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Denitrification activity and denitrifying population dynamic in the soil of a wooded riparian strip

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Summary

The EU "Nitrates Directive" (Directive 91/676/EEC) and the WFD (Water Framework Directive 2000/60/EEC) introduced a series of measures designed to reduce and prevent water pollution caused or induced by nitrates from agricultural sources.

Riparian zones, located at the interface between terrestrial human activities and waterways, play a key role as a buffer system protecting aquatic ecosystems from excessive nitrogen loads. There are several mechanisms through which excess nitrogen is removed in riparian buffer zones: some act as temporary sinks, for instance soil storage, assimilation and retention by plants and microbes, while the denitrification process permanently removes nitrogen from the soil in a gaseous form.

Within the lower plan of Venice Lagoon watershed, a newly afforested riparian buffer, irrigated with freshwater from the Zero River, was realized; inside this afforested area, a pilot experimental scale system was established. The experimental forest buffer received almost continuous sub-surface water flow with the aim of enhancing nitrate removal through denitrification.

The objectives of this research were to verify the potential capacity of this buffer system in removing the excess of nitrogen from river water and to increase knowledge on the processes there involved, with particular emphasis on denitrification.

To achieve these objectives the following specific activities were performed: (i) the quantification of the combined nitrogen removal rates from water which flows through the woody buffer area; (ii) the measure of the denitrification process in soils, both "*in situ*" conditions (DNT) and potential denitrification (DEA) and its relationship with the main environmental limiting factors (hydrology, soil, climate, vegetation); (iii) a specific study on denitrifying community, focused on *nirK* gene, in the soil of the riparian buffer compared to that of a neighbouring agricultural area.

The main results of this work demonstrates that a buffer strip 15 meters wide can remove an excess of nitrate not only at concentrations typical of freshwater bodies (less than 5 mg/L N-NO_3), but also with higher peaks (until 25 mg/L), with a reduced effectiveness during colder seasons. It was also demonstrated that microbial denitrification plays a key

role in nitrogen removal and that higher denitrification rates were reached in the soil layer often saturated by the perched aquifer. As expected, the potential denitrification rates generally decrease with soil depth, depending on the distribution of the microbial population. In general, organic carbon availability resulted as the most limiting factor.

Moreover, it was established that in the riparian buffer under study both denitrification potential and the *nirK*-type denitrifying community distribution significantly differ from a contiguous agricultural soil. Even if there is still a limited understanding of the relationships between denitrification activity and denitrifying community structure and/or abundance we observed that denitrifying community composition could affect potential denitrification in soils characterized by different management practices (i.e. riparian forested soils and agricultural soils).

Riassunto

La "Direttiva Nitrati" (91/676/EEC) e la "Direttiva Acque" (2000/60/EEC) hanno introdotto per gli stati dell'Unione Europea una serie di misure per ridurre e prevenire l'inquinamento delle acque dovuto all'azoto di origine agricola.

Le fasce tampone riparie sono dei sistemi che si frappongono fra le aree agricole ed i corsi d'acqua e giocano un importante ruolo nel proteggere gli ecosistemi acquatici dai carichi azotati. Ci sono diversi processi attraverso cui l'azoto viene rimosso dalle acque che attraversano questi sistemi: alcuni, come la sedimentazione nei suoli e l'assorbimento e la ritenzione operato da piante e batteri, agiscono come zone di accumulo temporaneo; il processo di denitrificazione è invece in grado di rimuovere l'azoto in modo permanente trasformando l'azoto nitrico in azoto molecolare gassoso.

Nella parte terminale del bacino scolante della laguna di Venezia è stata realizzata un'area filtro forestale irrigata con le acque del fiume Zero e al suo interno è stato allestito un sito sperimentale. L'area tampone viene alimentata in modo continuo attraverso un sistema di scoline di irrigazione e di drenaggio che favorisce la formazione di un deflusso sub-superficiale che crea delle condizioni favorevoli allo svolgimento del processo di denitrificazione.

L'obiettivo di questo progetto di ricerca è quello di monitorare la capacità di questo sistema a svolgere l'azione depurante nei confronti delle acque che lo attraversano e di comprendere le dinamiche dei processi che favoriscono tale rimozione, con particolare attenzione al processo di denitrificazione. Per poter indagare tali aspetti sono state condotte una serie di attività: (i) un bilancio complessivo delle quantità di azoto (nelle sue diverse forme) rimosse dalle acque che defluiscono attraverso il sistema; (ii) una misura dei ratei di denitrificazione "*in situ*" (DNT) e potenziale (DEA) e della loro dipendenza dai principali fattori ambientali (idrologia, caratteristiche dei suoli, andamento climatico e sviluppo vegetazionale); (iii) un confronto fra la distribuzione delle comunità batteriche denitrificanti, contenenti il gene *nirK*, presenti nei suoli del'area tampone riparia e di una limitrofa area agricola.

I risultati conseguiti hanno dimostrato come una fascia tampone dell'ampiezza di 15 metri sia in grado di garantire un'elevata rimozione di azoto, sia nel caso venga attraversata da

acque con concentrazioni tipiche dei corsi idrici superficiali (minori di 5 mg/L di $N\text{-}NO_3$), sia in presenza di picchi di azoto più elevati (fino a 25 mg/L di $N\text{-}NO_3$). Tale capacità depurante subisce un'inibizione nel corso delle stagioni caratterizzate da basse temperature.
E' stato inoltre dimostrato che il processo di denitrificazione svolge un ruolo chiave nell'azione depurativa e che risulta particolarmente attivo nella zona di suolo attraversata dal deflusso sub-superficiale. In termini di denitrificazione potenziale, come atteso, si è osservata una riduzione dell'attività passando dallo strato di suolo superficiale a quelli più profondi; ciò è in relazione alla diversa distribuzione delle popolazioni microbiche nei suoli. La disponibilità di carbonio organico è il fattore limitante più importante per il processo di denitrificazione.
Confrontando i suoli dell'area tampone boscata con quelli di una limitrofa area agricola, sia la denitrificazione potenziale, sia la composizione delle comunità batteriche denitrificanti contenenti il gene *nirK* sono risultate significativamente diverse. Anche se il livello di comprensione delle relazioni esistenti fra l'attività di denitrificazione e la struttura/abbondanza della comunità microbiche è ancora poco approfondito, dai risultati di questo lavoro si è osservata una possibile relazione fra questi aspetti: il diverso uso del suolo influenza la composizione delle comunità microbiche e ciò, a sua volta, influenza la capacità di denitrificazione potenziale di un suolo.

1 INTRODUCTION

1.1 The riparian zones

1.1.1 Introduction

Riparian zones are a type of ecotone, or boundary between terrestrial and aquatic ecosystems. They play a key ecological role as well as a buffer system (see following section 1.2) between terrestrial human activities and waterways (Peterjohn and Correl, 1984; Jordan *et al.*, 1993; Wengler and Fowler, 2000; Carline and Walsh, 2007). The particular ecological value of the riparian zones is determined by the overlap of functions and properties of both the aquatic and the terrestrial ecosystem.

Naiman *et al*., (1988) noted that ecotones can display a greater variation in the characteristics of the systems they connect; rather than being averages of the two systems, they are something unique. The space-time dynamism that characterizes these areas depends largely on the frequency and severity of hydrological events as well as by the distance from the river, with a shift from riparian areas dominated by fluvial processes to ecosystems with characteristics associated to those of terrestrial environments.

In general, ecotones are characterized by:

- *high spatial and temporal variability*. The processes of flooding, drying, erosion and sedimentation result in an extremely variability in space and time, said a mosaic or "patches" (Naiman *et al.*, 1988). Each patch is distinguished by the characteristics of soil, water content and morphology and depending on these factors it could sustain different biotic communities;
- *high productivity*. The high productivity of the plant community depends on the high level of humidity of the soil as well as on the presence of deep soils, on the high availability of nutrients and on the long and narrow shape that reduces intra and inter-specific competition for solar radiation and nutrients (Pinay *et al.*, 1992);
- *high biodiversity*. Like many other ecotones, riparian buffer zones support an exceptional level of biodiversity (Odum, 1978; Gregory *et al.*, 1991; Malanson, 1993) due to natural disturbance regimes, a diversity of habitats and small-scale climatic variation (Naiman *et al*., 1993). E.g. Gregory and Ashkenas, 1990 found that riparian forests in the Willamette National Forest support approximately twice the number of

species than are found in upland forests. Riparian zones also support many rare species (Naiman *et al*., 1993).

Despite this, riparian areas are a declining habitat. Malanson, (1993) estimates that 70% of natural riparian communities have been lost; in some areas losses may be as high as 98%.

1.1.2 Functions of the riparian zones

Riparian zones are complex and fascinating ecosystems that perform a variety of functions of vital importance to the environment and to the society, whose very existence depends on the quality of the environment. Vegetated riparian zones provide habitats, such as breeding grounds, nesting sites and other, for a variety of terrestrial and aquatic wildlife species as well as the unique habitat requirements of many threatened and endangered plants and animals.

In the same time they contribute to the stabilization of stream banks and floodplains (Tabacchi *et al.*, 1998; Abernethy and Rutherford, 1999; Wenger and Flower, 2000; Zaimes *et al.*, 2004).

Riparian forests reduce solar heating of stream water by shading, especially in low order streams (Brown and Krygier, 1970). Any riparian vegetation provides cooling by evapotranspiration of soil water and shallow groundwater (Beschta, 1984; Theuer *et al.*, 1984; Sinokrot and Stefan, 1993). Through shading, riparian trees contribute to maintaining healthy water ways by moderating light and temperature regimes (Rutherford *et al.*, 1999; Yamada *et al.*, 2007).

Riparian vegetation is also an important source of organic matter to the channel. Indeed, most stream channels are partially heterotrophic ecosystems which rely on organic matter inputs from the riparian zones (Conners and Naiman, 1984).

Finally, as better described in the following chapters of this work, riparian vegetation also traps sediments and helps remove nutrients, such as phosphate and nitrogen and other pollutants like pesticides entering from shallow groundwater and surface runoff coming from adjacent agricultural areas (Groffman *et al.*, 1992; Vought *et al*., 1994; Dillaha and Inamdar, 1996; Schoonover *et al.*, 2005).

1.2 Riparian buffer zones and their role in nitrogen removal

1.2.1 The contamination of waters by nitrates

The contamination of surface and ground waters by nitrates is a major factor affecting estuarine eutrophication (Howarth and Marino 2006; Hakanson *et al.,* 2007) and drinking water supplies in many European countries (EEA, 2005). Eutrophication leads to environmental impacts such as toxic algal blooms, oxygen depletion, fish kills and loss of biodiversity (Vitousek *et al.*, 1997). The USEPA (United States Environmental Protection Agency) considers nitrogen one of the primary stressors in aquatic ecosystems (USEPA, 2002a).

The control of water pollution, especially nitrates, was an important concern of the Nitrates Directive (91/676/EEC). The WFD (Water Framework Directive 2000/60/ECC) has the specific aim of enhancing the status of all European water systems. The WFD (Art. 10) confirms and reinforces the need to reduce non-point pollution using the same strategy and the same actions proposed by the Nitrates Directive.

Agriculture is a significant source of combined nitrogen release to the environment, because fertilizer inputs to crops are generally higher than the amount of nitrogen required to maximize plant productivity (Driscoll *et al.,* 2003). According to recent studies, agricultural practices are typically responsible for 50-80% of the total nitrogen load to ground and fresh water (EEA, 2005; JRC, 2006). Nitrate concentration in a number of intensive agricultural areas exceeds the maximum value of 50 mg NO_3/L for drinking water. Although surface-water quality trends have generally stabilized during the last few years, more effort is required to achieve the objectives of the Nitrates Directive. Regional estimates of the application rate of nitrogen from manure exceed 170 kg ha^{-1} $year^{-1}$ at the local level in several European countries. Approximately 16.5 million tons of nitrogen was applied to European soils in 2003, with 7.6 million tons per year derived from animal husbandry (mainly cows, pigs, poultry and sheep) and 8.9 million tons from mineral fertilisers.

1.2.2 Riparian zones as interface between agricultural areas and rivers

Riparian zones, located at the interface between terrestrial human activities and aquatic ecosystems, play a key role as a buffer system (Lowrance *et al.*, 1983; Lowrance *et al.* 1984; Peterjohn and Correll, 1984; Hunter and Faulker 2001; Spruill 2004; Carline and Walsh, 2007; Pinay *et al.* 2007; Gumiero *et al.*, 2011). A buffer zone can be defined as a transition area from one ecosystem to another, in this case from an agro-ecosystem to an aquatic ecosystem. The spatial distribution of riparian forests relative to agricultural fields is likely to affect their functioning and sustainability in controlling nitrogen fluxes. Similarly, the hydraulic connectivity between these riparian buffer and the landscape sources of nitrogen fundamentally influences their efficiency (Haycock *et al.*, 1997; Lowrance *et al.*, 1997; Sabater *et al.*, 2003). Indeed, farming systems constitute the driving force in undermining or enhancing both spatial distribution and connectivity of riparian ecotones within the agricultural landscape (Fig. 1.1). Hence, the spatial and functional sustainability of riparian ecotones under varying farming practices needs to be evaluated in order to propose the most efficient landscape design which would reduce nitrogen fluxes under a given climatic and farming constrains.

Fig. 1.1 - Spatial distribution of riparian forests relative to agricultural fields.

The efficiency of a riparian zone in regulating nitrogen fluxes is not a function of the surface area of the riparian zone but rather a function of the hydrological length of the contact between the riparian zone and the upland drainage basin (Decamps *et al.* 2004; Pinay *et al.,* 2006). As a consequence, increasing the contact between water and soil sediment increases nutrients retention and processing. Therefore the best strategy is to prioritize and conduct riparian protection and rehabilitation through all rural catchments, particularly near headwaters. Low order streams (Fig. 1.2) are considered the most suitable for controlling nitrogen fluxes because of their great interaction potential with both riparian and agricultural areas (Decamps *et al.*, 2004; Pinay *et al.*, 2006). However, in natural or semi-natural floodplains, riparian buffer can reduce the in-stream nitrate concentration of high-order water courses.

Fig. 1.2 – Buffer zone along a ditch draining water from the adjacent field.

1.2.3 Hydrology of the riparian buffer zones

Since the water quality effects on the riparian buffer zones are highly dependent upon the volume and pathway of water movement through this zone, it is obvious that an understanding of hydrology is important (Burt, 1996).

