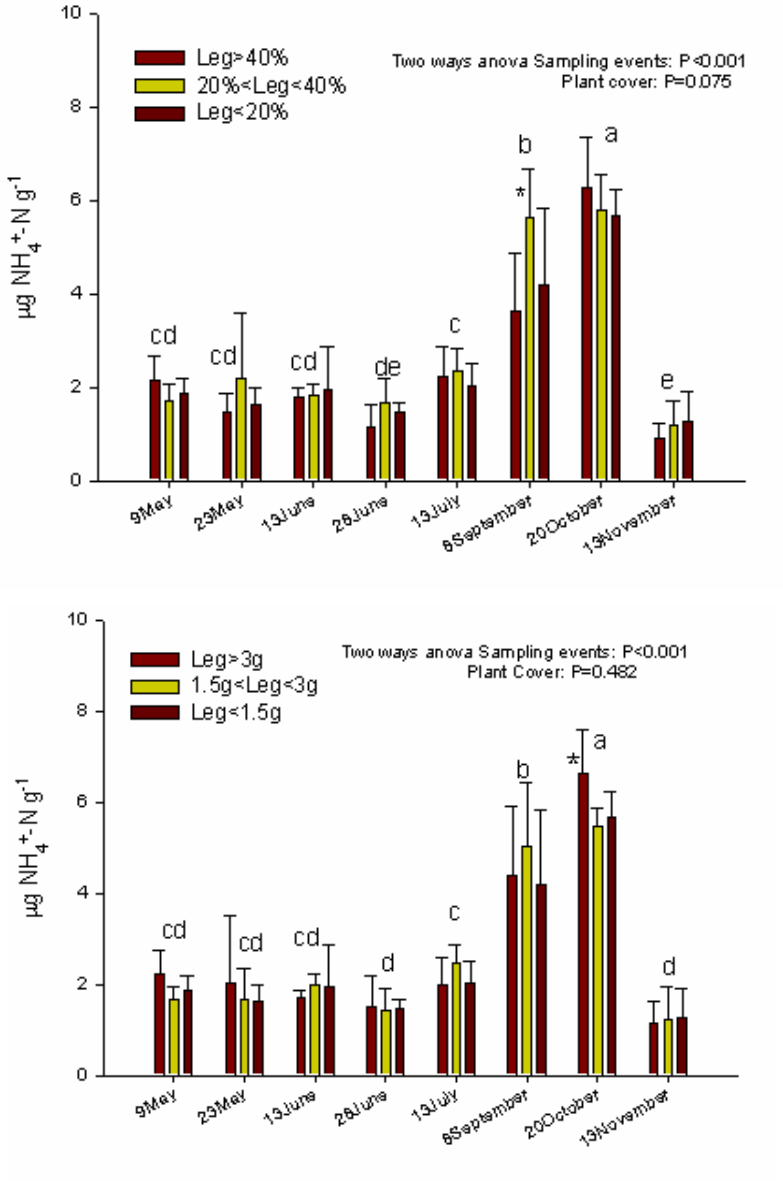


**Figure 5-14.** Soil ammonium ($NH_4^+$) content in different sampling dates (avg ± 1 st dev). Different letters indicate significant differences in time, asterisk indicate a significant difference of a single group within one single date.

Soil nitrate content (**Fig 5.15**) was the mineral N form which predominated in the site (10 folds higher than N-$NH_4^+$). The highest amounts (57.7 µg $NO_3^-$-N $g^{-1}$ dry soil ) of nitrate were measured in July, intermediate values on previous sampling months and lowest amount were measured on October and November. The plots with the highest biomass of leguminous plants showed higher values of nitrate, although only in one occasion the result was statistically significant (P=0.004) (**Fig.5.15b**).

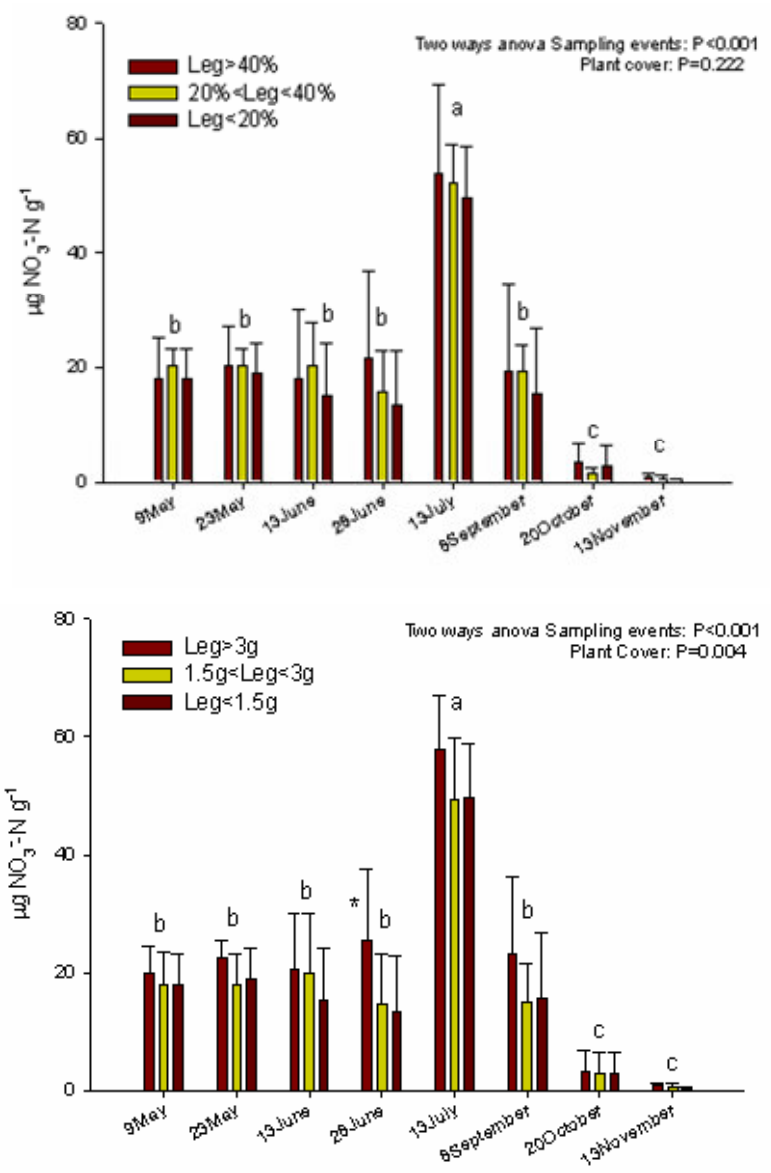


**Figure 5-15.** Soil nitrate ($NO_3^-$) content in different sampling dates (avg ± 1 st dev). Different letters indicate significant differences in time, asterisk indicate a significant difference of a single group within one single date.

Also soil organic α-$NH_2$ (**Fig.5.16**) was not different under the different groups, and had a less clear seasonal trend, with highest values at the beginning of may at peak of plant biomass and lower values at the end of May. The parameter showed a very high spatial variability.

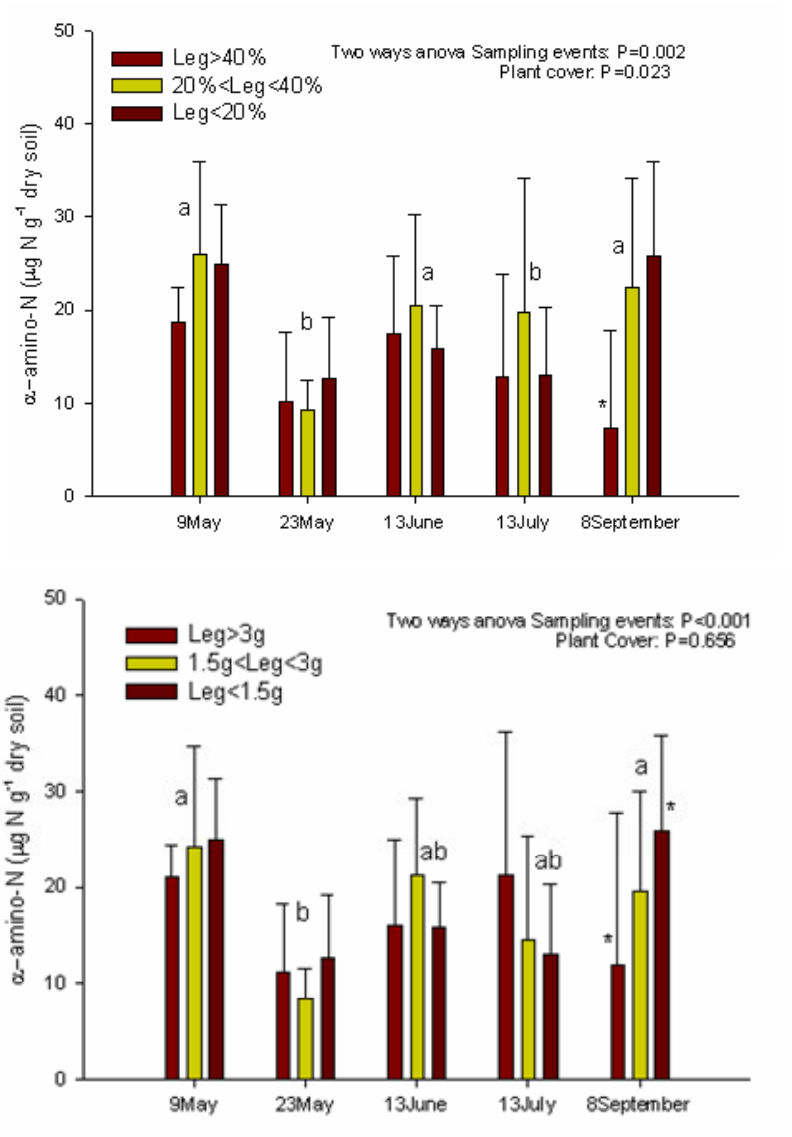


**Figure 5-16.** Soil organic α-$NH_2$ content in different sampling dates (avg ± 1 st dev). Different letters indicate significant differences in time, asterisk indicate a significant difference of a single group within one single date.