In riparian zones the nitrogen removal and transformation processes occur in the soil layer affected by the roots (rhizosphere). Indeed, in the rhizosphere plant can play their direct (assimilation) and indirect (support for microbial processes) action on nitrogen removal (Peterjohn and Correl, 1984). According to this, if the rhizosphere is bypassed by the water flows, nitrogen can't be intercepted and transformed. For example if the local groundwater passes beneath the rhizosphere or the whole stream system at too great a depth (Fig. 1.3) the riparian zone cannot interact (Staver and Brinsfield, 1991).

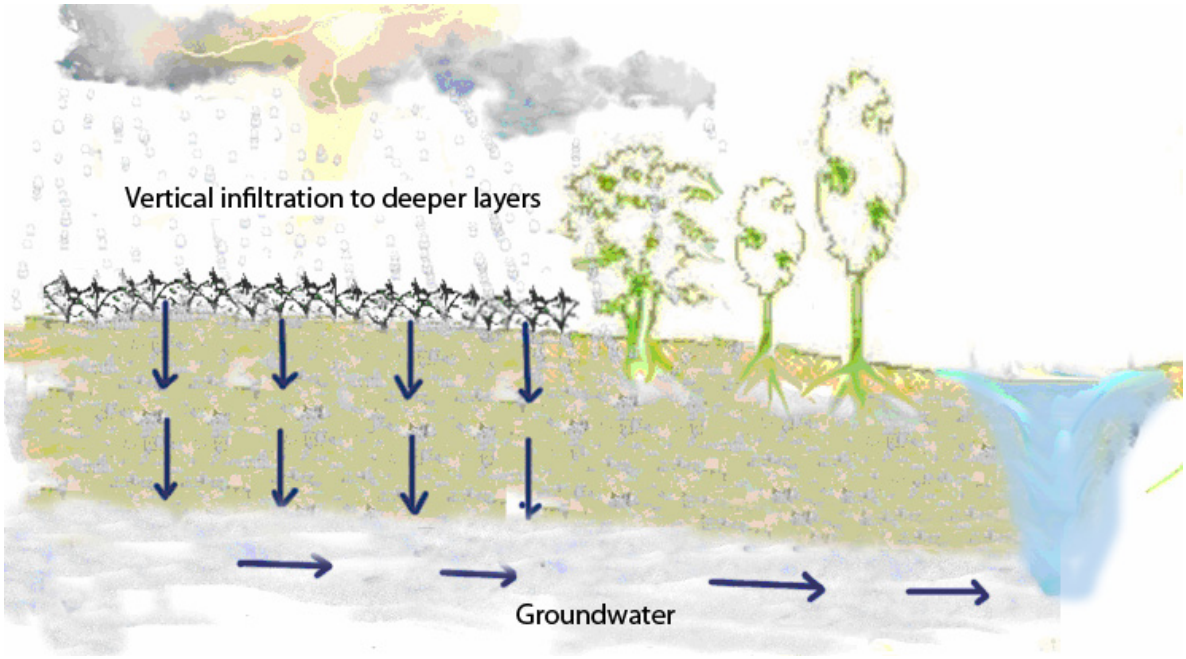


Fig. 1.3 – Vertical infiltration transport nutrients to the groundwater. Due to the deeply incised channel, the groundwater moves from the watershed to channel deep in the soil bypassing the rhizosphere.

The capability of buffer zones to attenuate pollutants will depend upon the mechanisms by which these pollutants reach surface waters. Mainly three transport processes can occur:

- *surface runoff*. Surface runoff can occur through several mechanisms. It may result when the surface soil becomes saturated (saturation excess) which is common where flow pathways converge as a result of topography. It may also occur (Fig. 1.4) when rainfall intensity exceeds the infiltration capacity of the soil (infiltration-excess) a process that is common in poorly drained clay-rich soils (Muscutt *et al.*, 1993). Riparian zones can be interested also by the runoff generated by flood waters coming from the stream channel.

  Surface runoff can be a major transport mechanism for water soluble pollutants, particularly when land beside a stream has been grazed, or fertiliser or livestock waste have been applied to the land during or prior to rain events. Surface runoff can also be a conduit for sediment and particulate pollutants. In both cases riparian vegetation can play an important role in removing and retaining particulates. Even if a fraction of surface runoff water could bypass the buffer system, usually the increased friction with soil surfaces can cause reduced velocity and consequent sedimentation of particulates. Herbaceous vegetation and the layer of litter it deposits on the soil surface are much more effective at slowing the velocity of surface waters. The fine roots of the plants, which area concentrated on or near the surface, and the microbial communities on the surface of the soil, litter and above-ground plant organs also are able to assimilate dissolved nutrients from the surface waters (Peterjohn and Correl, 1984). In some cases overland storm flows entering the riparian buffer zones, due to the reduced velocity, infiltrate the soil and became subsurface flow or groundwater (Correll *et al.*, 1996).

  Subsurface runoff. Subsurface flow is frequently the major pathway of N transport in catchment runoff and high concentrations commonly occur in artificial subsurface drains (Muscutt *et al.*, 1993). Intensive agriculture is often accompanied by subsurface drainage especially in clay-based soils. Indeed, in presence of permeable layers of soil (due both by their texture and by agricultural practices, like tillage) favour the vertical infiltration of rainwater and irrigation. If these waters meet a layer with lower permeability, they tend to form, occasionally, hypodermic sub-surface runoff directed towards the drainage network (Fig. 1.4). These outflows, can cross the rhizosphere conveying the buffer system dissolved pollutants

intercepted on their way. On occasion, subsurface flows may re-emerge, and discharge down slope as surface runoff.

In other cases subsurface flow could be generated by the water table (Fig. 1.5); depending on its level, the groundwater could be constantly or occasionally in touch with the rhizosphere. Also in this case lateral movements prevail, usually directed from agricultural areas to the stream channel but, in some periods (e.g. during floods), also directed by the rivers to the perifluvial areas.

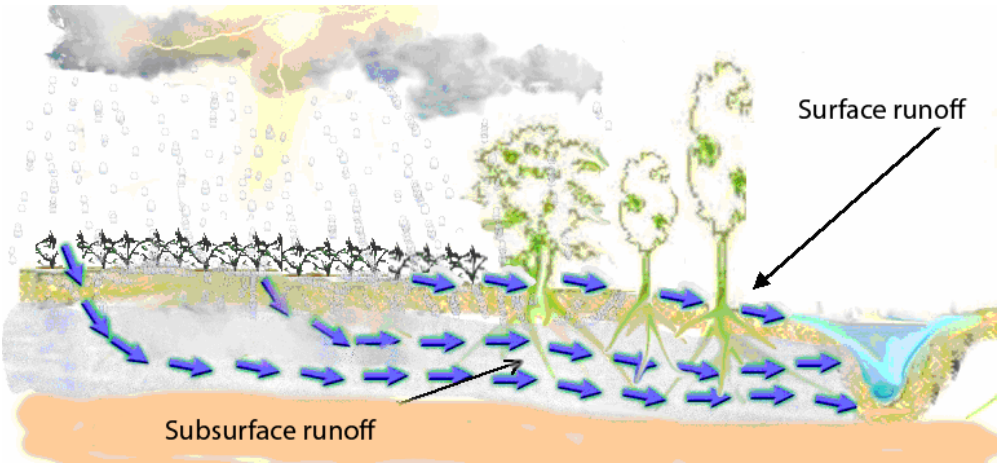


Fig. 1.4 – The presence of permeable layers of soil (due both by their texture and by agricultural practices, like tillage) favour the vertical infiltration of rainwater and irrigation. If these waters meet a layer with low permeability, they tend to form hypodermic sub-surface runoff directed towards the drainage network. When rainfall intensity exceeds the infiltration capacity of the soil also surface runoff could be generated.

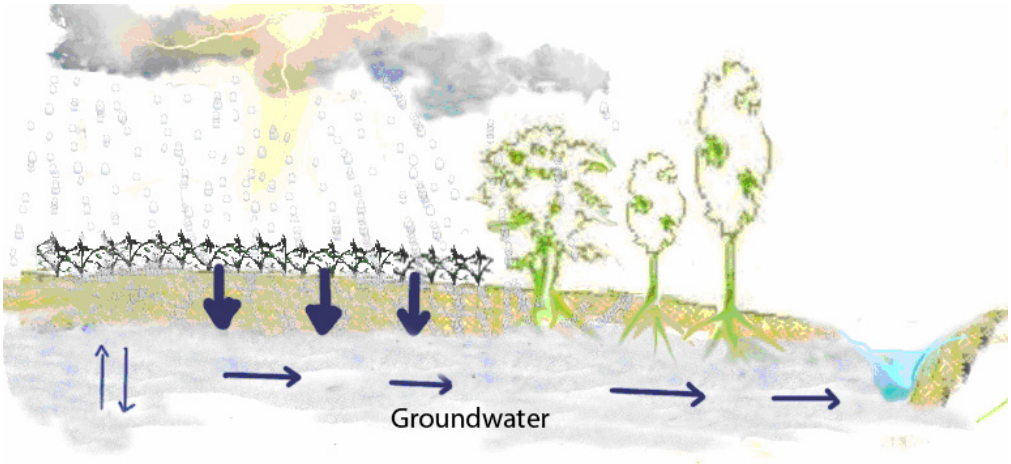


Fig. 1.5 – Depending on it vertical movements, groundwater could interest constantly or occasionally the rhizosphere. Also in this case lateral movements prevail, usually directed from agricultural areas to the stream channel.

The major climatic control factors are the components of the hydrological cycle: precipitation, runoff and evapotranspiration (ET) (Correll and Weller, 1989). ET is in turn, governed primary by such factors as vegetation, humidity, temperature, wind and sunlight. Thus, to some extent, the riparian vegetation has a feedback to the hydrological cycle. In term of balance, the output of the riparian zone equals precipitation plus surface and groundwater inputs minus ET minus infiltration to deeper layers.

1.2.4 Mechanisms of nitrate removal or transformation

There are several mechanisms through which excess nitrogen is removed in riparian buffer zones: some act as temporary sinks, for instance soil storage, assimilation and retention by plants and microbes, while the denitrification process permanently remove nitrogen from the soil in a gaseous form (Hefting and de Klein, 1998; Hedin *et al.,* 1998). The two processes, vegetational/microbial uptake of available nitrogen and denitrification, can work together to provide a buffer zone protecting aquatic ecosystems from excessive nitrogen loads (Lowrance *et al*. 1984; Peterjohn and Correll, 1984; Pinay *et al.*, 1993; Haycock *et al.* 1997; Lowrance *et al.* 1997; Pinay *et al.* 2000; Sabater *et al.* 2003; Pinay *et al.* 2007; Gumiero *et al.*, 2011). Few studies have accurately measured the amount of nitrate removed by each one of these mechanisms. Denitrification is most often invoked as the primary mechanism of nitrate retention (Cooper, 1990; Schipper *et al.*, 1993; Vidon and Hill, 2004); however the extreme spatial and temporal variability of denitrification rates in riparian buffer make it very difficult to determine accurate fluxes (Correl, 1991; Weller *et al.*, 1994). According to some studies (Jacobs and Gilliam, 1983; Peterjohn and Correl, 1984) assimilation by woody vegetation could be the primary mechanism of nitrate removal from groundwater during the growing season, while the flux of organic nitrogen delivered to the forest floor as litter could be gradually mineralised and denitrified at the soil surface during the other periods.

Recently, new technique (Dhont *et al.*, 2003) was successfully developed to quantify groundwater NO_3^- retention processes in a riparian zone using the variation of natural abundance of ^{15}N in NO_3^-. According to this research, the relative importance of denitrification and plant uptake to groundwater NO_3^- retention was 49 and 51% during spring, 53 and 47% during summer and 75 and 25% during autumn respectively.

1.2.5 Efficiency on nitrogen removal

The first studies which directly measured nitrate concentration decreases in groundwater as it moved through riparian zones along streams were in the Coastal Plain of North Carolina (Gilliam *et al.*, 1974; Gambrell *et al.*, 1975). Later the literature has not always been unanimous in highlighting this aspect. For example, on their 1994 literature review, Desbonnet *et al*., (1994) concluded that total nitrogen removal rates for buffers are good, but nitrate reductions are variable and low. Today, there is significant evidence that this was not a valid conclusion. A number of studies either not included in the Desbonnet *et al.*, (1994) review or published more recently show significant nitrate reductions. For examples, a series of studies conducted by Wenger, 1999, Lowrance *et al.*, 1983; Lowrance *et al.*, 1984; Jacobs and Gilliam, 1983; Correl, 1983; Peterjohn and Correl, 1984 led to a mass balance for total nitrogen retention in different experimental sites of 74 Kg N/ha year corresponding of 89% of inputs, 26 Kg N/ha year or 67% of inputs and 30 Kg N/ha year or 85% of inputs. As reported in Gilliam *et al.*, 1986, following experimental activities in France (Pinay and Décamps, 1988; Pinay *et al.*, 1989), in New Zealand (Cooke and Cooper, 1988; Cooper, 1990; Schipper *et al.*, 1994) in Rhode Island (Groffman *et al.*, 1992; Hanson *et al.*, 1994) and in England (Haycock and Burt, 1993a, 1993b; Haycock and Pinay, 1993) found very similar and high nitrogen removal rates. Fennesy and Cronk, 1997 reviewed riparian buffer literature with a focus on nitrogen reduction and concluded that riparian buffers of 20-30 m can remove nearly 100% of nitrate. More recently (Dhont *et al.*, 2004; Balestrini *et al.*, 2011; Gumiero *et al.*, 2011) described very high removal rates even in presence of narrower buffer strips.

1.2.6 Influence of vegetation type

There is still considerable uncertainty on the exact role of riparian vegetation and the relative efficiency of various type of vegetation on the effectiveness of riparian buffer zones. As reported in Correl, (1997), studies on the North Carolina Coastal Plain found that fields could be cropped right up to the stream channel and nitrate removal would still occur efficiently, if controlled drainage structures were used to prevent the drying of the riparian

soils (Gilliam *et al.*, 1979, 1986). Groffman *et al.*, (1991) reported that denitrification potentials in surface soils of grassed riparian buffers were somewhat higher than in forested ones. On the contrary Haycock and Pinay, (1993) found that poplar forested riparian buffer zones were more effective than grass, especially in the winter. Osborne and Kovacic, (1993) found that forested riparian buffer were more effective than grass for nitrate removal, but less effective for removal of phosphate and dissolved organic phosphorus from groundwater. Again, Correl *et al.*, (1996) in a comparison of two adjacent sites, one grassed and one forested, found that they had similar nitrate removal efficiencies. The European project NICOLAS (Nitrogen Control by Landscape Structures in Agricultural Environment, see Burt *et al.* 2002 and Hefting *et al.*, 2005) compared the effectiveness of different buffer systems present in an European network of monitoring sites; the obtained conclusions stated that there was no evidence of differences, in term of efficiency, between grass, forested and grass-forested buffers.

Some authors see an advantage in the use of woody vegetation in relation to its capability to explore deeper layers of soil, picking up the nitrates and then releasing them, as a result of the process of mineralization and nitrification, to the ground available for denitrifying bacteria (Hanson *et al.,* 1994). According to other authors the most important role of woody vegetation concerns its capability in providing by its roots the organic carbon in the deeper subsoils, where it is needed for effective denitrification in groundwater. A number of studies suggest that forest ecosystems determine a flow of carbon from two to three times higher than in grassland ecosystems (Fogel and Hunt, 1983). Other authors, considering only the organic matter supply by the root system, demonstrated that the total amount of organic matter added to the soil every year by tree root systems is lower than the one resulting from the meadow areas (Troeh and Thompson, 1993). In both cases, in the long term vegetation is necessary to maintain the organic matter in soils, which is needed for maintaining bacterial activities.

Recent researches (Bremer *et al.*, 2009; Dandie *et al.,* 2011) based on the study of microbial communities open a new perspective on the role of vegetation, demonstrating that the presence as well as the combination of different plants affected the composition of the denitrifiers.

Even if this uncertainty, it is well accepted that grass or dense herbaceous vegetation is more effective at trapping particulates from overland storm flows (Osborne and Kovacic, 1993; Parsons *et al.*, 1994), but that woody vegetation may be more effective at removing nitrate from groundwater. It is also clear that buffer strips realized combining grass and forested areas could guarantee the best efficiency.

1.2.7 Relationship between width and efficiency

One of the challenging problems is to determine the correct width of buffer strips. Numbers of studies underline different answers to this issue depending on the prevailing type of flow (surface runoff or subsurface flow, see pharagraph 1.2.3). Reduction of various forms of nitrogen in surface runoff is reasonably well correlated with buffer width. Dillaha *et al.,* 1988, found that 4.6 m and 9.1 m grassed filter strips were moderately effective in removing total nitrogen from surface runoff from a simulated feed lot, but ineffective in removing nitrate. Other studies of similar design (Dillaha *et al.*, 1989 and Magette *et al*. 1989) yielded similar results. Total nitrogen removal efficiencies in all studies increased with buffer width. Similarly, Vought *et al.*, (1994) reported surface nitrate reductions of 20% after 8 m and 50% after 16 m for grass buffers in Sweden. They concluded that a buffer strip of 10-20 m will, in most cases, retain the major part of the nitrogen and phosphorus carried by surface runoff. A study by Daniels and Gilliam, (1996) determined that grassed buffers of 6 m width and combination grass-forested buffers of 13 m and 18 m width retained 20-50% of ammonium and 50% of both total nitrogen and nitrate. Because sites had different characteristics it is not possible to determine whether width was a factor. The studies summarized above, only studied surface flow, not subsurface flow. Since in many cases most nitrate passes through buffers in the subsurface flow, studies that ignore it may greatly underestimate (or, in some cases, overestimate) nitrate reduction.

Many studies have found that nitrate reduction in subsurface flow is high, although the optimal buffer width depends on factors such as the hydrologic pathway and denitrification potential. Hanson *et al.*, (1994) reported that a 31 m wide riparian buffer reduced shallow groundwater nitrate concentrations by 94%, from 8 mg/L to 0.5 mg/L. Mander *et al*., (1997) found total groundwater nitrogen removal efficiencies of 81% and 80% for riparian

buffer sites of 20 m and 28 m width, respectively. Another research (Hubbard and Lowrance, 1994) determined that buffers less than 15 m wide can remove significant amounts of nitrate in surface and subsurface flows. Osborne and Kovacic, (1993) reported that a 16 m wide forested buffer reduced shallow groundwater nitrate levels of 10-25 mg/L to less than 1.0 mg/L with a maximum of 96% reduction. Coming to more recent studies, Gumiero *et al.*, (2011) determined than in a newly afforested subirrigated riparian buffer 15 m wide, 74.5 Kg N/ha year (63%) were removed. In this study is reported that a considerable reduction of nitrate concentration was observed even at 3-4 metres from the irrigation ditch. Similar results are reported in Balestrini *et al.*, (2011), were in a number of monitored riparian sites high removal percentage (95-100%) was found.

In summary, looking at literature, if hydrological conditions are suitable, we can conclude that buffer strips ranging between 10 to 30 meters wide can assure very high efficiency in term of nitrogen removal.

1.3 Denitrification in riparian zones

1.3.1 Denitrification process

Denitrification is most often considered as the primary mechanism of nitrate retention in riparian buffer (Cooper, 1990; Schipper *et al.*, 1993; Vidon and Hill, 2004). Over the past two decades, research has focused on denitrification on riparian zones because this process permanently removes nitrogen from the soil in a gaseous form (Knowles, 1982).

More precisely, the denitrification part of the N-cycle transforms nitrate (NO_3^-) into N_2 gas. Denitrification is a reductive process and thus is a form of respiration; it occurs in four stages: NO_3^- to NO_2^- (nitrite), NO_2^- to NO (nitric oxide), NO to N_2O (nitrous oxide) and N_2O to N_2 (Fig. 1.6).

All steps within this metabolic pathway area catalysed by complex metalloenzymes (reductases) with characteristic spectroscopic and structural features (Berks *et al.*, 1995). The conversion of NO_3^- to NO_2^- is catalysed by Nar, membrane-bound NO_3^- reductase; the conversion of NO_2^- to NO by the periplasmic protein Nir, NO_2^- reductase; the following steps from NO to N_2O and from N_2O to N_2 are catalyzed by Nor, NO-reductase and Nos, N_2O-reductase respectively (Fig. 1.6). For each step there may be more than one kind of reductase (van Spanning *et al.*, 1997).

Fig. 1.6 – Stages of denitrification process and corresponding enzymes involved.

In general, the proteins required for denitrification are produced only under (or close to) anaerobic conditions, and if anaerobically grown cells are exposed to O_2 then the activities of the proteins are inhibited (Knowles, 1982).

If denitrification is not carried through to completion, N_2O can be produced (Firestone *et al.*, 1980). N_2O is a greenhouse gas and emissions may contribute to adverse environmental effects (Groffman *et al.*, 1998).

1.3.2 Nitrite reductase (Nir)

Nitrite reductase (Nir) is a key enzyme in the dissimilatory denitrification chain, catalysing the reduction of NO_2^- to NO (Hendriks *et al.*, 2000).

Purification and characterization of Nir from several bacterial sources have shown that there are two distinct classes, containing either copper (CuNir) or heme (cd_1Nir). The genes coding for CuNir and cd_1Nir are called *nirK* and *nirS* respectively. The enzyme containing Cu and heme never coexists within the same bacterial species. *NirS* has been suggested to be more common, while *nirK* is found in a wider phylogenetic range of Bacteria and Archaea (Coyne *et al.*, 1989; Coyne and Tiedje, 1990).

1.3.3 Molecular tools to assess the diversity and density of denitrifying Bacteria in their habitats

Denitrifiers are commonly found in many natural environments such as soil, marine and freshwater sediments as well as in wastewater treatment systems. Some cultivation-based studies (Gamble *et al.*, 1977) found that the genera *Pseudomonas, Ralstonia, Alcaligenes, Paracoccus, Rhodobacter, Rubrivivax, Thauera, Burkholderia, Bacillus* and *Streptomyces* have been pointed out as the dominant denitrifires in various environments. However, cultivation is known to be highly selective for certain organisms and the lack of appropriate tools to study these bacteria in the environment have limited our knowledge of denitrifier ecology. Today molecular tools are being developed to assess both diversity and numbers of denitrifying population in different ecosystems (Wallestein *et al.*, 2006).

The ability to denitrify is sporadically distributed both within and between different genera and cannot be associated with any taxonomic group (Hallin *et al.*, 2007). Therefore, existing techniques to study the ecology of denitrifiers are based on the use of the functional genes in denitrification pathway or their transcripts as molecular markers of this community (Philippot *et al.*, 2006). DNA extraction followed by PCR amplification of denitrifications genes has been the most common way to start-off the analysis of denitrifier

communities. An increasing number of attempts are available and concerns in particular the amplification of partial *nirK*, *nirS* and *nosZ* genes (Throbäck *et al.*, 2004; Sharma *et al.*, 2004).

To obtain the genetic fingerprints of denitrifiers communities resolving PCR-amplified denitrification genes, several different techniques are available. In particular terminal restriction fragment length polymorphism (T-RFLP) and denaturating gradient gel electrophoresis (DGGE) are widely used.

1.3.3.1 DGGE

The use of DGGE to fingerprints denitrifier communities in the environment is rather new, although the technique has been exploited since around 1990. DGGE of partial 16S rDNA has been successfully employed for analysis of community DNA even in such complex environments as soil (Smalla *et al.*, 2001). However, the use of DGGE with functional genes is still in its beginnings. DGGE of *nirS, nirK* and *nosZ* fragments was evaluated to analyze denitrifier communities from different environments (Throbäck *et al.*, 2004). DGGE separates gene fragments of the same size on a plyacrylamide gel cast with a gradient of increasing concentration of the denaturants formammide and urea. The presence of several melting domains in the *nirS, nirK* and *nosZ* genes is known to hamper band resolution and typically results in cloudy bands (Kisand and Wikner, 2003). To avoid complete denaturating of the PCR-amplified fragments and to minimize the effects of multiple melting domains, a GC-clamp is added to one of the primers.

For successful DGGE analysis, the optimum fragment size is about 500bp. This limits the amount of sequence information and restricts the possibility of finding appropriate PCR primers. DNA fragments with different sequences can sometimes have similar mobility characteristics and more than one *nirK* or *nosZ* sequence was detected in some bands when the method was evaluated (Throbäck *et al.*, 2004). These were in most cases closely related and thereby difficult to separate accurately. Therefore, conclusions on denitrifier diversity exclusively based on DGGE patterns can be ambiguous. One advantage is that DGGE not only provides one fingerprints of the communities, but also allows sequencing of the bands appearing on the gel after excising them.

1.3.4 Factors controlling denitrification in riparian soils

The most important factors controlling denitrification in riparian soils are:

- *the presence of an electron donor or energy source for denitrifying bacteria, mostly available organic carbon*; organic C sources are available in most riparian topsoil in the form of soil organic matter, litter and decaying plant roots. Most subsoil contain little organic C. The transport of dissolved organic carbon from upper soil to subsoils may occur and may increase denitrification there. Even if organic carbon is generally assumed to be the energy source used by microbes in denitrification, there are other less studied denitrification pathways as well (Mariotti *et al.*, 1988; Correl and Weller, 1989; Jordan *et al.*, 1993). Some of these pathways include chemoautotrophic denitrification in which the reduction of nitrate is coupled with processes like manganese, sulphide or iron oxidation (Burgin and Hamilton, 2007).

- *anoxic conditions*; the O_2 content of a soil is largely influenced by rainfall, irrigation, groundwater table, soil texture and plant root microbial respiration. In topsoils generally, denitrification rates increase after rainfall and decrease again when the soil dries out. The chance for anoxic conditions results higher in soil with low porosity (clay and loamy soil) than in soils with a coarse structure as in sandy soils (Ruser *et al.*, 2006). The water-filled pore space, i.e. the percentage of the soil pores filled with water, is often used as an indicator for anoxic conditions. Anoxic conditions may also occur if the rate of O_2 consumption in the soils exceeds that of supply of O_2. High O_2 consumption are found when the respiration activity in the soil is high, e.g. after application of an easily degradable source of organic carbon (manures or crop residues). In this case, local high rates of O_2 consumption may cause enhanced denitrification in microsites (the so called hotspots) also in dry soils.

  The O_2 status of the subsoils may vary widely, depending mostly on water table depth and on soil organic matter content.

- *NO_3^- availability in the soil.* The sources of nitrate in riparian buffer includes the organic N deriving from plants and litter decomposition, which could be mineralized to NH_4^+ and, by nitrification, to NO_3^-, the atmospheric deposition, the biological N_2 fixation and

the input deriving from NO_3^- leached and transported by surface runoff and groundwater. The NO_3^- content strongly varies in time, because of the different sources and sinks.

1.3.5 Quantification of denitrification losses

Quantifying denitrification is difficult and no standard, absolute methods exist. Difficulties are linked to the characteristics of this process which presents high variability in time and space. In addition, the major end product N_2, cannot easily be detected because of its high back ground concentration of almost 80% in ambient air. A shynthesis of the main approaches used to quantify denitrification losses are presented in Munch and Velthof, (2007).

The most frequently used measurements methods are the acetylene-inhibition method (Yoshinari and Knowles, 1976) and ^{15}N-labelling technique (Mengis *et al.*, 1999).

Even if the C_2H_2-inhibition technique is the most widely used method to measure denitrification activity there are several disadvantages of using this technique (see also additional specifications at paragraph 5.2.2.1):

- C_2H_2 also blocks nitrification, by which denitrification may be made slow down when nitrification is the major source of NO_3^- in soils;
- C_2H_2 diffusion into the soil and N_2O out of the soil may be a problem, especially in wet or heavy-textured soils;
- often mostly intact soil cores are taken from the field and incubated in closed bottles containing C_2H_2; this practice may disturb the soil structure and O_2 may diffuse into soil possibly affecting denitrification; this is particularly true in subsoils samples;
- microorganisms may adapt to C_2H_2 or even use it as alternative energy source;
- due to the high temporal and spatial variability of denitrification in the fields usually many samples are required.

The fate of ^{15}N-labelled substrate (fertilizer, manure, crop residue) can be measured, including ^{15}N-labelled N_2 and/or N_2O fluxes. The main disadvantages of this method concerns:

- only denitrification from ^{15}N can be measured. Denitrification from other N sources is not included (e.g. N mineralized from organic matter);
- it is assumed that ^{15}N is homogeneously distributed throughout the soil. A heterogeneous distribution may lead to errors;
- analysis are in general more expensive and requiring specialized laboratories.

2 OBJECTIVES

2.1 General objectives

Over the past decades nutrient loads delivered to the Venice Lagoon have attracted considerable concern, resulting in the establishment of a series of nitrogen and phosphorus reduction targets by the local government (Regional Authority) in 1995.

Several actions were undertaken to achieve these objectives, one of which was the conversion of a cultivated area of about 30 ha to a forested buffer strip, irrigated with freshwater from the Zero River. Inside this afforested area, a pilot experimental scale system was established in order to find the most suitable conditions for enhancing denitrification activity. The monitoring activity is based on the study of the efficiency of the riparian forest buffer strips in reducing nitrogen loads flowing into the water bodies, and from there to Venice Lagoon.