N mineralization and nitrification rates (**Fig. 5.17**) showed similar trend, with lower rates on June and October, and higher in others sampling events. The high spatial variability obscured possible significant differences between groups with different cover of leguminous.

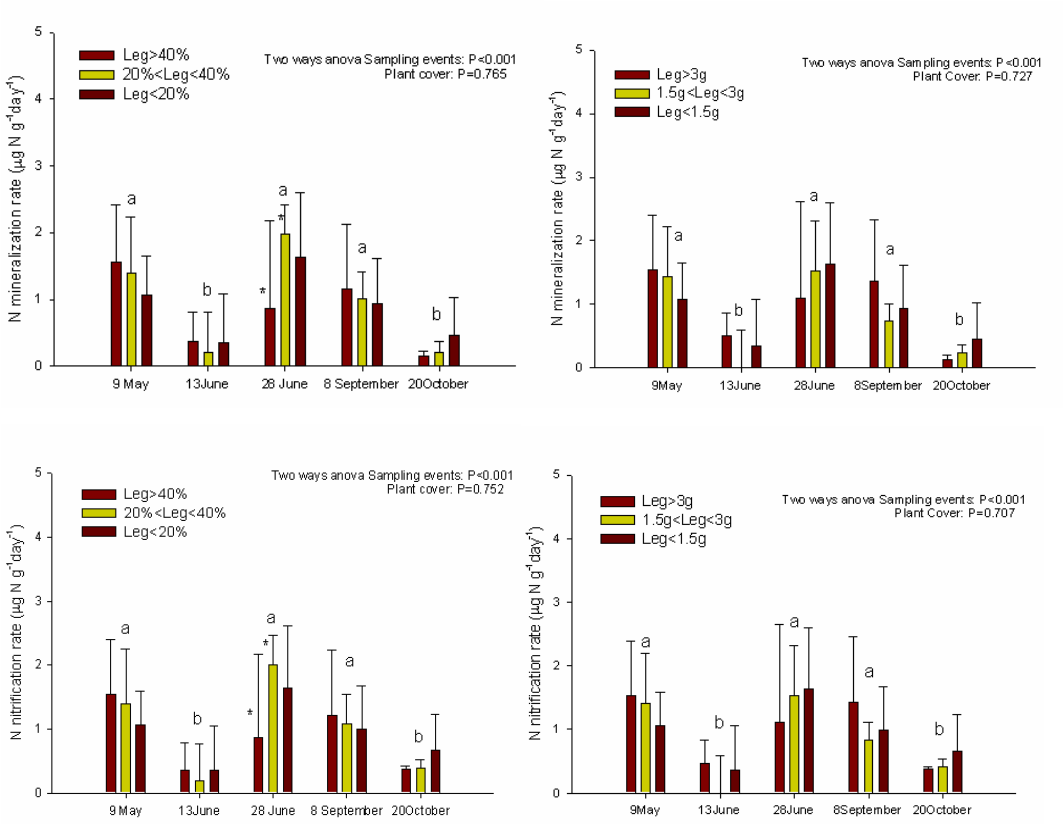


**Figure 5-17.** N mineralization, and nitrification rates in different sampling dates (avg ± 1 st dev). Different letters indicate significant differences in time, asterisk indicate a significant difference of a single group within one single date.

Denitrification enzyme activity (DEA) (**Fig. 5.18**) was similar from May to October with a single higher value measured in July. No differences among groups were observed, although as a general trend the highest the biomass of leguminous the lower was the DEA.

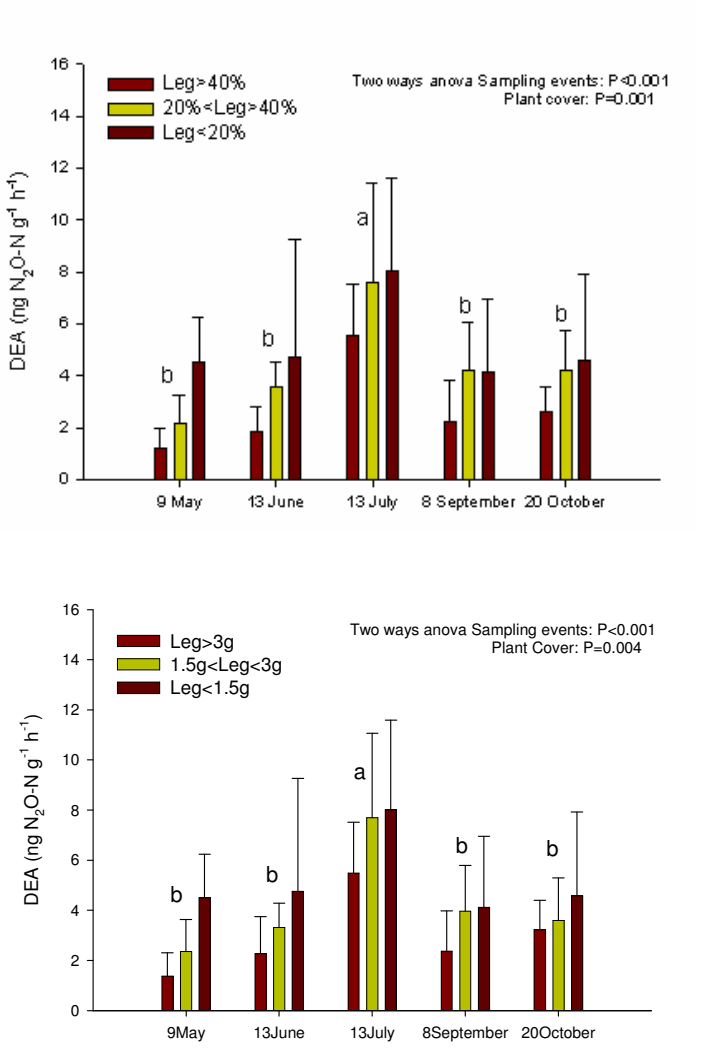


**Figure 5-18.** Denitrification enzyme activity (DEA) for each event of sampling, according two ways of representation (avg ± st dev).

Overall, the significant increase of DEA was associated to a significant increase of $NO_3$ (**Fig 5.19**) according the equation reported in the graph. In the plots with major percentage or major amounts of $N_2$-fixing species, at high nitrate content correspond low denitrification activity.

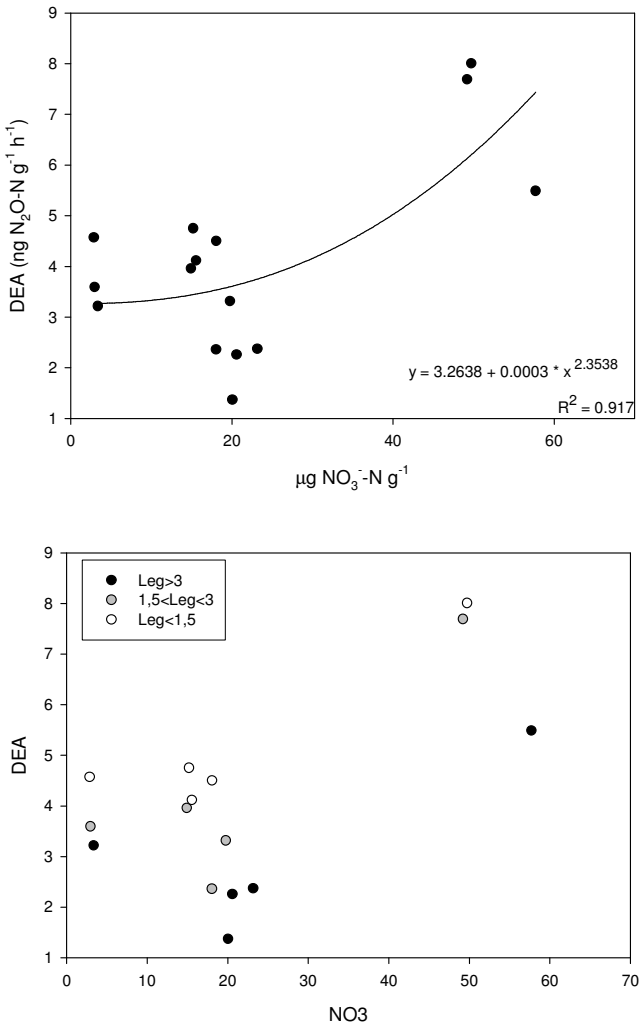


**Figure 5-19.** Denitrification enzyme activity versus $NO_3^-$

## 5.3.3 GASES FLUXES

### *5.3.3.1. NITROUS OXIDE*

$N_2O$ fluxes were extremely low in all sampling events (**Table 5.3**), never reaching 1g $m^{-2}$ $day^{-1}$, even in the period when significant mineral N and denitrification potential activity was measured. The enormous variability did not allow to identify significant difference among groups or dates. The highest values were measured when the soil was wetter.