General aims of this research and monitoring activity are:

- to increase knowledge on the processes which allow the riparian forest buffer strips to act as a buffer thus reducing the concentration of the main nitrogen compounds carried by the water flows running through;
- to quantify the nitrogen removal amount and the trend during the maturation phase of the riparian forest system;
- to identify the most appropriate management strategies of the buffer strips and water flow, in order to maximize the efficiency of these systems supporting the microbial processes involved in nitrogen removal.

2.2 Specific objectives

Specific objectives of this doctoral research project are:

- to quantify total nitrogen removal rates from water which flows through the woody buffer area;
- to study the denitrification process in “in situ” conditions (DNT), considering relationship with main environmental limiting factors (hydrology, soil, climate, vegetation);

- to explore under controlled laboratory conditions the soil potential denitrification activity (DEA) considering the role of different limiting factors: if anoxic conditions occur and if a non-limiting amount of nitric nitrogen or/and organic carbon are added;
- to study the effects of buffer area management on population dynamic of denitrifying bacterial communities; this research was conducted studying specific denitrification genes (*nirK*) encoding nitrite reductase;
- to compare the denitrification rates measures with composition, biomass and distribution of bacterial community (evaluated by another linked doctoral research project, Rahman, 2011).

This research focus mainly on the monitoring period 2007-2010 but in order to reach the specific objectives also data collected between 1999 and 2002 were considered.

3 EXPERIMENTAL SITE

3.1 Introduction

The experimental site is located inside the Pilot Demonstrative Farm “Diana”, in the municipal district of Mogliano Veneto (North East part of Italy, Venice Lagoon catchment), managed by Veneto Agricoltura. It was built within the project promoted and carried out by the local drainage authority “Consorzio di Bonifica Dese Sile” (from 2010 renamed “Consorzio di Bonifica Acque Risorgive”) and titled "Environmental restoration actions along the low course of Zero River for the reduction of nutrient input into Venice Lagoon", funded by Veneto Region through the “Plan for pollution prevention in the watershed flowing directly into Venice Lagoon”.

The experimental design was planned according to the protocols and methods of the European project NICOLAS (Nitrogen Control by Landscape Structures in Agricultural Environment - EC DGXII, 1997-2000 ENV4-CT97-0395; see also Burt *et al*. 2002)

3.2 The study area

North East Italy includes one of the major drained reclamation regions of the country and a considerable portion of the Venice Lagoon watershed area is located within this region (Fig. 3.3).

Over the past decades nutrient loads delivered to the Venice Lagoon have attracted considerable concern, resulting in the establishment of a series of nitrogen and phosphorus reduction targets by the local government (Regional Authority) in 1995. For Dese and Zero rivers (Fig. 3.2) a reduction of 150 x 10^3 Kg $year^{-1}$ of total N and 40 x 10^3 Kg $year^{-1}$ of total P were established.

To achieve these results, the Consortium planned a major river restoration project for the Zero river based on the identification of a series of natural key habitats to create or to restore (Fig. 3.1): a riverine lake, with the same function of an instream wetland, a wetland next a new tidal gate, new terraces created increasing the river section and covered by aquatic vegetation, a series of rainwater- and groundwater-fed shallow lakes, created out stream in an area previously used for the extraction of clay and a 30 ha forested buffer strip irrigated with freshwater from the Zero River.

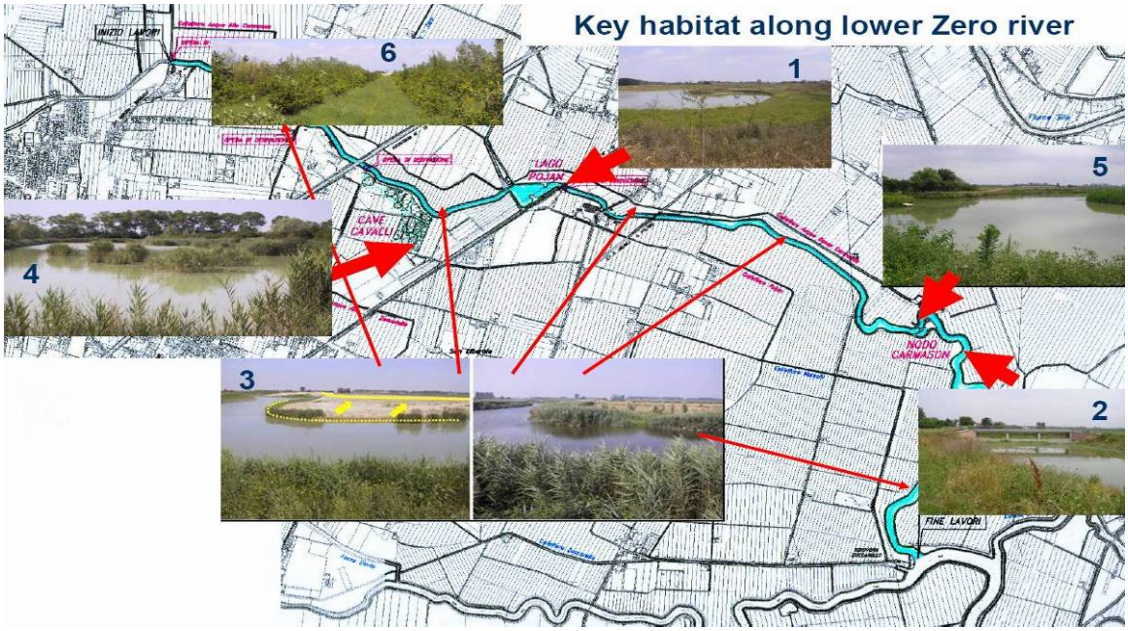


Fig. 3.1 - Location of the key habitat of Zero river restoration project: 1) riverine lake; 2) freshwater pond with the gate; 3) vegetated terraces in freshwater section 4) rainwater- and groundwater- fed shallow lakes 5) wetland next the tidal gate 6) riparian woodland.

The Zero joins the Dese River just before the latter flows into Venice Lagoon; it is a river 41.5 km long and fed by spring, with a 7,283 ha watershed, 94% of which is used for agriculture and 6% as urban areas. The watershed is mostly covered by herbaceous cultivations (corn, soy, wheat) farmed “alla ferrarese”, i.e. in regular plots, longitudinally convex with 1-4% steepness, 30-50 m large and 200-500 m long, bordered by lateral permanent drainage.

The soil (texture category “silty clay loam”) belong to the “Zerman soil consociation” according to the “Soils map of the watershed draining into Venice Lagoon” (ARPAV, 2004).

Fig. 3.2 - The Zero River in the reach adjacent to the experimental site.

3.3 Experimental site description

The experimental site is located within a much wider (about 30 ha) forested buffer zone, developed in lands previously used for arable crops, along the left bank of the lower course of Zero River (locality Bonisiolo) 15 km far from Venice (Fig. 3.3).

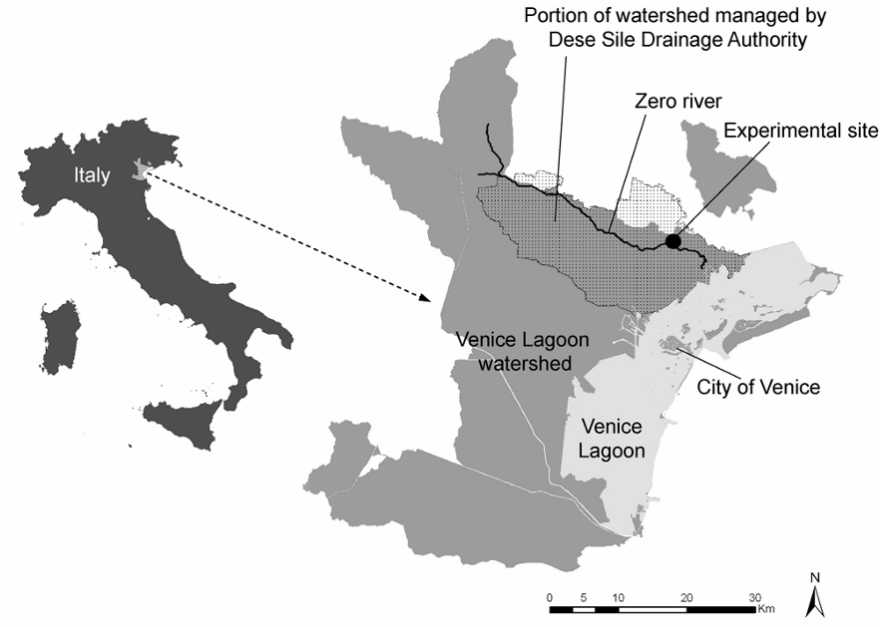


Fig. 3.3 - The experimental site, located on the left bank of the terminal reach of Zero River, in the watershed draining into Venice Lagoon. This portion of basin is managed from the drainage authority Consorzio di Bonifica Dese Sile (from 2010 renamed Consorzio di Bonifica Acque Risorgive).

The afforested area is divided in plots of the same size (0.35 ha each) and structure, but with different forest configuration (Fig. 3.4). Each plot is watered through a system of ditches carrying water (through a pumping system) from Zero River (Fig. 3.5).

Fig. 3.4 – Plan of the 30 ha wide forested buffer zone.

The experimental site was built in 1999 in two of these different plots (Fig. 3.5). It occupies a total area of about 0.70 ha (227 m long and 30 m wide). It required rebuilding the hydraulic structures (furrows facilitating sub-superficial water flow) and the water pumping plant, upgrading the meteorological station already existing in the Diana farm, installing the piezometric network, preparing the soil, planting the saplings (Fig. 3.12).

Two 5 m x 3 m grids of piezometers (1.5 m depth and 38 mm diameter each), were installed in each plot for a total of 30 piezometers. These were used to determine water table depths and to collect water samples.

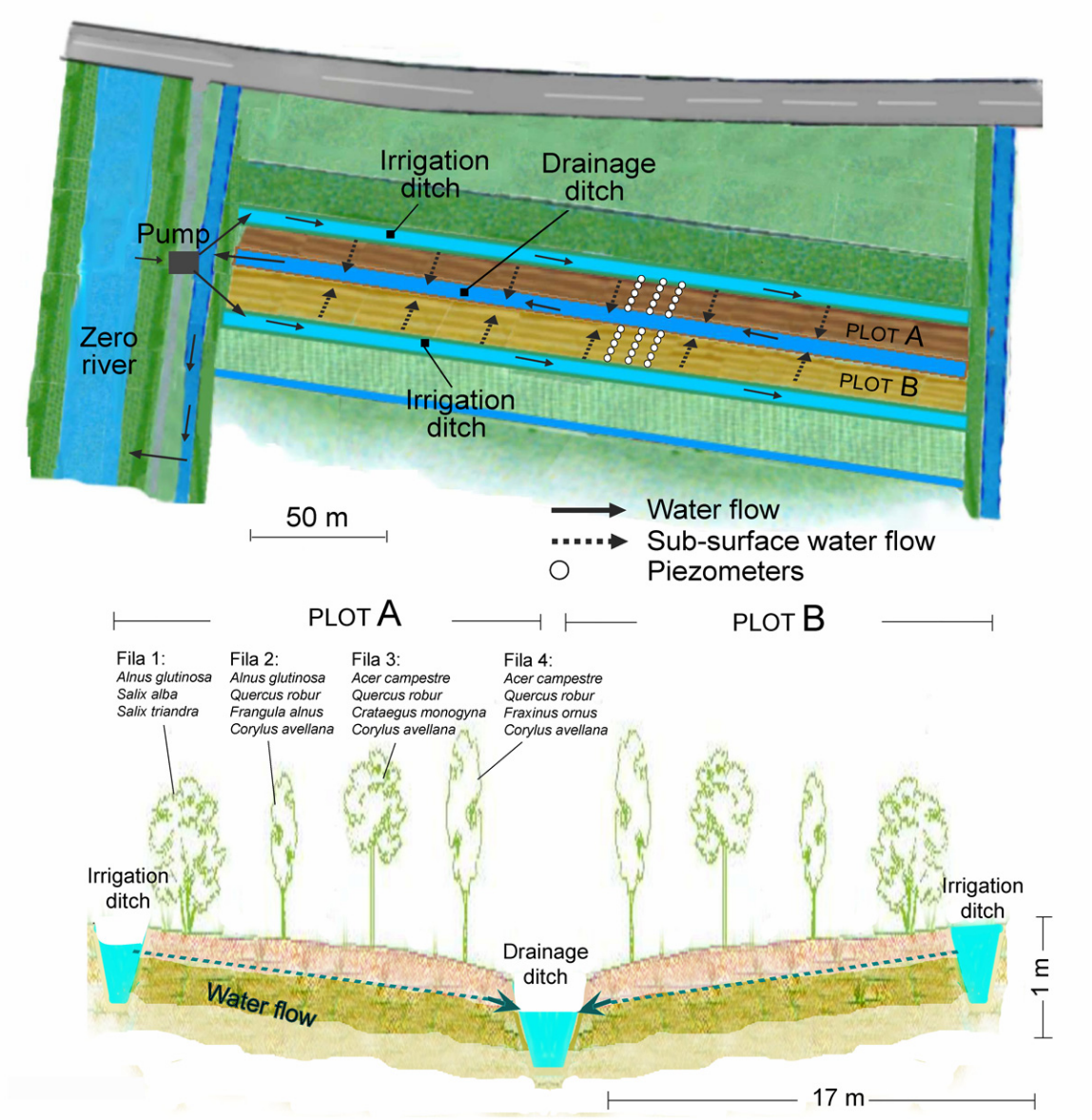


Fig. 3.5 - Plan (above) and section (below) of the experimental site: each of the 2 plots is watered through an irrigation ditch carrying water from the Zero River. Soil setting allows a difference in elevation among the irrigation ditches and the drainage ditch, resulting in a sub-surface flow of water running through the wooded buffer strips.

3.4 Soil characteristics

A soil profile was determined near the experimental site by digging a trench 150 cm deep (ARPAV, 2004). Soil is fine textured (according to textural classification USDA-SCS, 1984; Ritchie 1972), with a deep calcic horizon. In particular, the top layer of the soil horizon (Ap, to a 70 cm depth) is olive-brown, with silty clay loam texture (according to textural classification USDA-SCS, 1984), low limestone content and alkalinity. Underneath the top soil is a weathered subsoil (Bw) 20 cm thick, light olive-brown, with silty-clay texture, lower limestone content.

The following horizon is 30 cm thick, light olive gray with grey and yellow-brown streaks, loamy sand textured, highly calcic and strongly alkaline, characterized by limestone accumulation (calcic horizon Bk) forming irregular concretions or soft masses, of light colour. At 120 cm depth begins the Ckq substratum, with no structure and with colours and texture similar to the previous horizon.

This no structured and calcic layer (Bk and CKq horizons, 90-150 cm depth), with high content of loam and clay, due to its very low permeability, limits interactions between alluvial groundwater (about 1,6 m depth) and the near surface soil. The soil rooting depth is moderately high, limited by hydromorphic horizons, low drainage and permeability.

Zerman soil - ZMR1 (SINPA 13 profile) - Location: Diana farm (Bonisiolo) - 2001										
Horizon	Depth	pH	Particle size			Carbonates	Limestone	Organic C	Total P	Ks
	cm		Sand	Silt	Clay	%			mg/Kg	mm/hr
Ap1	0-40	8.0	12.9	51.4	35.7	4	1	0.9	22	0.88
Ap2	40-70	8.0	12.2	51.8	36.0	4	2	0.9	16	
Bw	70-90	8.1	7.4	52.3	40.3	1	1	0.3		1.20
Bk	90-120	8.6	10.5	63.4	26.1	15	13	0.2		0.08
Ckg	120-150	8.4	18.1	64.8	17.1	46	11	0.1		

Fig. 3.6 - Table summarizing the physical and chemical characteristics of the various soil horizons as surveyed in the experimental site in 2001 – Source ©2003-2007 ARPAV.

A new soil profile was done in 2011. The soil characteristics are summarized in the following Fig. 3.7.

Zerman soil - ZMR1 (VAR2P0023) - Location: Diana farm (Bonisiolo) - 2011											
Horizon	Depth	pH	Particle size			Carbonates	Limestone	Organic C	Total P	Ks	Bulk density
			Sand	Silt	Clay						
	cm		%			%			mg/Kg	mm/hr	g/cm^3
Ap1	0-50	8.0	13.0	58.5	28.5	10	3	1.0	17	1.41	1.63
Ap2	50-80	8.0	11.3	53.5	35.2	3	2	0.6		2.47	1.57
Bw	80-100	8.1	13.1	66.9	20.0	26	8	0.4		4.40	1.57
Bk	100-120	8.6	8.3	66.7	25.0	41	14	0.4		3.42	1.67
Ckg	120-140	8.4	4.2	69.1	26.7	45	15	0.3		3.47	1.67
Soil Taxonomy (KEYS 2010): Oxyaquic Eutrudepts fine-silty, mixed, mesic											
WRB (2006): Endogleyic Calcisols (Orthosiltic)											

Fig. 3.7 - Table summarizing the physical and chemical characteristics of the various soil horizons as surveyed in the experimental site in 2011 – Source © ARPAV.

3.5 Site hydrology

The experimental site was designed to rigorously monitor the hydrological fluxes and to carefully characterize the hydrology of the buffer system. Ridges and furrows facilitate sub-surface water flow throughout the field from the inlet point, represented by two irrigation ditches where water is pumped, to the parallel drainage ditches localized at lower elevation (Fig. 3.5). The average slope between irrigation and drainage ditches is 4% (Fig. 3.9). The drainage ditches are connected to a canal which brings back water to Zero River (Fig. 3.5).

The volume of the introduced irrigation water was continuously measured by a flowmeter (Maddalena – Datawater WMPE) inserted in the water supply line.

As a consequence of the irrigation (an average of 55,000 m^3 ha^{-1} $year^{-1}$, about three times rainfall), a perched aquifer was created on the undisturbed calcic layer located at around 90-150 cm depth.

Despite the soil is fine textured, in the layer between 0 to 90cm below soil surface, the vertical and lateral water flows are favoured by fissures due to the previous tillage activity and to the presence of trees radical apparatus (Fig. 3.8). These preferential flows allow

pumping in the system higher volumes of irrigation water than expected considering only saturated hydraulic conductivity (Ks). As a consequence water moves through the soil creating sub-surface flows with different velocity.

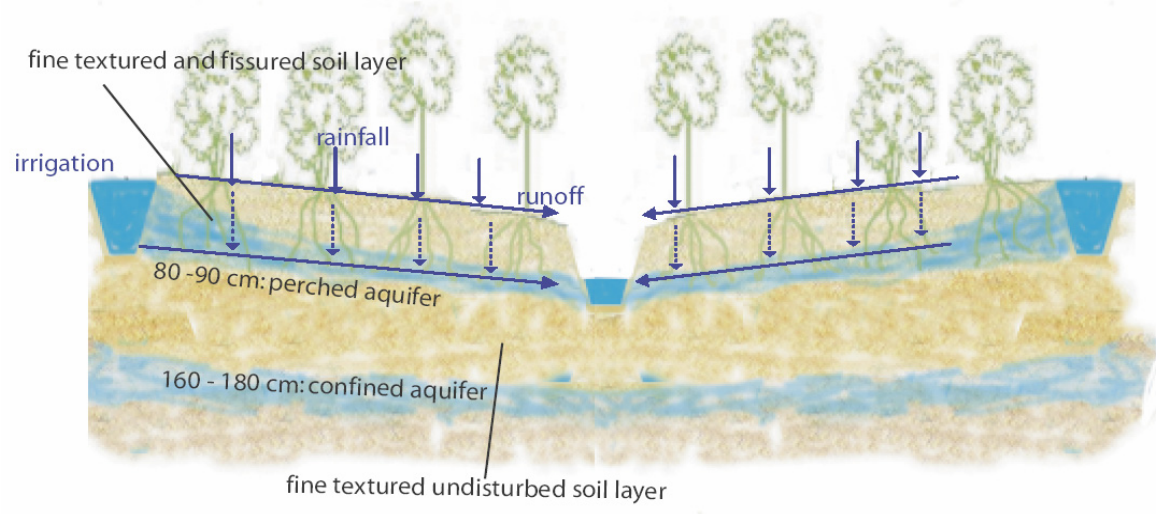


Fig. 3.8 – Scheme of buffer system hydrology. Arrows represent the preferential flow directions of irrigation and rainfall.

Sub-surface water depth was measured monthly (using phreatimeters: OTT Messtechnik – Electric contact gauge mod. KL 015) in the 30 piezometers during the entire period of study. The water level in the experimental site was always between 25 to 90 cm below the soil surface (Fig. 3.9). While the surface soil layer was subjected to the normal seasonal cycle, the medium and depth layers were often saturated.

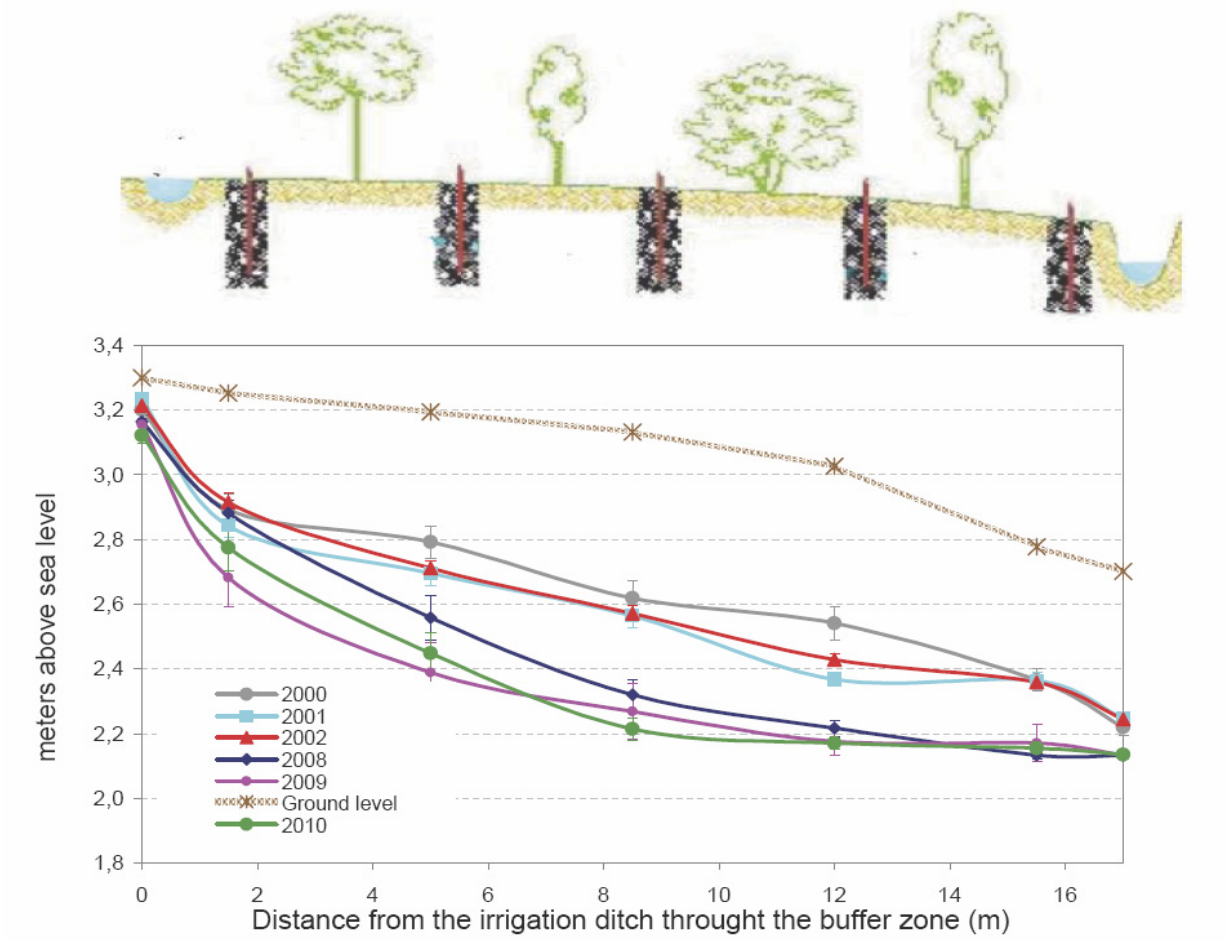


Fig. 3.9 – Ground level and mean annual water table elevation measured in plot A (monthly measures in ditches and piezometers) during the monitored years. These values are not significantly different to those from plot B. Bars represent standard error.

During 1999 – 2002, the water table elevation was measured continuously (one record every 15 minutes) by two pressure transducers connected to a data logger and inserted in two piezometers: one located near the irrigation ditch (IN) and one located near the drainage ditch (OUT). The variation of rainfall and irrigation volumes and the corresponding fluctuation of the water table are reported in the following Fig. 3.10.

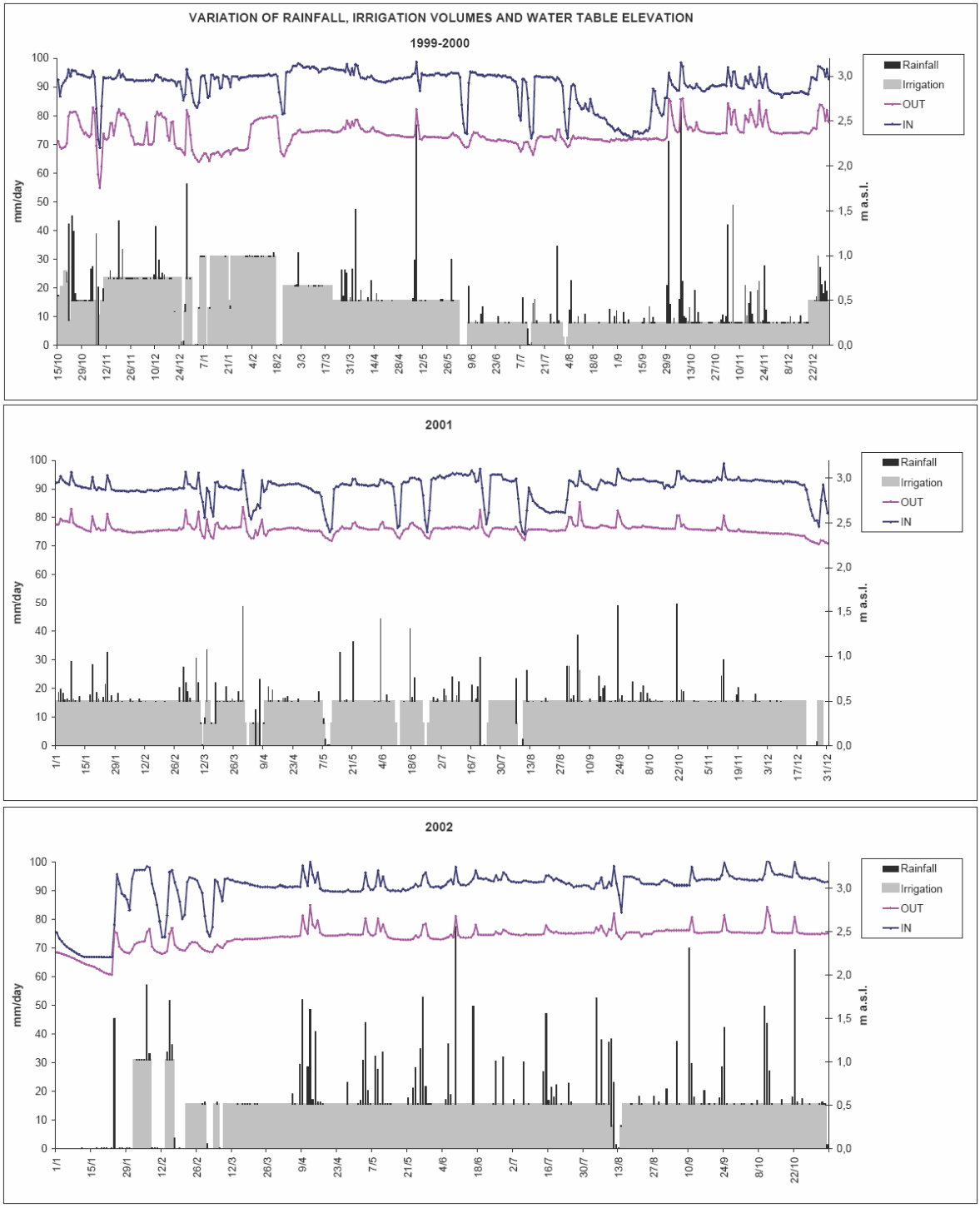


Fig. 3.10 - Daily variations of rainfall and irrigation volumes (cumulative mm/day) and the corresponding fluctuation of the water table measured in two piezometers: one located near the irrigation ditch (IN) and one located near the drainage ditch (OUT). The graph reports daily mean of data recorded every 15 minutes.