**Table 5-3.** $N_2O$ fluxes measured from field plots during 5 sampling events. (Avg ± 1stdev).

| Groups | $N_2O$ (mg $m^{-2}$ $day^{-1}$) | | | | |
|---|---|---|---|---|---|
| | 9th May 2006 | 23th May 2006 | 29th Sept 2006 | 20th Oct 2006 | 13th Nov 2006 |
| Leg>3g | 0.017±0.038 | 0.169±0.210 | 0.061±0.086 | 0.000±0.000 | 0.055±0.088 |
| 1,5g<Leg<3g | 0.084±0.134 | 0.064±0.132 | 0.000±0.000 | 0.000±0.000 | 0.268±0.362 |
| Leg<1,5g | 0.026±0.061 | 0.089±0.224 | 0.258±0.365 | 0.080±0.113 | 0.233±0.342 |

### *5.3.3.2. METHANE*

A $CH_4$ sink was observed in all events of sampling. Consumption rates were quite high during all the year with a peak in October. No significant differences were observed among groups of plots with different cover of N2-fixing species.

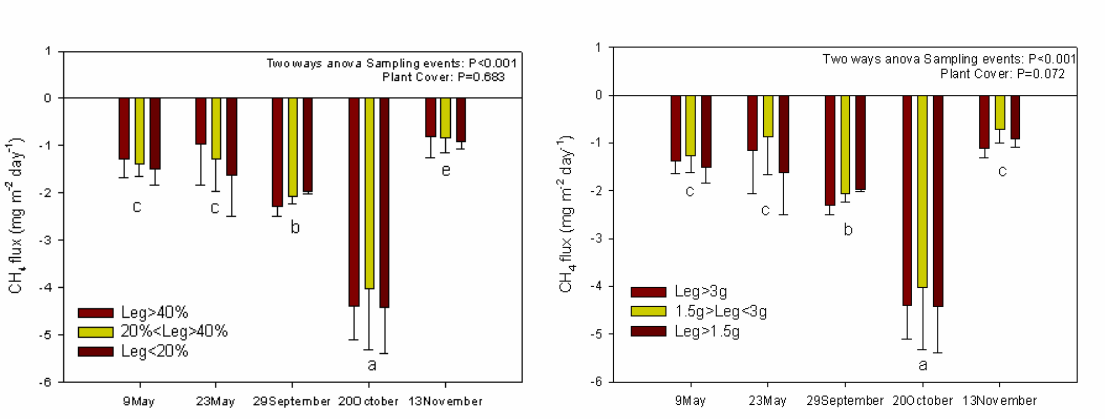


**Figure 5-20.** $CH_4$ flux in different sampling dates (avg ± 1 st dev). Different letters indicate significant differences in time, asterisk indicate a significant difference of a single group within one single date.

## 5.4 DISCUSSION

Leguminous herbaceous plants were present in the studied Mediterranean grassland with a cover density from 0 to >90%. The most abundant leguminous plants were *Medicago minima* and *Melilotus neapolitana*, which were used as representative of $N_2$-fixing species due their frequency and abundance in the studied areas. The percentages of $N_2$-fixation obtained using two different methodology was enough in agreement, given the very different appoach and assumption used ofr these two techniques. Overall, without giving any preference to any of the two techniques, the leguminous plants we studied seemed to fix a percentage of N which goes from 24 to 50% in *Melilotus neapolitana* and from 30% to 47 % in *Medicago minima*. Thus, a significant part of the plant nitrogen derives from mineral N in soil. Given a biomass peak of about 300 g $m^{-2}$, with an average N content of 4%, it can be calculated that the amount of N entering the system via $N_2$-fixation was between 28 and 60 kg of $N\text{-}N_2$ $ha^{-1}$ (total biomass). This N would reach the system, once that plants dye and decompose, and via root exudates.

Higher ranges of $N_2$ fixation have been reported by *Casals et al. (xxx)* in a Mediterranean grassland community developed after fire, with values of fixation ranging from 52% to 99% during the first 9 months after fire. Also values in croplands are generally reported to be higher. Narrow-leaf lupin for example has been found to derive up to 86% of its N content via $N_2$ fixation (*Unkovich et al., 1994*). The lowest levels of fixation (78%) were reported when soil was fertilized with 100 kg N/ha. Ranges of fixation for subterranean clover have been found in the order of 85-94% (*Bergersen and Turner 1983, Sanford et al. 1995*). Interestingly it has been observed that in absence of fertilizer N, the %Ndfa of subterranean clover was 79% but with addition of 50 to 100 kg fertilizerN/ha the %Ndfa fell to 48 amd 32%, respectively (*Quigley and Peoples. 1995*). This variation suggests that plants can significantly modify their $N_2$-fixation rates depending on the source of available N. In extreme cases in grazed pastures the %Ndfa of subterranean clover was found to be close to zero, i.e. complete dependence on mineral N. Medics (*Medicago* spp.) have been found to have a wide range of %Ndfa varying from a low 7% in grazed pastures to 71% (*Sanford et al. 1994*). *M. trunculata* was found to have low symbiotic tolerance of nitrate (*Butler and Ladd 1985*), showing greatly reduced nodulation in presence of early season $NO_3^-$.

The range of observed %Ndfa for the representative $N_2$-fixing plants at Castel Volturno seemed, hence, lower compared with many reported cases of undisturbed, or cultivated areas not receiving extra N. This means that from 70 to 50% of the N in the plants derives from soil mineral N. Considering the relatively low amount of total N present in this Mediterranean soil, it can be concluded that the studied $N_2$ fixing plants are very efficient to uptake mineral N, competing with other non fixing species and microbes.

The amount of N which enters this studied ecosystem via $N_2$ fixation is much lower compared with values reported for leguminous crops (*Unkovich et al. 1997*), however it still represents a significant source of N (from 28 to 60 kg of N-$N_2$ $ha^{-1}$). As a matter of fact, although we have tried to sample leguminous plants from areas mostly composed by leguminous cover, in natural grassland it is very difficult to obtain homogeneous layer of the same species, as instead we can have in croplands. This means that in most cases, as also evidenced in the second year of experiments, leguminous plants will be mixes with non – fixers. When legume and reference plants are grown in close association, a direct transfer of fixed N of near zero delta value could occur from leguminous to companion reference-specie via mycorrhizas interactions, which would lead to a lowering of the reference plant delta, below that of soil mineral N, with consequent under-estimation of the %Ndfa (*Unkovich et al. 1997*). In fact, the values of delta in reference plants, we found, were quite close to those of $N_2$ fixers. Also, plant growth in successive seasons will lead to have reference plants which might grow on soil where $N_2$ fixers have occurred the previous year. Thus references might exploit a mineral N pool where the delta signal is lowered by the entrance, via decomposition, of leguminous fixed N. This means that in natural environments the use of the $^{15}N$ natural abundance technique is much more difficult and subject to errors and under-estimation than in cultivated soils where leguminous can be planted at high density in areas which never hosted $N_2$ fixers before. Despite these considerations, the value of %Ndfa found using $^{15}N$ natural abundance was comparable to that found using the "N difference" technique. Thus, this value of %Ndfa, might be close to the real estimate. In fact, photos show very little nodulation in the roots of the analysed leguminous plants, but also, data from the second experiment did not really show significant differences of N pools and processes under different plant cover distribution. This suggests that the amount of extra N which arrives in the soil with the tissue of leguminous plants, where the % of N is higher than in the reference, is only in part a real external N input, the rest is due to exploitation of soil mineral N by the leguminous plants. In

fact, at the end of the growing season, when plants dyed (early summer), the amount of extra N which arrived to the soil did not seem sufficient to push soil processes, as in fact mineralization, nitrification and denitrification rates were not found to be significantly different under the three cover types. Slightly more mineral N was observed in some cases in the plots reached in leguminous plants. Probably the little excess of leguminous N entering the soils was: a) not fully exploited because limited by some other factors, most probably soil water content, b) the excess of N could be immobilized by microbes during the same decomposition process, being this soil quite N limited. Thus the overall effect of a higher N percentage being released from leguminous plant tissue and higher N being immobilized by microbes would result in un-significant variation of the net amount of N in soil under leguminous compared with areas covered by reference plants.

Almost of the plots presented a very high percentage of non-fixers, thus the effect of extra N in tissue of leguminous plants arriving to the soil was "diluted" by the presence of non-fixing plants. Maybe in order to have a much clear evidence of the effect of leguminous plant cover, a more homogeneous community would be needed, which is not the case in wild environments, with few exceptions. Similar results were found by *Scherer-Lorenzen et al (2003)* who studying a mid-European grassland observed that in the communities containing legumes, N losses decreased with increasing diversity, because higher species richness leaded to complementary uptake of extra mineralized N by non leguminous herbs. *Fillery (2001)*, in a review on the fate of biologically fixed nitrogen in legume based drylands farming systems, concluded that losses of mineralized N from leguminous residues by either leaching of $NO_3^-$ or denitrification are small in Mediterranean ecosystems, where the soil organic matter pool (abiotic and microbial biomass) is the main sink of this extra N. Indeed in the present experiment both $NO_3^-$ content and $N_2O$ emissions did not statistically differ among the different plant cover. This might be the result of low $N_2$ fixation, high plant diversity in the plots and limited soil water content for most of the period.

Also $CH_4$ fluxes did not seem to be affected by the different plant cover. Extra N in form of $NH_4^+$ might have exerted some lowering activity on $CH_4$ uptake. However, data show that: 1) mineral N was generally low and was immediately oxidised to $NO_3^-$, which was the predominant form of mineral N; b) that no difference in N mineralization occurred under different plant cover density. So $CH_4$ flux data are in agreement with these results. Overall, as

previously reported for this site (*Castaldi and Fierro, 2005*) $CH_4$ uptake rates were very high compared with other Mediterranean sites (see also chapter 3).

## 5.5 CONCLUSIONS

Overall data indicate that the investigated leguminous plant in the Mediterranean grassland of Castel Volturno represent a lower input of extra N in the system than expected on the base of literature data. This means that most of the N in these plants is recycled within the ecosystem.