The concentration of Chloride, a biologically inert conservative tracer (Altman and Parizek, 1995), was measured monthly to monitor dilution and dispersion (Sabater *et al.*, 2003).
The Chloride concentrations measured in the water collected from different piezometers and ditches during 1999-2002 changed little through the buffer (from 10 to 15 mg Cl L^{-1}.). Moreover the mean value of chloride concentrations of regional groundwater is about 55 mg/L (Regional Environmental Agency, unpublished data). So the lack of changes in the chloride concentration within the buffer and the large amount of water flowing into the shallow groundwater system, make the input of deeper groundwater to the shallow flow system unlikely. Therefore, it was assumed that dilution from existing groundwater was minor.
To better describe the hydraulic behaviour of the system, tracer experiments (Dierberg and DeBusk, 2005) with the organic fluorescent tracer Rhodamine WT (RWT) were performed in 2008. Two hundred grams of diluted (21.33%) RWT were injected into the irrigation ditch and subsequently 99 samples were collected every 60 minutes from the discharge ditch by an ISCO 6700 automatic sampler (Teledyne Isco Inc., Lincoln, USA). All samples were filtered through a 0.45 μm fibreglass filter and RWT concentration was determined by a fluorometer (SCUFA ® Turner Designs Inc., Sunnyvale, California, USA).
The RWT injection, although carried out after the time period being reported, provides further evidence that the irrigation water was moving rapidly through the shallow perched aquifer and not seeping out into the alluvial aquifer. Indeed, comparisons of mass balance in the irrigation and drainage ditches, a loss of only 9.6% of RWT was registered, with an average travel time through the shallow groundwater from the irrigation to the drainage ditch of 24.3 hours. These results indicate that the deep seepage out of the shallow aquifer into the underlying alluvial aquifer is negligible.
According to this evidences the water balance of the afforested buffer area could be estimated using the following formula:

$$I + R - ET = D$$

where: *I = Irrigation volume; R = Rainfall; ET = Evapotranspiration; D = Drainage back to river*

Irrigation volumes, rainfall, evapotranspiration and water balance for the monitored years are reported in the following Tab. 3.1.

Tab. 3.1

Year	Irrigation volume (m-cu ha^{-1} $year^{-1}$)	Rainfall (m-cu ha^{-1} $year^{-1}$)	Evapotranspiration (m-cu ha^{-1} $year^{-1}$)	Drainage back to river (m-cu ha^{-1} $year^{-1}$)
1999 - 2000	51,917	7,562	7,274	52,205
2000 - 2001	48,060	8,888	6,963	49,985
2001 - 2002	48,600	11,450	9,611	50,439
2003-2004	9,730	10,666	7,906	12,490
2004 - 2005	18,144	8,126	7,908	18,362
2007 - 2008	59,986	8,570	6,962	61,574
2009	53,788	10,900	8,400	56,288
2010	55,787	14,680	7,843	62,624

3.6 Vegetation

Several tree and shrub species (white willow (*Salix alba* L.), almond willow (*Salix triandra*), black alder (*Alnus glutinosa* (L.) Gaertner), pedunculate oak (*Quercus robur* L.), field maple (*Acer campestre* L.), common hazel (*Corylus avellana* L.), common hawthorn (*Crataegus monogyna* Jacq.), manna ash (*Fraxinus ornus* L.), black dogwood (*Frangula alnus* L.) were planted in spring 1999 using 2-3 years old harvested plants and were arranged in four parallel rows for each plot as indicated in Fig. 3.5. The chosen forest configuration was: 1.5 m for shrubs and 3.5 m for trees spaced along the row and 3.5 m wide inter-rows, for a total of 4 rows for each plot.

Plants developed in different way in each row. In particular the row 1, located near the irrigation ditch, reached highest biomass values (Fig. 3.11).

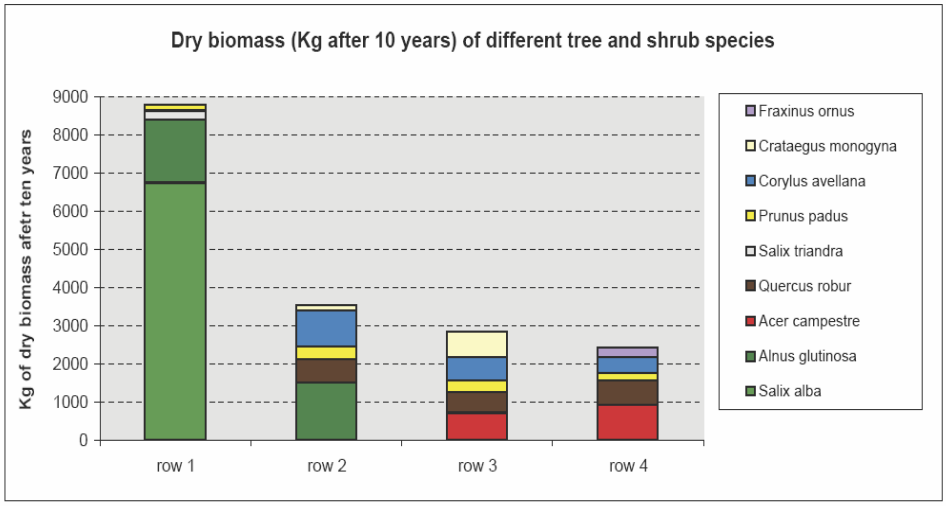


Fig. 3.11 – Dry biomass, expressed as total Kg for each different species after ten years from planting, in the four different rows in plotA.

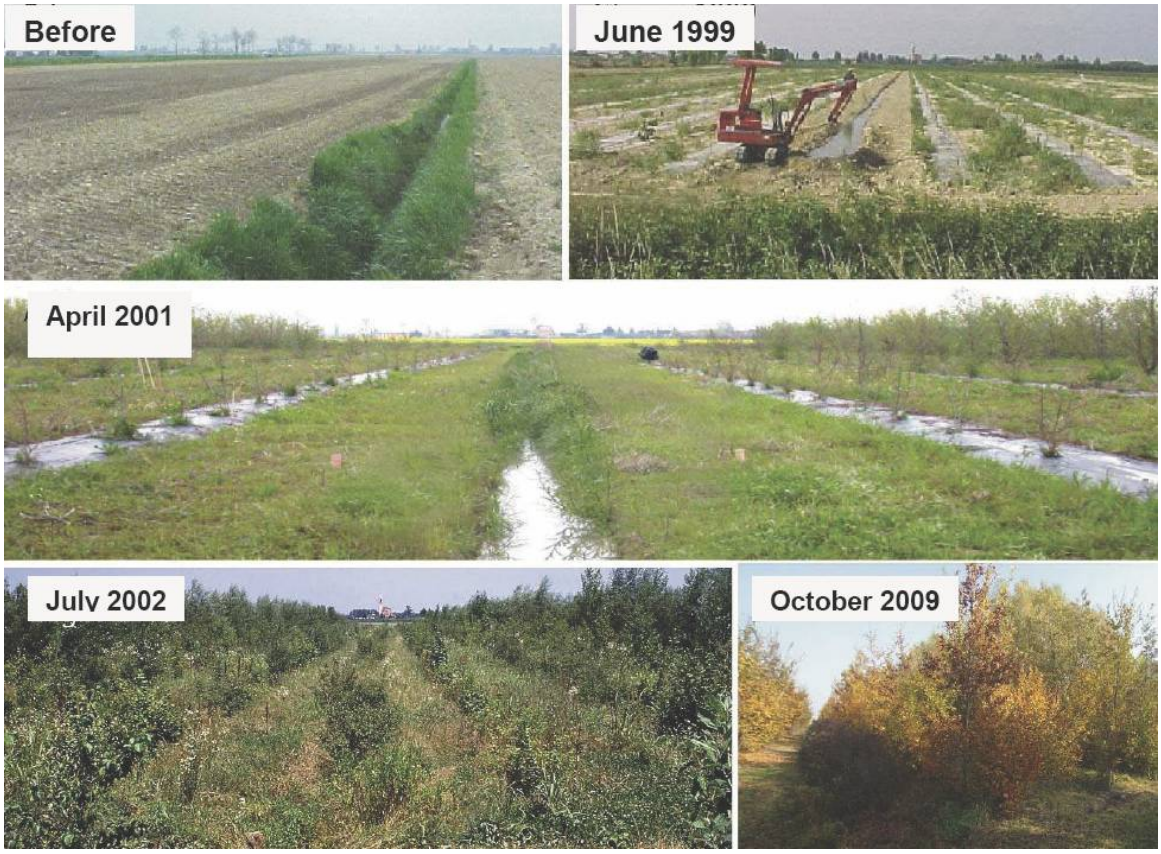


Fig. 3.12 – Pictures allow comparing the quick transformation occurring in the experimental site from the initial condition of agricultural area with newly-planted saplings to a forested buffer area.

3.7 Climate

The climate is sub-continental with temperatures ranging from a daytime average of 1°C in January to 23°C in July and August. The mean value of rainfall is 900 mm per year, with higher peaks in Autumn and Spring and lower values in Winter and Summer.

Rainfall, T. max, T. min registered during the monitored years are reported in the following Fig. 3.13 and Fig. 3.14.

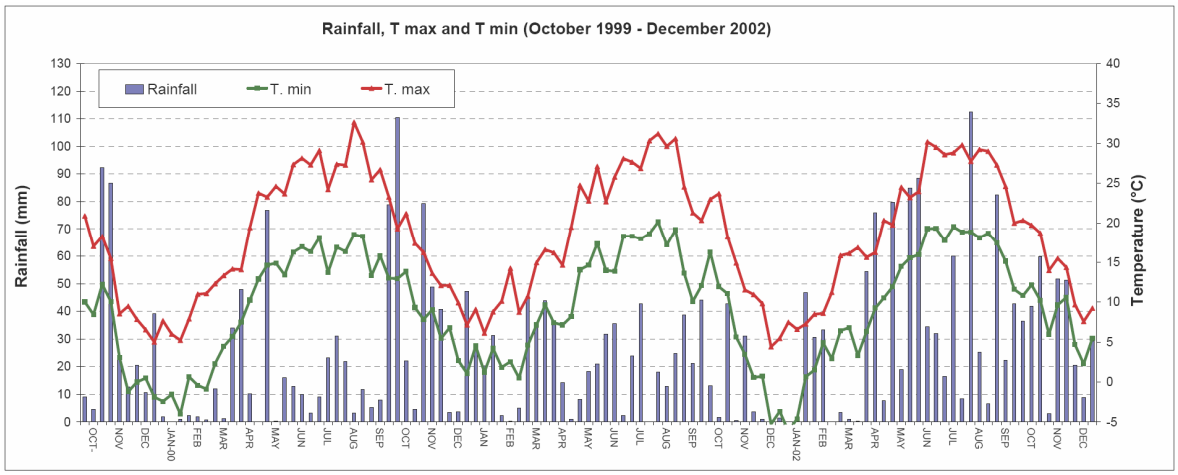


Fig. 3.13 – Rainfall, T. min and T. max registered during the period October 1999 to December 2002 in the experimental site. The rainfall is calculated as sum of 10 days long periods; T. max and T. min is the mean values registered during the same 10 days long periods.

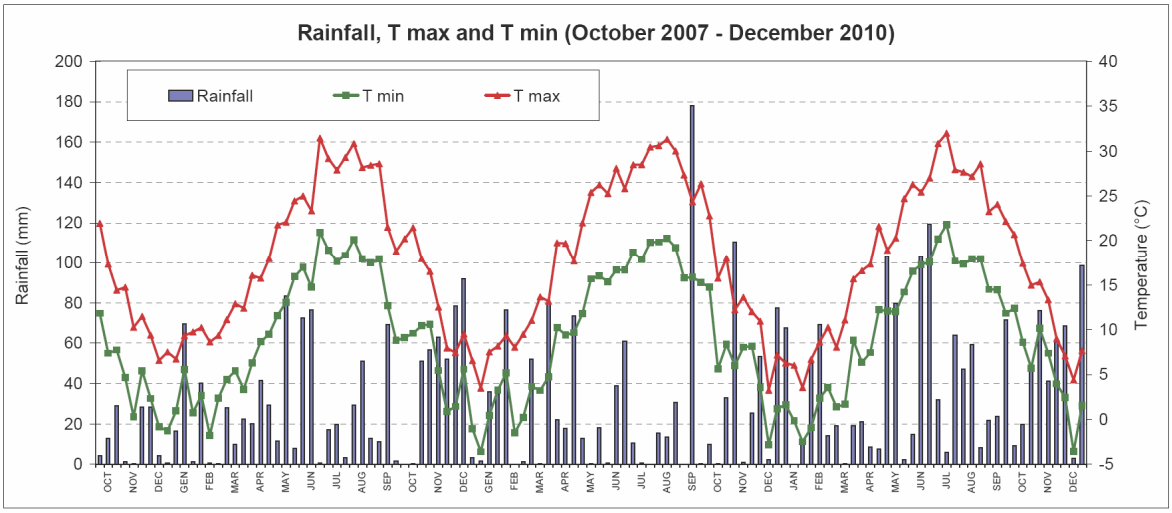


Fig. 3.14 - Rainfall, T. min and T. max registered during the period October 2007 to December 2010 in the experimental site. The rainfall is calculated as sum of 10 days long periods; T. max and T. min is the mean values registered during the same 10 days long periods.

4 NITOGEN REMOVAL IN WATER

4.1 Introduction

4.1.1 Monitoring plan

During the first period (October 1999 - September 2002) the monitoring activity was planned in order to quantify the nitrogen removal from the water pumped from the Zero river and moving through the wooded buffer strips to the drainage ditch. According to this, high frequency (daily and monthly) sampling were performed as well in the Zero river water, as in the ditches and piezometers.

During 2007 – 2008, a similar but less detailed (seasonal sampling) survey was performed in order to see if significant variations in nitrogen removal effectiveness have been happened.

Considering the reduced fluctuations and amount of the nitrogen concentrations in the water of the Zero river and the high effectiveness of the buffer system in removing nitrogen, during 2009 and 2010 the buffer system was forced in order to monitor its response in presence of higher peaks of nitrate in the input water. Four seasonal peaks of nitrogen, 7 days long, were induced by increasing nitrate concentration from about 1,5 - 2 to about 20 - 25 mg/L $N\text{-}NO_3$.

4.1.2 Nitrate addition

To increase nitrate concentration in the irrigation ditch, a solution of diluted KNO_3 (mineral fertilezer “Poni” NK 13-46, by Haifa Chemicals, Israel) was prepared in a tank with a capacity of 500 L and added at the top of the irrigation ditch using a peristaltic pump (Type MSC-WM5, ISMATEC SA, Switzerland) synchronized with the irrigation system pumps (functioning for 1 hour every 3). The total amount of $N\text{-}NO_3$ added during each weekly campaign is reported in the following Tab. 4.1.

Tab. 4.1 – Total amount of $N\text{-}NO_3$ added during the seasonal campaigns in 2009 and 2010. The $N\text{-}NO_3$ concentrations measured in the water of the irrigation ditch before and during the addition are also reported.

Period	Total amount of N-NO3 added (Kg)	mg/L $N\text{-}NO_3$ in the irrigation ditch water before the addition	mg/L N-NO3 in the irrigation ditch water during the addition period (after complete mixing)
23-30 March 2009	31.00	1.70	19.14
11-18 May 2009	46.00	1.50	27.17
28 July – 03 Aug. 2009*	10.50	1.50	8.25
28 Sept. - 05 Oct. 2009	41.00	1.30	24.43
02-09 March 2010	38.00	1.70	22.90
10-17 May 2010	35.00	1.80	21.03
12-19 July 2010	44.50	1.50	26.05
08-15 November 2010	35.50	1.70	21.60

* Low addition due to technical problems

4.1.3 Sampling activity

From 2000 to 2002 the experimental site was monitored in both plots A and B; during 2008-2010 only in plot A.

During the first monitoring period (October 1999 - September 2002), the water pumped from the Zero River into the irrigation ditches was sampled daily as a single discrete sample by using an automatic sampler (American Sigma – Portable sampler 900 standard, with 24 one L bottles, Hach Company, Loveland, Colorado, USA). The irrigation and drainage ditches and the piezometers were sampled monthly by grab sampling of the general shallow flow. Piezometers were pumped using a hand pump (Kartell –MR 50 H ca. 240ml, Kartell S.p.A., Milan, Italy) first to remove 2 well volumes and then sampled after water had recharged the well.

During the second monitoring period (2007-2010) the water was collected seasonally using the same procedures and instruments. In 2009-2010, during the 7 days long periods of nitrate addition, the water from irrigation and drainage ditches was sampled every two days with a mean of 12 total samples for each season.

4.1.4 Water analysis

Field measurements were made of pH (pH meter handylab 1, Schott-Geräte GmbH, Mainz, Germany), temperature (°C), and Electrical Conductivity (EC) using a Schott-Geräte Conductivity meter handylab LF with integrated temperature sensor.

Water samples for analyses were filtered through a 0.45 µm PVDF filter in the laboratory and analyzed within 24-36 h for $N\text{-}NO_3$, $N\text{-}NO_2$, $N\text{-}NH_4$, total Nitrogen, and chloride.

Dissolved anions (Cl, $N\text{-}NO_3$) were determined by ion liquid chromatography (Pfaff *et al.*, 1997). Dissolved $N\text{-}NO_2$ was determined by the Griess-Illosvay method and spectrophotometric measurements (APHA AWWA WEF, 2005a). Dissolved $N\text{-}NH_4$ was determined by the Indophenol blue Method and spectrophotometric measurements (APHA AWWA WEF, 2005b). Dissolved total N was determined with the persulphate oxidation method (Valderrama, 1981) followed by nitrate analysis. Nitrate was reduced to nitrite by cadmium reduction and determined as explained. Organic N was determined by calculation ($Norg = Ntot - N\text{-}NH_3 - N\text{-}NO_2 - N\text{-}NO_3$).

4.1.5 Water and mass balance

For all the monitored periods, the water balance has been estimated daily using the following formula (for details on hydrological measurements methods see paragraph 3.5):

$$I + R - ET = D$$

where: *I = Irrigation volume; R = Rainfall; ET = Evapotranspiration; D = Drainage back to river*

To obtain the *INPUT*, the measured concentration of different nitrogen forms in the irrigation ditch was multiplied by the irrigation volumes (I).

To obtain the *OUTPUT* the measured concentration of different nitrogen forms in the drainage ditches were multiplied by the drainage volumes (D).

The *RETENTION* rates were obtained by the following equation:

$$RETENTION = 1 - (INPUT/OUTPUT)$$

and expressed as a percentage.

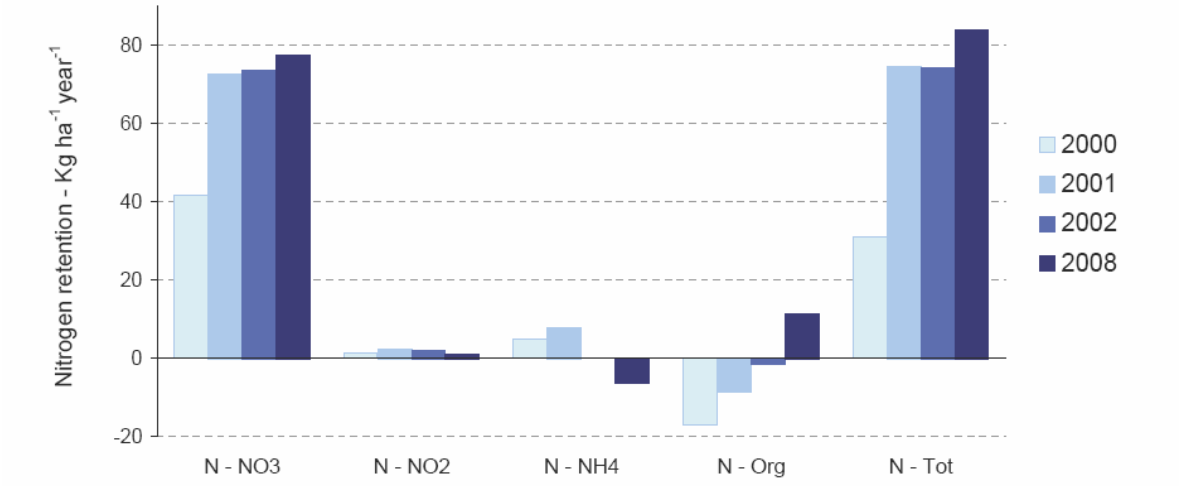
To obtain weekly, monthly, seasonal or annual balances, for the not-monitored days the data were interpolated as follow: the daily INPUT was obtained multiplying the measured irrigation volume by the concentration measured during the sampling date considered representative for that period (for example the one done during the same month or week); the OUTPUT was obtained multiplying the INPUT by the RETENTION rate calculated for that representative date.

4.2 Results

Monitoring from 2000 to 2002.

The total nitrogen entering in the system during the first three years was calculated as ranging from 116 to 135 Kg ha^{-1} $year^{-1}$ (Fig. 4.1).

The nitrate retention capacity increased strongly (from about 40 to 86 %) from the first to the third year (Fig. 4.1), which means from 41.6 to 73.6 Kg ha^{-1} $year^{-1}$. The same trend was evident for total nitrogen, with about 23% removed in the first year and more than 60% during the second and third years. A weak removal of $N\text{-}NO_2$ was observed during the whole period. $N\text{-}NH_4$ and N-Org had a higher annual variability, with the outputs sometimes exceeding the inputs. Note that (Fig. 4.1) the leaching of organic nitrogen in the course of the first year (-152%) decreased considerably in the second and third years (-87% and -11%, respectively).



	2000				2001				2002				2008			
	Input	Output	Retention		Input	Output	Retention		Input	Output	Retention		Input	Output	Retention	
	kg ha^{-1}year^{-1}			%	kg ha^{-1}year^{-1}			%	kg ha^{-1}year^{-1}			%	kg ha^{-1}year^{-1}			%
N - NO_3	107.6	66.0	41.6	**39**	85.0	12.3	72.7	**86**	85.2	11.6	73.6	**86**	102.0	24.5	77.5	**76**
N - NO_2	1.9	0.4	1.5	**77**	2.6	0.3	2.3	**88**	2.5	0.3	2.2	**87**	2.2	1.0	1.2	**53**
N - NH_4	14.7	9.6	5.1	**35**	18.7	10.8	7.9	**42**	18.0	18.1	0.0	**-0.1**	6.4	12.6	-6.3	**-99**
N - Org	11.2	28.2	-17.0	**-152**	9.6	18.0	-8.4	**-87**	12.0	13.3	-1.3	**-11**	43.3	31.8	11.5	**27**
N - Tot	**135.3**	**104.1**	**31.2**	**23**	**116**	**41**	**75**	**64**	**118**	**43**	**74**	**63**	**154**	**69.9**	**84**	**55**

Fig. 4.1 - Combined nitrogen in terms of input, output and retention rates during 2000 – 2002 and 2008.

The Fig. 4.2 shows N-NO_3 concentration in the input water from the river Zero, through the 15 metres of the buffer zone to the drainage ditch, for the monitored years. It is evident that the system did not remove nitrate during the first six months of monitoring. During the following months a considerable reduction of nitrate concentration was observed even at 3-4 metres from the irrigation ditch. This performance was more evident during the warm season (April/May to November), while in the winter period (from December to March) the system was less effective.

Monitoring 2008. During 2008 the retention percentages were similar to those of 2001 – 2002 (Fig. 4.1) and 84 Kg ha^{-1} year^{-1} of total nitrogen were removed. One of the most significant results was the capability to remove even the organic nitrogen instead of leaching it; a percentage removal of 27% was recorded during 2008. On the other hand a significant increase of N-NH_4 leaching was observed, with the output values which resulted twice as the inputs.

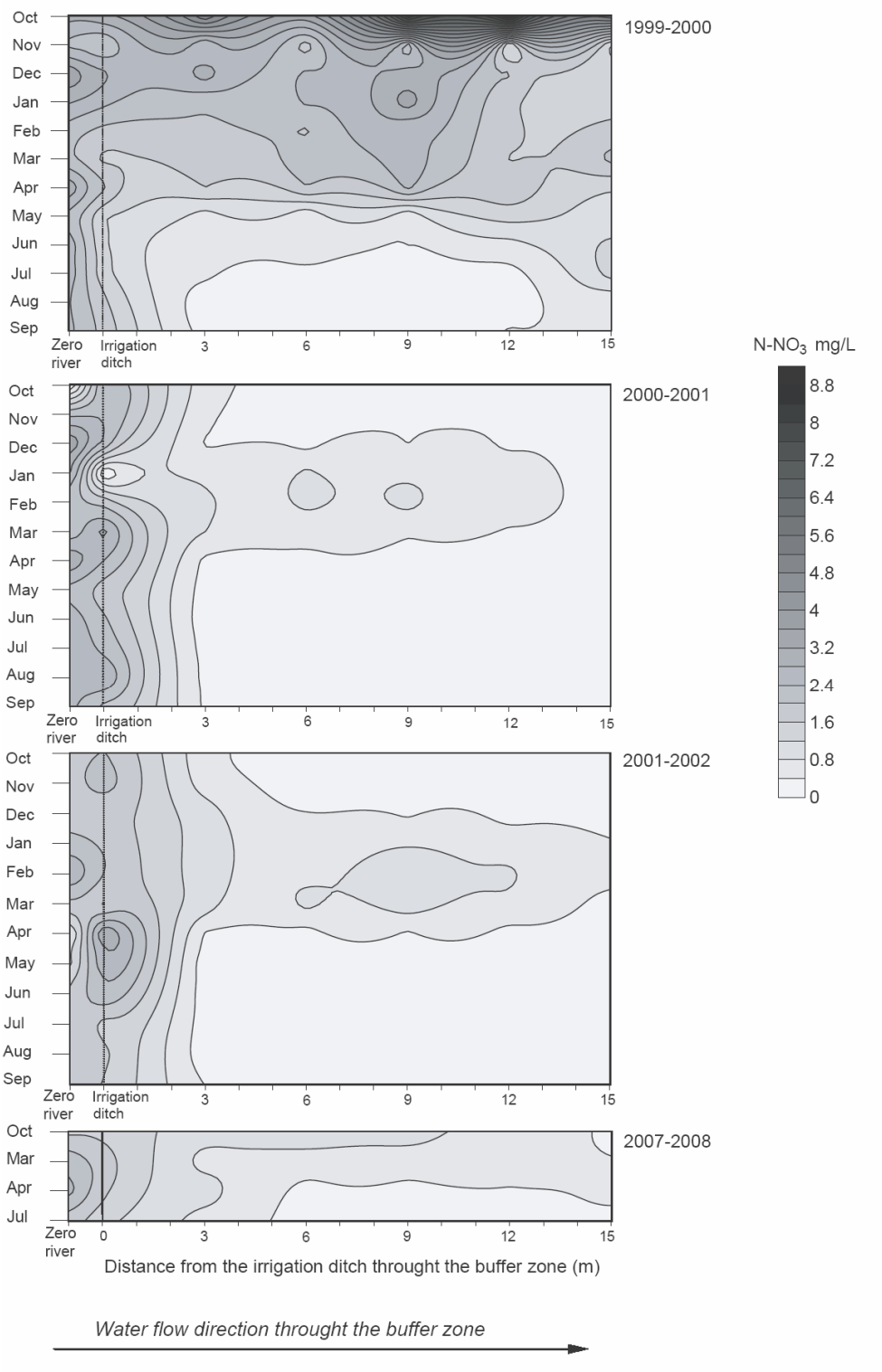


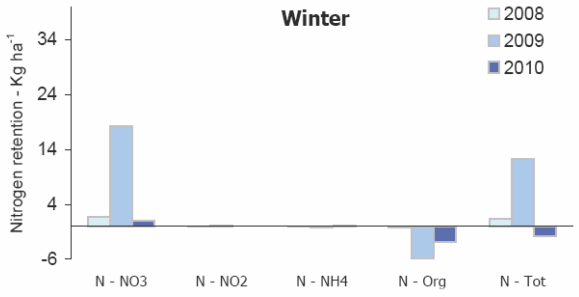
Fig. 4.2 - $N\text{-}NO_3$ concentration in the input water from the river Zero, through the 15 meters of the buffer zone to the drainage ditch, for the monitored years. The grey scale on the right indicates the $N\text{-}NO_3$ concentration in mg/L. Data processing by the software "Surfer® Version 8.01". SSG-Surfer.com, a Division of Scientific Software Group, Sandy, Utah, USA.