As a consequence of the low $N_2$ fixation rates, high plant diversity and limited soil water content N losses, is form of $NO_3^-$ or gaseous products, associated to the presence of leguminous plants can be assumed to be negligible in this Mediterranean grassland.

# 6 POTENTIAL EMISSIONS OF NO AND $N_2O$ FROM MEDITERRANEAN ECOSYSTEMS: COMPARISON IN FUNCTION OF SITE CHARACTERISTICS

## 6.1 INTRODUCTION

Nitric oxide and nitrous oxide are two gases which are produced by soil microorganisms involved in the nitrogen cycle. They can make a significant fraction of total N gaseous loss from terrestrial ecosystems. The environmental impact of nitrous oxide is mainly related to its greenhouse gas effect, having this gas a radiative warming potential 310 times higher than $CO_2$ (120 year projection, mass base, *IPCC 2007*), whereas NO is involved in tropospheric chemical reactions and is considered a source of indirect emissions for $N_2O$ (*IPCC 1998*). Both gases are by products of the nitrification process and are intermediate products in the denitrification process (*Firestone and Davidson, 1989; Williams et al. 1992; Conrad, 1996*). Reported key controlling factors for NO and $N_2O$ emissions in terrestrial ecosystems are availability of substrate, gas diffusivity and $O_2$ concentration in soil pores, both strictly related to the water filled pore space (WPFS), soil pH, soil temperature (*Slemr and Seiler, 1984; Skiba et al., 1997; Yamulki et al., 1997; Skiba et al., 1998; Smith et al., 1998; Bollmann and Conrad, 1998; Ormeci et al., 1999; Van Dijk and Duyzer, 1999; Skiba and Smith, 2000; Ludwig et al., 2001*). Nitrification is mainly led by aerobic autotrophic microorganisms, and consequently the process rate is mainly controlled by the availability of $NH_4^+$ and by soil aerobicity. In natural ecosystems hence nitrification generally correlates with mineralization and ammonification rates (*Granli e Bøckman, 1994*). Also soil pH, however, seems to affect significantly the rate of nitrification. Denitrification is carried out by heterotrophic anaerobic microorganisms, and hence is mainly controlled by $NO_3^-$ and $NO_2^-$ availability, soil C content and quality, and soil aerobicity. In upland soils the denitrification process occurs in "hotspot" characterized by intense respiration activity associated to available C, which creates anaerobic spots within an aerobic soil matrix. The $NO/N_2O$ stechiometric ratio can change significantly

in function of soil characteristics, given that these gases are emitted from two different sources, which are differently controlled by environmental parameters. The best known and frequently reported controlling factor is the soil water content. Usually NO emissions are reported to peak around 5-20% of water filled pore space, to decline exponentially thereafter till an almost complete disappearance at about 60% WFPS. On the other hand, $N_2O$ is generally reported to be very low at water content below 60%, where only nitrification is the dominant source, whereas it increases exponentially above 60% WFPS, peaking at about 90% WFPS, having as the main source the denitrification process (*Lemke et al., 1998*). Thus the ratio NO/$N_2O$ decreases significantly with increasing WFPS. An equal good amount of literature is available for the effect of exogenous substrates (synthetic or organic fertilization) on NO and $N_2O$ emission rates in agricultural ecosystems (*Dick et al., 2001; 2006; Vallejo et al., 2005; Sánchez-Martín et al., 2008*). Much less information exist on factor controlling potential NO and $N_2O$ emissions in natural ecosystems, especially regarding NO emissions. Most of the significant literature on NO emissions comes from tropical seasonally-dry ecosystems (*Davidson et al., 1993; Matson and Vitousek, 1996; Hall and Matson, 1999*), whereas few data exist on NO and $N_2O$ emissions from seasonally-dry temperate forest and shrublands, in particular in Mediterranean ecosystems. A frequently reported observation, from studies in tropical savannas, is the production of flushes of NO and $N_2O$ at the off-set of the rainy season, although no univocal answer has been given for which factors control the length and magnitude for this N gaseous flush (*Austin, 2004*). Mediterranean woodland are also exposed to significant periods of strong aridity, when the top soil, where most of the microbial activity takes place, reaches soil water contents as low as the wilting point. Isolated rain events often interrupt these dry periods, with a frequency and intensity which is quite variable. However, in the recent year, summer and autumnal rains are getting more and more characterized by less frequent and more intense events. Hence also Mediterranean ecosystems are probably interested by frequent and significant pulses of N gas emissions.

In the present work we compared the potential emissions of NO and $N_2O$ in several Mediterranean woodlands with different soil characteristics in order to improve our understanding on the influence of ecosystem characteristics on: a) the magnitude of NO and $N_2O$ fluxes and their ratio; b) the length of the gaseous pulse following soil rewetting.

The experiment was carried on in controlled conditions of laboratory, by incubating the soil in reconstituted cores at the same temperature and soil water content. This allowed to get

independent from these two controlling factors, which influence on gas emissions is already well known, and to investigate instead the effect on gas emissions of intrinsic characteristics of the investigated soils such as availability of substrate, soil pH, texture, soil organic C and N, net rates of mineralization and nitrification, denitrification enzyme activity and other peculiar environmental factors associated to the Mediterranean sites. In this respect we compared four different woodlands characterized by different soil types and vegetation, and for one site we also used a typical chronosequence of Mediterranean ecosystem after disturbance (open herbaceous community-shrubland-woodland). We also tested the potential of these natural ecosystems to respond to extra nitrogen addition in function of their soil characteristics, by fertilizing the soil with $NH_4NO_3$.

## 6.2 MATERIALS AND METHODS

### 6.2.1 SITES OF STUDY DESCRIPTION

Four Mediterranean woodland sites dominated by of evergreen broadleaf species were chosen for the present work Lecceto (L), Tolfa (T), Roccarespanpani (R) and CastelVolturno (CV).

All the sites were located in the Italian peninsula. **Lecceto (L)** is a 30 years old coppice site located at 305 m (a.s.l.) close to Siena (Toscany) (43°3' N; 11°29' E), characterized by Mediterranean climate with warm summer (June – September) (average annual precipitation 870mm, average air temperature 12°C). The soil is classified as Xerochrept (Soil Taxonomy, 1975). The main dominant arboreus species is *Quercus Ilex* (average plant canopy height 8-9 m), with few isolated trees of *Quercus Robur, Quercus Cerris, Fraxinus Ornus*. **Roccarespampani (R)** is a 20 years old coppice site dominated by *Quercus cerris* (other isolated trees of *Ruscus, Crataegus monogina*, overall less than 15% cover) located at 120-190 m a.s.l. in a flat area close to Viterbo (42° 23' N; 11° 51' E). The soil is a Cambisol (Volcanic tuff is parent material) with a sandy clay texture. The total annual precipitation is 936 mm, the mean annual temperature is 14.4°C. **Tolfa (T)** site is located not far from the Tyrrenian coast in central Italy (42°11'N 11°56'E) in a flat central plateau located at 220m

a.s.l.. The area is characterized by a typical Mediterranean climate, a mean annual precipitation around 650 mm and a mean annual temperature of 15°C. The site is a coppiced woodland of *Arbutus unedo, L.* (65%-90% cover). *Erica arborea, L.* (13% cover) is also well represented at the site, while other species as *Fraxinus ornus, L.* (8%), *Quercus pubescens, Willd.* (5%) and *Quercus cerris, L.* (4%) are present as isolated trees. The geology of the site is characterized by the presence of emerging eruptive deposits that, by the action of hydrothermal processes, gave origin to alum mineral beds. The soil is classified, according to the USDA system, as an Andisol. The **CastelVolturno** site, part of a Nature Reserve, is a flat coastal area located about 30 km North of Naples, in Southern Italy (40° 57'N; 1° 33'E). The climate is typically Mediterranean, with a total annual precipitation of 760.3 mm and mean annual temperature of 15.8°C. Soil is classified as a Calcaric Arenosol (FAO, 1998). In this site three different successional stages, typical of a Mediterranean secondary succesion, following disturbance, were identified: **(CVg)** grassland community typical of open areas, which follows a fire event (about 10 years ago) and is kept open by the Reserve operators by means of cutting; **(CVs)** a shrubland community about 2-3 meters tall, which represents an intermediate successional stage, characterized by a continuos plant cover dominated by *Quercus ilex L., Phillyrea angustifolia L., Pistacia lentiscus L., Cistus spp, Myrtus communis L., Rosmarinus officinalis L.,*; **(CVw)** a climax woodland stand of *Quercus ilex L.*. The main soil characteristics for the six study areas are reported in **Table 6.1**.

**Table 6-1-.** Main soil characteristics (± 1 stdev) in the investigated sites.

| **Site** | Soil pH | Total C g C $100g^{-1}$ dry soil | Total N g N 100 $g^{-1}$ dry soil | Water Holding Capacity g $g^{-1}$ | Bulk density g $cm^{-3}$ |
|---|---|---|---|---|---|
| **CVg** | 6.75 ± 0.14 | 2.41 ± 0.57 | 0.32 ± 0.03 | 0.375 ± 0.031 | 1.333 ± 0.132 |
| **CVs** | 6.84 ± 0.10 | 3.85 ± 0.48 | 0.41 ± 0.03 | 0.458 ± 0.115 | 1.133 ± 0.143 |
| **CVw** | 7.25 ± 0.15 | 6.98 ± 1.36 | 0.49 ± 0.07 | 0.435 ± 0.075 | 1.194 ± 0.015 |
| **T** | 3.42 ± 0.08 | 10.75 ± 2.78 | 0.78 ± 0.36 | 1.488 ± 0.143 | 0.429 ± 0.028 |
| **L** | 6.24 ± 0.06 | 10.20 ± 2.36 | 0.49 ± 0.14 | 0.549 ± 0.164 | 0.949 ± 0.079 |
| **R** | 5.82 ± 0.17 | 3.32 ± 0.72 | 0.38 ± 0.08 | 0.443 ± 0.119 | 1.029 ± 0.115 |

### 6.2.2 EXPERIMENTAL SET-UP

In the present work we compared NO and $N_2O$ emissions and related soil N processes from four Mediterranean evergreen woodlands: Lecceto (L), Roccarespampani (R), Tolfa (T) and Castel Volturno (CVw); we also compared at one site (CastelVolturno) three successional stages: open grassland areas (CVg), shrubland (CVs), mature stand of Quercus ilex woodland (CVw). At each site soil was sampled from the top 0-10 cm from 10 different spots located on a grid of 1m length. Soil subsamples were well mixed and sieved with a 2 mm mesh sieve. On the same day soil was oven-dry at 37 °C using a ventilated oven to speed up the process of water loss, which occurred within 48 hours.

Soil samples of 100 g each were repacked into clear Perspex columns (20 cm height, 5.5 cm inner diameter) sealed at the base. Soil was left to accumulate by gravity without further manual compression, just avoiding the occurrence of empty spaces. For each column the exact soil height was recorded in order to calculate the correct headspace volume.

All the soil samples were incubated at 50% of water saturation, at 20°C, conditions which could typically occur during the early autumn period at the offset of rain. In order to simulate the effect of rain on gaseous emissions from dry soils, condition which typically occurs in the soil top centimetres during the dry Mediterranean summers, we did not pre-incubate the soil in moist conditions.

Two series were compared: the five Mediterranean soils (L, R, T, CVw, CVs, CVg) amended with sole water, and the same five soils amended with water plus $NH_4NO_3$ (100 μg N $g^{-1}$ dry soil). This second set was investigated in order to evaluate the potential of each of the studied soils for NO and $N_2O$ emissions in conditions where N is not limiting. In fact, in our natural soil the main source of N is represented by the ammonium produced by the mineralization and the $NO_3$ produced in the subsequent nitrification process. So the rate of these two processes controls the rate of gaseous losses. The two series hence represent the actual capacity of the soils to emit NO and $N_2O$ at the set water and temperature conditions (no N addition) and the potential capacity of the soils to emit NO and $N_2O$ in presence of extra N (+ N addition). For each soil and each treatment we used three experimental replicates for the analysis of N gaseous emissions.

NO and $N_2O$ production was measured 1hour, 3 days, 7 days and 10 days after water addition, as described in the following paragraph. Soil cores were left uncapped in between gas sampling events and eventual water losses were corrected by weighting the soil cores everyday and adding the lost water.

As we were also interested to measure the amount of mineralized and nitrified nitrogen corresponding at the same moment of gas sampling, we prepared a parallel set of soil cores which were not used for gas analysis but were exclusively used for the extraction of mineral N, determination of soil water content and denitrification enzyme activity. So for each soil and for each treatment we prepared a set (on triplicate) to be extracted 1 hour, one set at 3 days, one set at 7 days and one set at 10 days after water addition. This avoided the disturbance of soil cores where gas was going to be measured.

### 6.2.3 SOIL AND GAS ANALYSIS

Soil physical-chemical properties and soil biological processes related nitrogen cycle were measured in each sampling event. Soil water content, pH, organic carbon, total nitrogen and carbon, mineral nitrogen, organic $\alpha$-$NH_2$, mineralization rate, nitrification rate, denitrification enzyme activity and gases fluxes are described in detail in the chapter 2.

### 6.2.4 STATISTICAL ANALYSIS

A one way analysis of variance was used to evidence significant differences among different sites or among different treatments. When the difference was significant ($P<0.05$) an "all pairwise" comparison was carried on using the “Student-Newman-Keuls test”. Best subset regression analysis was used to find which linear combination of independent variables best contributed to predict the dependent variable. Simple linear regression or multiple linear regression were performed to find the relationship between the independent variables and the dependent variable. All the statistical analyses were performed using Sigma Stat package (Jandel Scientific).

## 6.3 RESULTS

### 6.3.1 GASES FLUXES ALONG A MEDITERRANEAN SUCCESSIONAL GRADIENT

In natural conditions, all of three stages of secondary succession, open grassland, shrubland and woodland of *Quercus ilex* showed higher NO emission rates than $N_2O$ (**Fig. 6.1**).

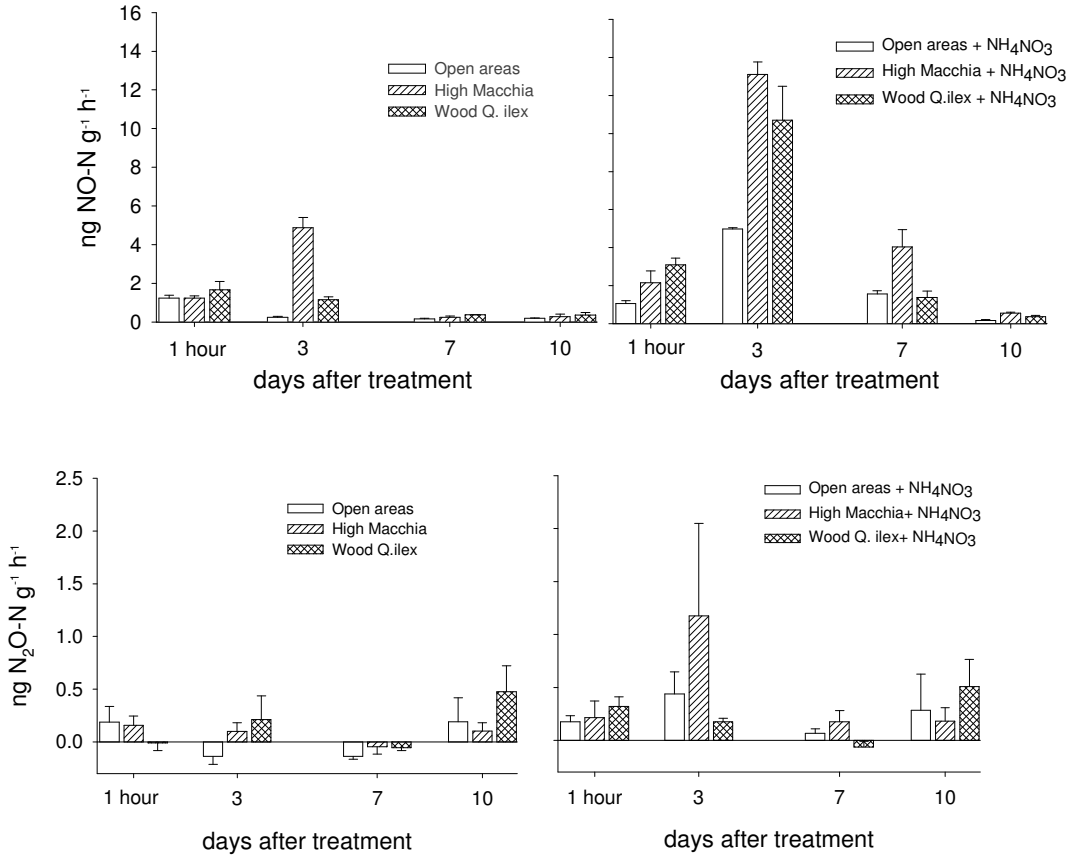


**Figure 6-1.** NO and $N_2O$ flux measurements without addition of $NH_4NO_3$ on the left and with addition of $NH_4NO_3$ on the right. Error bars represent standard deviation for three replicates.

No emission peaked at day 3 in the shrubland, an in the first day for the other two stages. After 7 days values were everywhere extremely low. $N_2O$ emissions were low and without a particular trend (Fig 6.1). In presence of extra-N, in all stages NO emissions increased, and NO peaks were observed after 3 days. After 10 days the extra flux went back to background conditions (without addition of mineral N). $N_2O$ fluxes also stimulated by N addition, althought to a much lesser extent. As for NO, also for $N_2O$, peaks of emissions were observed after 3 days, the highest being again in the shrubland.

### 6.3.2 GASES FLUXES FROM WOODLAND ECOSYSTEMS

Comparing the 4 woodland ecosystems, NO emissions without extra N were again higher than $N_2O$ emissions (**Fig. 6.2**) in all sites. The highest NO flux was generally recorded immediately after water addition. $N_2O$ emissions did not have a clear temporal trend, with the exception of Rocca. Adding extra-N, $N_2O$ strongly increased in two sites, Lecceto and Rocca, where they peaked at day 3. At Rocca $N_2O$ emissions were still significantly higher than in the other sites after 10 days, whereas at Lecceto the $N_2O$ flush did not last more than 3 days.

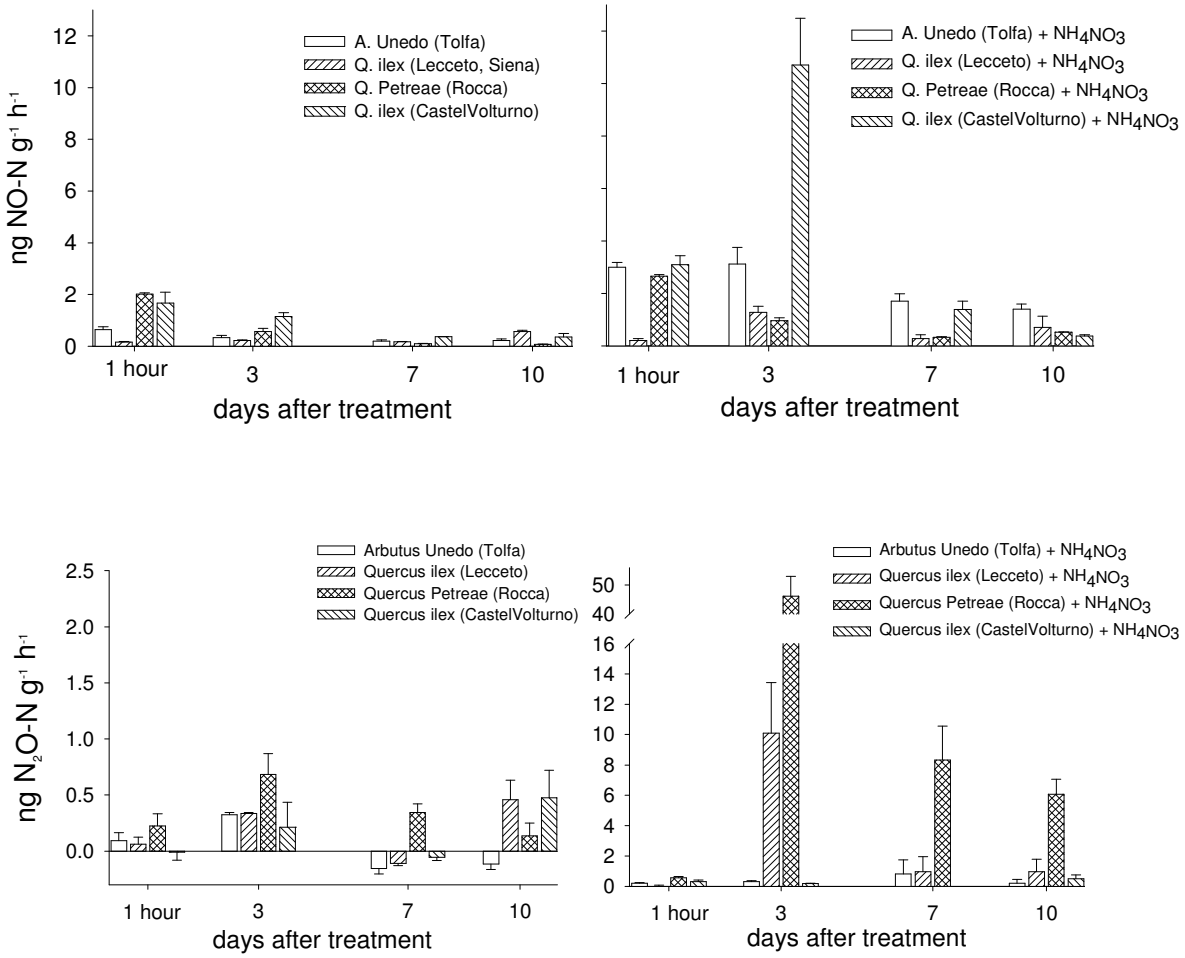


**Figure 6-2.** NO and $N_2O$ flux measurements without addition of $NH_4NO_3$ on the left and with addition of $NH_4NO_3$ on the right. Error bars represent standard deviation for three replicates.

At Tolfa and Castel Volturno NO emissions remained higher than $N_2O$ emissions, despite the higher overall gas production. CV presented the highest flux of NO, which occurred after 3 days but fastly decreased back to background levels. Overall the $N_2O$ emissions were quite low when no extra N was added (maximum value of 0.8 ng $N_2O$-N $g^{-1}$ $h^{-1}$) and were exceptionally high (45 ng $N_2O$-N $g^{-1}$ $h^{-1}$) and long lasting at Rocca when N was added. In **Table 2** are reported NO/$N_2O$ ratios for the analysed sites.

**Table 6-2.** NO-N/$N_2O$-N ratios calculated for the six soils from NO-N and $N_2O$-N emissions measured after 1 hour, 3, 7, and 10 days after wetting the soil with sole water (-N) or water plus $NH_4NO_3$ (+N). No value (-) indicates absence of negative $N_2O$ emissions.

| | 1 hour | | 3 days | | 7 days | | 10 days | |
|---|---|---|---|---|---|---|---|---|
| | - N | +N | - N | +N | - N | +N | - N | +N |
| **CVg** | 6.6 | 6.0 | - | 11.4 | - | 24.4 | 0.9 | 0.6 |
| **CVs** | 7.8 | 10.0 | 49.7 | 11.1 | - | 23.2 | 2.6 | 3.0 |
| **CVw** | - | 9.7 | 5.4 | 60.8 | - | - | 0.7 | 0.7 |
| **T** | 6.8 | 13.5 | 1.0 | 10.0 | - | 2.1 | - | 6.8 |
| **L** | 2.6 | 9.1 | 0.7 | 0.1 | - | 0.3 | 1.2 | 0.7 |
| **R** | 9.0 | 4.6 | 0.8 | 0.02 | 0.3 | 0.03 | 0.5 | 0.08 |

A clear dominance of the NO gaseous N form is evidenced immediately after water addition with fluxes of NO from 3 to 10 times higher than $N_2O$. In the CV site the ratio in general goes increasing with time. N addition increase this ratio only at Tolfa, where we know the limits for $N_2O$ production from chapter 3 and 4. In the other two woodland site the ratio instead decreases with N addition compared to –N treatments.

### 6.3.3 MINERALIZATION, NITRIFICATION AND DENITRIFICATION ALONG A MEDITERRANEAN SUCCESSIONAL GRADIENT

The highest mineralization and nitrification rates were measured in the mature stand of *Quercus ilex* (**Fig. 6.3**) When N was added, however, was the site which showed the highest immobilization rate. Difference were however never statistically significant. Maquis and shrubland instead behaved very similarly. In all cases N addition stimulated N immobilization.

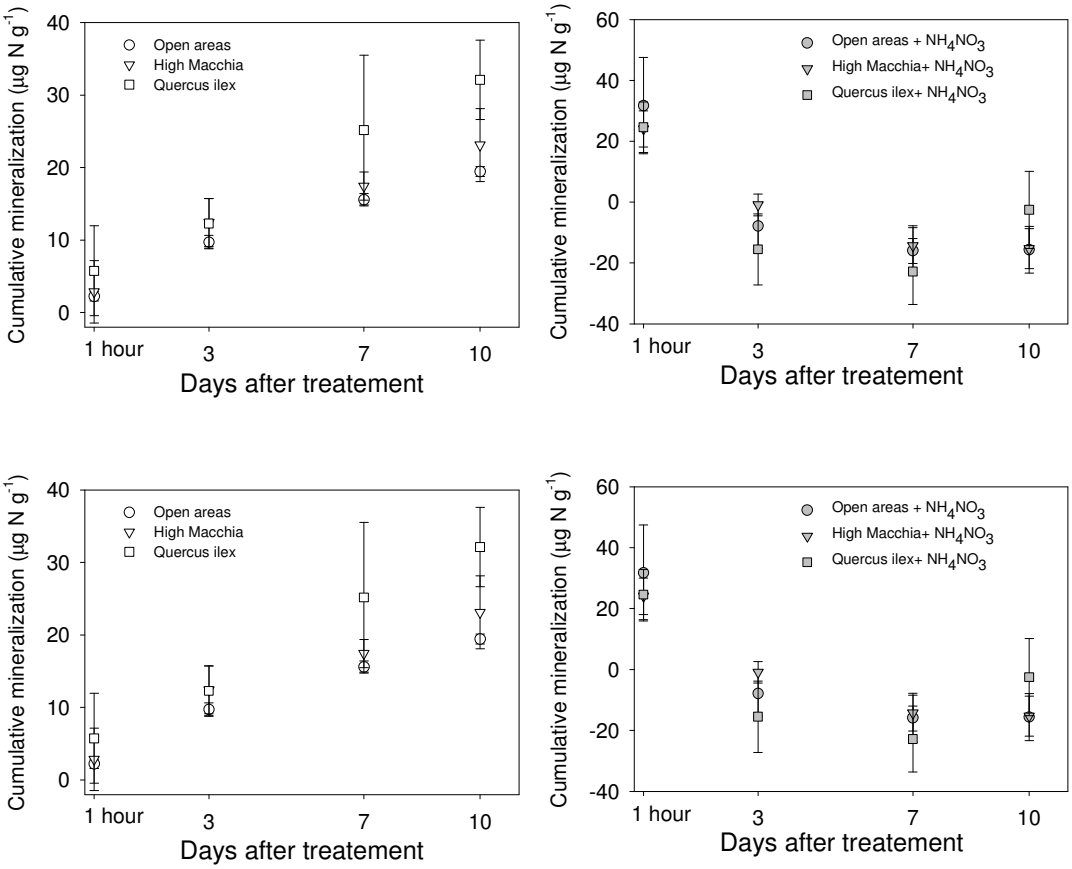


**Figure 6-3.** Cumulative mineralization and nitrification measured in three successional stages with and without N addition (right and left). Error bars represent standard deviation for three replicates.

Denitrification enzyme activity measured at the end of the incubation did not result stimulated by N addition. (**Fig. 6.4**). Values were lowest in the maquis, and resulted significantly lower than in the grassland. The woodland presented intermediate value but no significant difference with the other two stages.

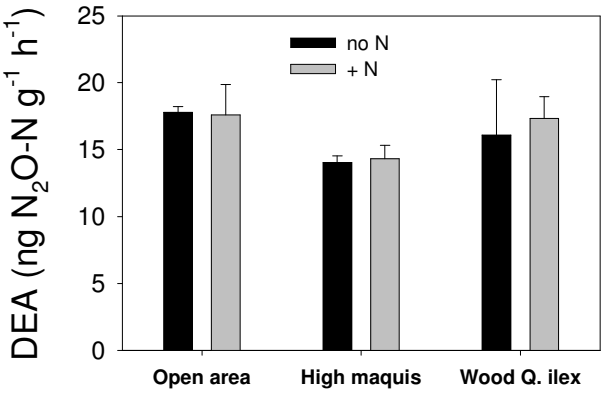


**Figure 6-4.** Denitrification enzyme activity, measured along a successional gradient, with and without N addition (gray and black bars). Error bars represent standard deviation for three replicates.

### 6.3.4 MINERALIZATION, NITRIFICATION AND DENITRIFICATION AMONG FOUR DIFFERENT MEDITERRANEAN ECOSYSTEMS

**Fig. 6.5** reported cumulative data of mineralization and nitrification in the four different woodland ecosystems. With addition of sole water to dry soil, Tolfa showed on average the highest mineralization values and no nitrification, as explained in chapter 3 and 4. Rocca and Castel Volturno showed a similar increase of mineralization, mostly linear over time similarly to nitrification. Lecceto site showed the lowest mineralization rates and extremely low nitrification rates. Addition of $NH_4NO_3$ (100µg $g^{-1}$ dry soil) induced a slight increase of mineralization rates in Tolfa and Rocca and immobilization at Lecceto and Castel Volturno, a similar trend was observed for nitrification.

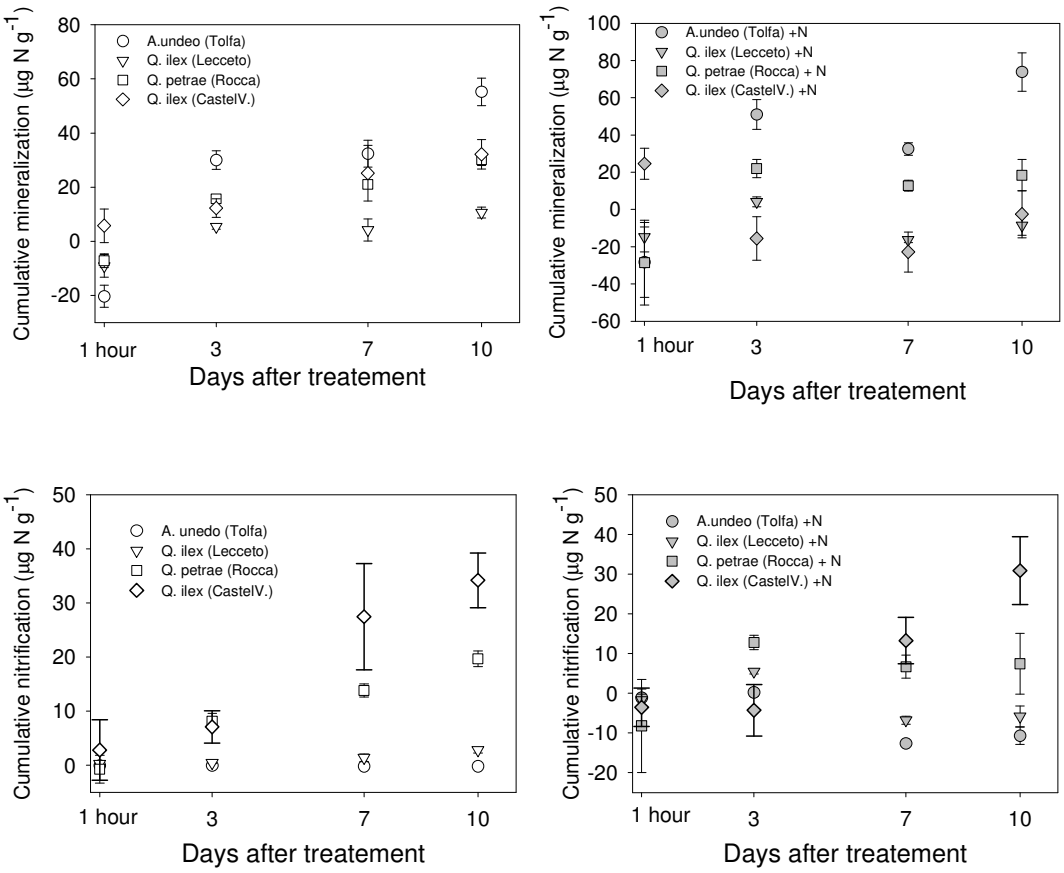


**Figure 6-5.** Cumulative mineralization and nitrification measured in four different Mediterranean sites with and without N addition (right and left). Error bars represent standard deviation for three replicates.

Denitrification enzyme activity (**Fig. 6.6**) was measured after 10 days. Denitrification rates were very different in the 4 Mediterranean analyzed sites. Castel Volturno site showed the highest values, an intermediate value was measured for Tolfa and Rocca, and thw lowest value was reported for Lecceto ($L^a<R^b<T^{bc}<C^{cd}$, $P<0.05$, One-Way ANOVA). Also in this case N addition did not stimulate denitrification enzyme production over 10 days.

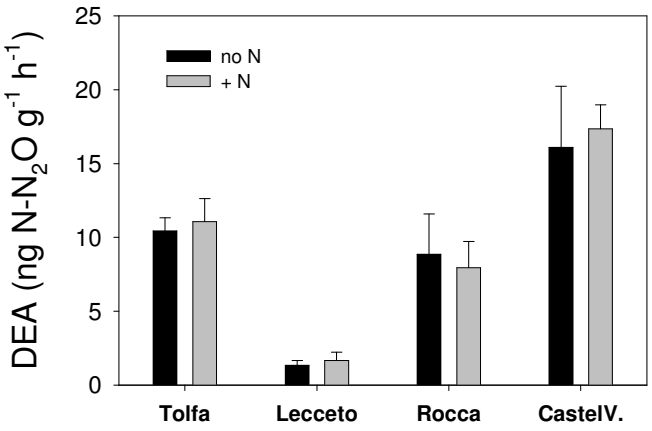


**Figure 6-6.** Denitrification enzyme activity, measured in four different Mediterranean sites, with and without N addition (gray and black bars). Error bars represent standard deviation for three replicates.

### 6.3.5 CORRELATIONS WITH SOIL CHARACTERISTICS

In order to correlate gas emissions with soil characteristics the former were cumulated over the period of incubation. Data are presented in **Fig.6.7**. The figure nicely show that the 4 woodlands have an opposite trend for NO and $N_2O$ emissions. The site which emit more of a gas emit also less of the other. In the chronosequence the result is similar for the two stages. This appears clear when extra N is added but without extra N all the site present an overall low emission of $N_2O$.

NO emissions were not correlated to any biological or chemical-physical soil parameter, or at least no clear trend was observed.

The only characteristics which resulted correlated with $N_2O$ emissions were soil total N and the C/N ratio. In the case the only meaningful correlation was with $N_2O$ fluxes in absence of extra N, which might have masked the effect of soil N.

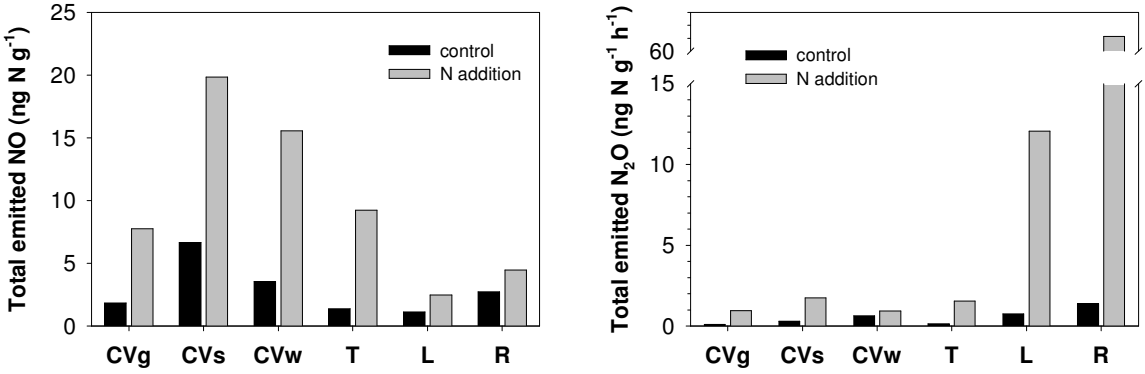


**Figure 6-7.** Total NO and $N_2O$ flux measurements without addition of $NH_4NO_3$ (black) and with addition of $NH_4NO_3$ (gray).

$N_2O$ emissions (without N addition) linearly increase with total soil N content if the site of Tolfa and Rocca are excluded (**Fig. 6.8**). In fact we know that Tolfa has basically no $N_2O$ emissions as consequence of factors other than soil N. A similar trend was observed for C/N ratios.

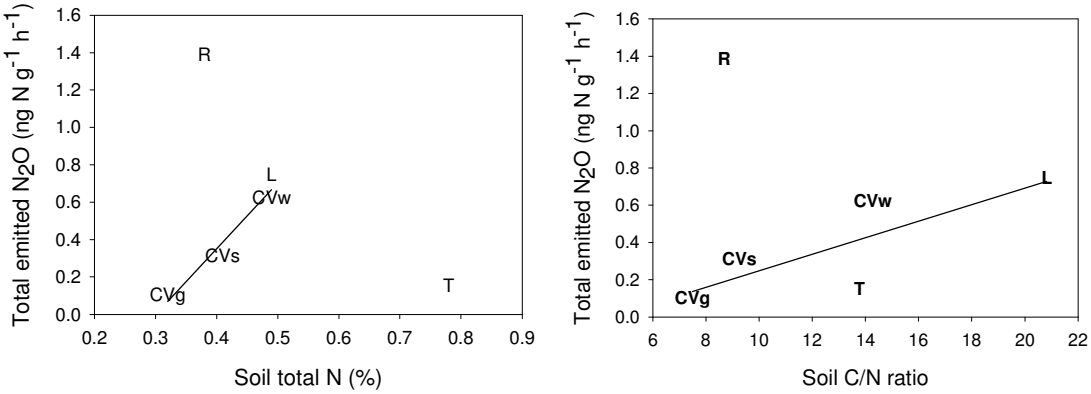


**Figure 6-8.** Total emitted $N_2O$ versus soil total N (in the left) and versus C/N (in the right).

Mineralization activity seemed to be influence by the CN ratio, increasing for increasing ratio up to a certain value of C/N around 14, to decrease for higher values (**Fig. 6.9**). Having however only one point after the limit of 14 we cannot be sure of this trend. The first part of the curve suggests that increasing C over N stimulates mineralization, but this is probably due to the fact that the soils in question have quite low contents of organic matter (**Table 1**), so

that an increase in ratio reflect an increase of substrate to optimal conditions for microbial growth and activity. Over C/N of 14 probably the amount of C starts to become critical in terms of biomass growth (immobilization) over activity (net mineralization, N release).

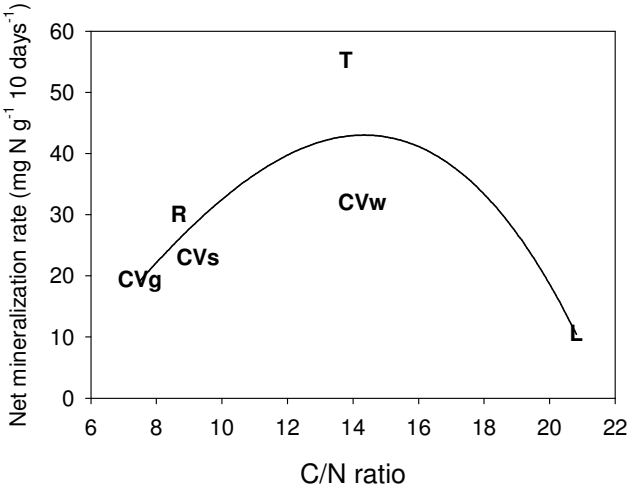


**Figure 6-9.** Net mineralization rate versus C/N ratio.

Mineralization did not appear to be affect by soil pH. In fact the most acid site, Tolfa showed the highest values of mineralization (**Fig 6.5**). On the contrary an exponential decreasing trend of nitrification with decreasing pH was observed (**Fig 6.10**). Nitrification seemed drastically reduced already at pHs between 6 and 7. The same behaviour was observed in presence of absence of extra N.

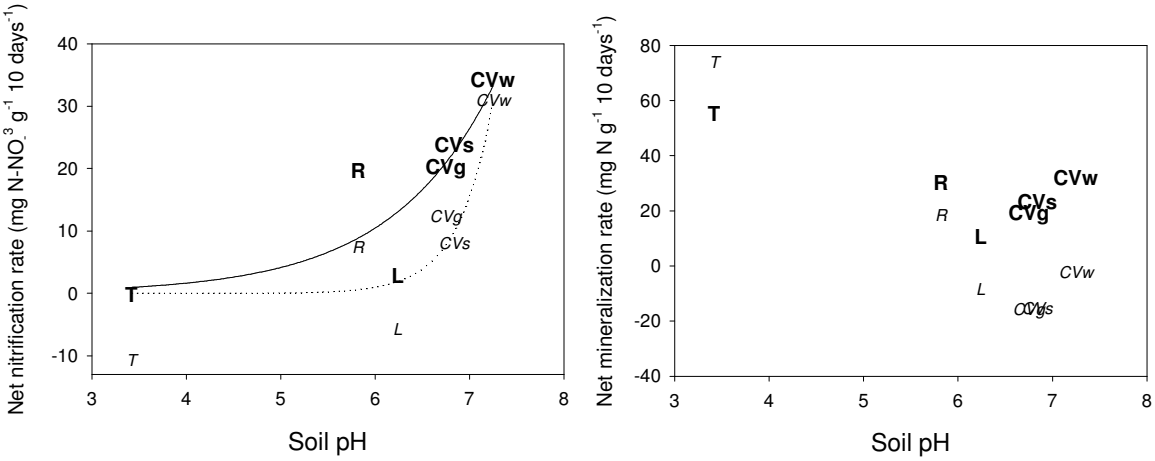


**Figure 6-10.** Net nitrification rate (left) and net mineralization rate (right)versus soil pH.
plain line: $y = 0.04 \cdot e^{0.93 \cdot x}$ $r^2$=0.91 symbols bold, -N plots;
dotted line: $y = e^{2.76 \cdot x}$ $r^2$= 0.82 (light symbols + N treated plots.

## 6.4 DISCUSSION

A predominance of NO emissions respect to $N_2O$ were measured in all studied sites in controlled conditions of temperature and soil moisture. Theoretical models (*Davidson 1991; Potter et al. 1996*) assume a maximum NO release at soil moisture contents of 10–60% WFPS and a maximum $N_2O$ release at higher soil moisture contents, i.e. 70–80% WFPS. Thus at the incubation condition of 50% WFPS, we would have expected a dominance of $N_2O$ over NO fluxes (*Davidson 1992; Schuster & Conrad 1992; Davidson et al. 1993; Hutchinson et al. 1993; Skiba et al. 1993*). Thus other factors were limiting $N_2O$ production in the soil. This was true also when N was added to the soil, in some cases, such as the Castel Volturno reserve, where N addition stimulated NO production more than $N_2O$. Addition of sole mineral N in fact might not be sufficient to stimulate significant denitrification activity, which is the main process responsible for $N_2O$ production in terms of yield. Denitrification in aerobic soils in fact requires enough organic matter so to stimulate microbial activity which from one end provides the substrate via mineralization and subsequent nitrification, in the other end it lower the oxygen content in the soil favouring redox conditions for $N_2O$ production (*Smith 1990*). On the contrary, being NO a by product of nitrification, led by autotrophic microorganisms, it

doesn't require low oxygen tension but just sufficient activity to provide microbes with $NH_4^+$ substrate. Although there was not a clear correlation, as a general trend the sites which showed the highest nitrification activity were also those characterized by the highest NO emissions. Another factor that could be important is soil texture, in fact it regulate the distribution of water in soil aggregates and the formation of oxic and anoxic sites and thus oxidative or reductive processes in soils. At soil water condition considered, an intermediate value, close to field capacity, soil micro-pores are water-filled, so to ensure microbial activity without stress and soil macro-pores are air-filled, so to ensure oxygen available and dissolved substrates. A fine-texture soils, has a smaller pores and thus, more anoxic sites are created at the same soil moisture conditions than in a coarse-texture soil (*Groffman & Tiedje 1991; Parton et al. 1996*). Then, soil capacity to retain water is very different in the studied sites, in fact water losses between sampling events were more pronounced in a sandy soil, as Castel Volturno reserve soil. Thus, texture in this soil result in an additional effect to low organic matter to favour oxidative process as nitrification, as result by high soil nitrate content and to favour NO emissions.

In Castel Volturno site, potential N emissions doesn't increase along the gradient of successional stages, but after 3 days, a peak was observed in intermediate stage of shrubland. Greater availability of C in the mature site could result in major nitrogen immobilization, with consequent competitive ability versus nitrification and denitrification and then low N emissions. Nitrogen mineralization was correlated exponentially with C/N ratio, and this factor become critical in soil of mature stand of *Quercus ilex* of Castel Volturno reserve.

The site of Lecceto, in opposite of Castel Volturno, has high soil total carbon and fine texture, and in presence of extra-N emit high amount of $N_2O$ confirming the effect of soil characteristics when N is not limiting. In undisturbed condition, at 50% WHC, it emits equal and little amount of NO and $N_2O$ gases, in fact the comparison among the different sites has showed that the sites with high emissions are in linear positive relationship with soil total N content (N%). The soil pH doesn't affect NO and $N_2O$ emissions in studied sites, while seems to be drastically a very important factor for nitrification rate.

Tolfa site, despite elevated soil total carbon and nitrogen content and texture, doesn't emit $N_2O$. According to dynamic of nitrogen cycle studied in previous research in this site, allelophatic compounds limit nitrification activity and soil nitrate content, affected indirectly $N_2O$ emissions. The "conservative" nitrogen cycle works energy in less mobile forms of $NH_4^+$

and organic N and block N losses also in presence of extra-N. However, NO emissions were detected but they cannot attribute to nitrification process , but could be result by abiotic processes.

The site of Rocca, in opposite to Tolfa, represent a positive exception. It has total organic carbon and nitrogen comparable to Castel Volturno site, but stronger and longer $N_2O$ emissions were observed when N is added to soil. Major investigations are needed to explain these results.

## 6.5 CONCLUSIONS

As we could expect, Mediterranean sites are very scarse sources of N gaseous compounds. Generally, it seem depend by a limiting factors of C and N-poor soil characteristics, but in some case it can be attribute to N-conservation strategy (see Tolfa site). Texture and soil organic matter became driving factors for $NO/N_2O$ ratio in presence of an excess of available forms of nitrogen, influencing also a nitrification and denitrification processes that are responsible for gases production. Sandy soil and with low C content (Castel Volturno) emit more NO than fine-texture soil with high C content (Lecceto). A sandy-loam and C-poor soil of Rocca emit highest peaks of $N_2O$, indicating that others factors, but non soil pH, must be take in account.

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