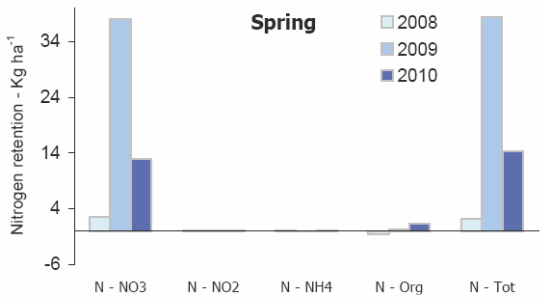
Monitoring from 2009 to 2010

The retention rates measured seasonally and during the seven days period of nitrate addition on 2009 and 2010 in comparison with the standard condition (2008) are reported in the following Fig. 4.3. During 2009 the nitrate retention rates ranged bet Kg ha^{-1}. In term of percentage removal during all the seasons the values resulted very similar to the ones registered in standard conditions (2008). In term of Kg ha^{-1} the retention increased from 1.6 - 2.6 to 18 - 38 Kg ha^{-1} per week.

Whereas the total nitrogen is given almost entirely by nitrate, similar values in term of total nitrogen retention were measured with the exception of winter 2009 when significant losses of organic nitrogen were registered.

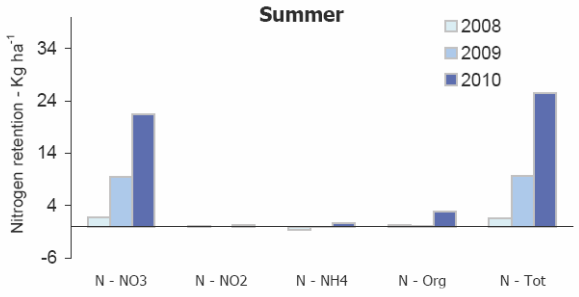
A different situation was observed during 2010, when a significant and general decrease of the nitrate retention rates were measured, ranging between 2% (in winter) and 46% (in summer) and corresponding respectively to a removal of about 2 and 21.44 Kg ha^{-1} per week. Also in this case total nitrogen followed the same trend. Again, during the winter period high losses of organic nitrogen were observed; due to this, the output of total nitrogen in this case exceeded the input.

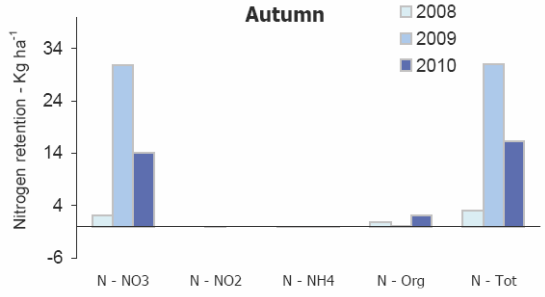




WINTER	RETENTION 2008		RETENTION 2009		RETENTION 2010	
	kg ha^{-1}	%	kg ha^{-1}	%	kg ha^{-1}	%
N - NO_3	1.66	55%	18.22	54%	0.95	2%
N - NO_2	-0.03	-55%	0.05	32%	0.00	7%
N - NH_4	-0.02	-119%	-0.14	-43%	0.13	68%
N - Org	-0.22	-34%	-5.79	-461%	-2.92	-87%
N - Tot	1.39	37%	12.35	35%	-1.83	-4%

SPRING	RETENTION 2008		RETENTION 2009		RETENTION 2010	
	kg ha^{-1}	%	kg ha^{-1}	%	kg ha^{-1}	%
N - NO_3	2.56	80%	38.01	79%	12.93	34%
N - NO_2	0.07	80%	0.08	32%	0.07	41%
N - NH_4	0.15	46%	-0.03	-27%	0.11	73%
N - Org	-0.53	-65%	0.36	13%	1.25	76%
N - Tot	2.25	51%	38.42	75%	14.36	36%





SUMMER	RETENTION 2008		RETENTION 2009		RETENTION 2010	
	kg ha^{-1}	%	kg ha^{-1}	%	kg ha^{-1}	%
N - NO_3	1.76	66%	9.56	75%	21.44	46%
N - NO_2	0.06	69%	-0.01	-21%	0.25	55%
N - NH_4	-0.54	-204%	0.03	25%	0.77	96%
N - Org	0.36	39%	0.06	6%	2.99	86%
N - Tot	1.64	42%	9.64	70%	25.46	49%

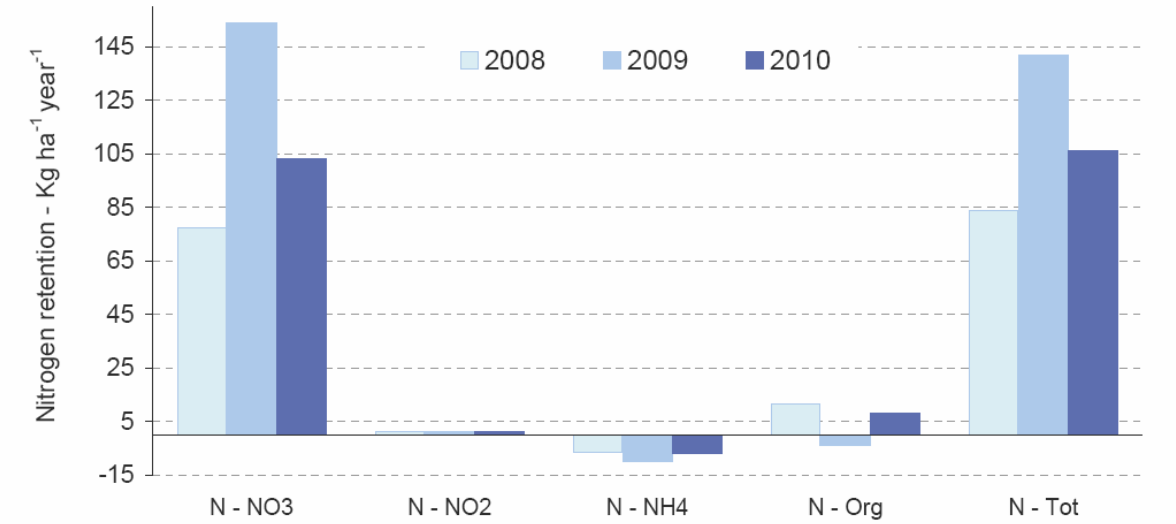
AUTUMN	RETENTION 2008		RETENTION 2009		RETENTION 2010	
	kg ha^{-1}	%	kg ha^{-1}	%	kg ha^{-1}	%
N - NO_3	2.21	96%	30.87	71%	14.18	37%
N - NO_2	0.01	53%	0.03	45%	0.01	25%
N - NH_4	-0.10	-182%	-0.06	-32%	-0.02	-
N - Org	0.95	49%	0.21	16%	2.15	64%
N - Tot	3.07	71%	31.05	69%	16.32	39%

Fig. 4.3 - The retention rates measured during the four seasonal campaigns of nitrate addition (periods 7 days long), during 2009 and 2010 in comparison with the standard condition (2008).

Annual budget of combined nitrogen in terms of input, output and retention rates during 2008 – 2010 are reported in the following Fig. 4.4. Due to the seasonal additions of nitrate, the input increases from about 150 Kg ha^{-1} year^{-1} of total N in 2008 (which represents the standard condition) to 272 and 290 Kg ha^{-1} year^{-1} in 2009 and 2010 respectively. Even if a so significant increase of the inputs occurred, during 2009 the retention rates did not substantially changed. It means that the buffer system was able to remove more than 140 Kg ha^{-1} year^{-1} of total nitrogen instead of the usual 75 – 85 Kg ha^{-1} year^{-1} of the previous

years. The removal of total nitrogen increased also during 2010, reaching a value of about 106 Kg ha^{-1} $year^{-1}$, despite the percent retention of total nitrogen resulted only 37%.

If compared with the results registered during 2000 – 2002 (Fig. 4.1), during 2008 – 2010 we can observe a strong increase of N-NH_4 leaching, with the outputs which constantly exceeded the inputs. The capability to remove even the organic nitrogen instead of leaching it, like during 2001 – 2002, was evident during 2008 and 2010, while small losses were still registered during 2009.



	2008				2009				2010			
	Input	Output	Retention		Input	Output	Retention		Input	Output	Retention	
	kg $ha^{-1}$$year^{-1}$			%	kg $ha^{-1}$$year^{-1}$			%	kg $ha^{-1}$$year^{-1}$			%
N - NO_3	102.0	24.5	77.5	**76**	226.9	72.7	154.2	**68**	241.7	138.2	103.4	**43**
N - NO_2	2.2	1.0	1.2	**53**	2.5	1.4	1.1	**45**	2.7	1.3	1.4	**51**
N - NH_4	6.4	12.6	-6.3	**-99**	6.9	16.6	-9.7	**-140**	6.8	13.5	-6.7	**-99**
N - Org	43.3	31.8	11.5	**27**	35.9	39.8	-3.9	**-11**	39.3	31.1	8.2	**21**
N - Tot	**153.9**	**69.9**	**83.9**	**55**	**272.2**	**130.5**	**141.8**	**52**	**290.4**	**184.1**	**106.3**	**37**

Fig. 4.4 – Annual budget of combined nitrogen in terms of input, output and retention rates during 2008 – 2010.

4.3 Discussion

There are several mechanisms through which excess nitrogen is removed in riparian buffer zones: some act as temporary sinks, for instance soil storage, assimilation and retention by plants and microbes, while the denitrification process permanently removes nitrogen from the soil in a gaseous form (Hefting and de Klein, 1998; Hedin *et al.,* 1998). The two processes, vegetational/microbial uptake of available nitrogen and denitrification, can work together to provide a buffer zone protecting aquatic ecosystems from excessive nitrogen loads (Lowrance *et al*. 1984; Peterjohn and Correll 1984; Pinay *et al.*, 1993; Haycock *et al.* 1997; Lowrance *et al.* 1997; Pinay *et al.* 2000; Sabater *et al.* 2003; Pinay *et al.* 2007; Gumiero *et al.*, 2011).

The results of this study underline how after only one year of conversion from arable land to wooded wetland, the experimental buffer system started to efficiently remove nitrogen loads flowing through, reaching 80-85% of nitrate removal already during the second year (see Fig. 4.1 and Fig. 4.2) corresponding to about 75 Kg ha^{-1} $year^{-1}$ of total N. Such a high effectiveness is similar to those registered in a number of previous studies (e.g. Lowrance *et al.*, 1983; Peterjohn and Correl, 1984; Pinay *et al.*, 1989 ; Hanson *et al.*, 1994; Dhont *et al.*, 2004; Balestrini *et al.*, 2011).

Our results also demonstrates that a maximum buffer strip width of 15 metres can remove an excess of nitrate at concentrations typical of freshwater bodies (less than 5 mg/L $N\text{-}NO_3$), and that narrower buffer strips (e.g. 5 metres wide with only one row of trees) are likely to be adequate (see Fig. 4.2). This was more evident during the warm season (April/May to November), while in the winter period (from December to March) the system was less effective and to reach a complete removal all the 15 meters of riparian buffer were necessary. This is not completely in accordance with previous studies (Osborne and Kovacic, 1993; Mander *et al*., 1997; Hanson *et al.*, 1994) which usually indicate the need of wider (between 15 to 30 meters) riparian buffer to reach so high nitrogen retention rates. To explain this, we have to consider that the studied buffer system is a semi-natural floodplains where water flows has been efficiently managed to support high nitrogen removal by microbial denitrification. In addition we have also to consider that nitrogen is supplied to the system with a low but constant amount, while in natural system nitrogen usually

reaches the riparian buffer through fluctuating and high peaks; constant and low inputs could facilitate the processes involved in nitrogen removal.

An increasing capability of the system to even remove the organic nitrogen instead of leaching it, like it was found to happen during the first 3 years, was observed during 2008 – 2010, while an opposite trend was recorded for $N\text{-}NH_4$, with a considerable increase of the losses during the last three years. It may be due to an increasing capability of the system to mineralize the organic nitrogen coupled to a not corresponding increase of the plant assimilation and bacterial nitrification activities.

The addition of seasonal and high peaks of nitrogen to the system during 2009 – 2010 gave conflicting results. While during 2009 the retention rates did not substantially changed if compared with standard years and the system resulted able to remove higher amounts of nitrate (until 140 Kg ha^{-1} $year^{-1}$), during 2010 it resulted less effective. To explain this result further studies are necessary, but we can suppose it could be related to the combination of different factors: i) a season (winter 2010) with very low temperature (see Tab. 5.2) which inhibited biological processes, ii) the cutting of the first row of trees done during October 2009 with a consequent contraction of the tree roots and iii) the lower values of denitrification rates mainly due to the lowering of the groundwater (see next Chapter).

5 DENITRIFICATION ACTIVITY

5.1 Introduction

The effective removal of nitrate within riparian zones is dependent upon the presence of conditions conducive to high denitrification rates as well as to the growth of vegetation. Denitrification capacity of the soil can be evaluated and possibly enhanced in order to increase nitrogen (N) removal. Exploitation of *in situ* denitrification (DNT) to reduce nitrate loads depends largely on local conditions such as the reduction capacity of the soils, the redox potential, temperature, nitrate concentration and organic carbon availability (Pinay *et al.*, 2006). Denitrification is most active in soils rich in organic matter and having high moisture content and low oxygen (Pinay *et al.*, 1995), all characteristics commonly found in riparian zones.

In this chapter we focus mainly on DNT and denitrification enzyme activity (DEA) to determine the processes responsible for reducing nitrogen in the whole studied system.

5.2 Materials and methods

Denitrification activity was evaluated following protocols and methods of the European project NICOLAS (Nitrogen Control by Landscape Structures in Agricultural Environment - EC DGXII, 1997-2000 ENV4-CT97-0395; see also Burt *et al*., 2002)

5.2.1 Sampling activity

In each plot (A and B) during 1999 – 2002 and only in plot A during 2007-2010, soil samples were collected using a manual drill (Fig. 5.2), from nine different places (three replications for each of the three zones) at three different depths (Surface (S), 0-15 cm; Medium (M) 40-55 cm; and Deep (D) 80-95 cm) (see Fig. 5.1 and Fig. 5.3). Samples were taken seasonally (every three months) for two phases 3 years long (phase 1, from October 1999 to October 2002 and phase 2 from October 2007 to November 2010) with 54 soil samples per season and 24 total sample dates. Zone 3 is close to the irrigation ditch, zone 2 is at mid-distance between the two ditches and zone 1 is close to the drainage ditch.

Only during the last 5 seasonal sample dates (October 2009 and March, May, July and November 2010) soil samples were collected also from three different places (three replications) at three different depths (Surface (S), 0-15 cm; Medium (M) 40-55 cm; and Deep (D) 80-95 cm) from an agricultural area, regularly tilled but left uncultivated during the last two year and bordering the woody buffer zone (Fig 5.4).

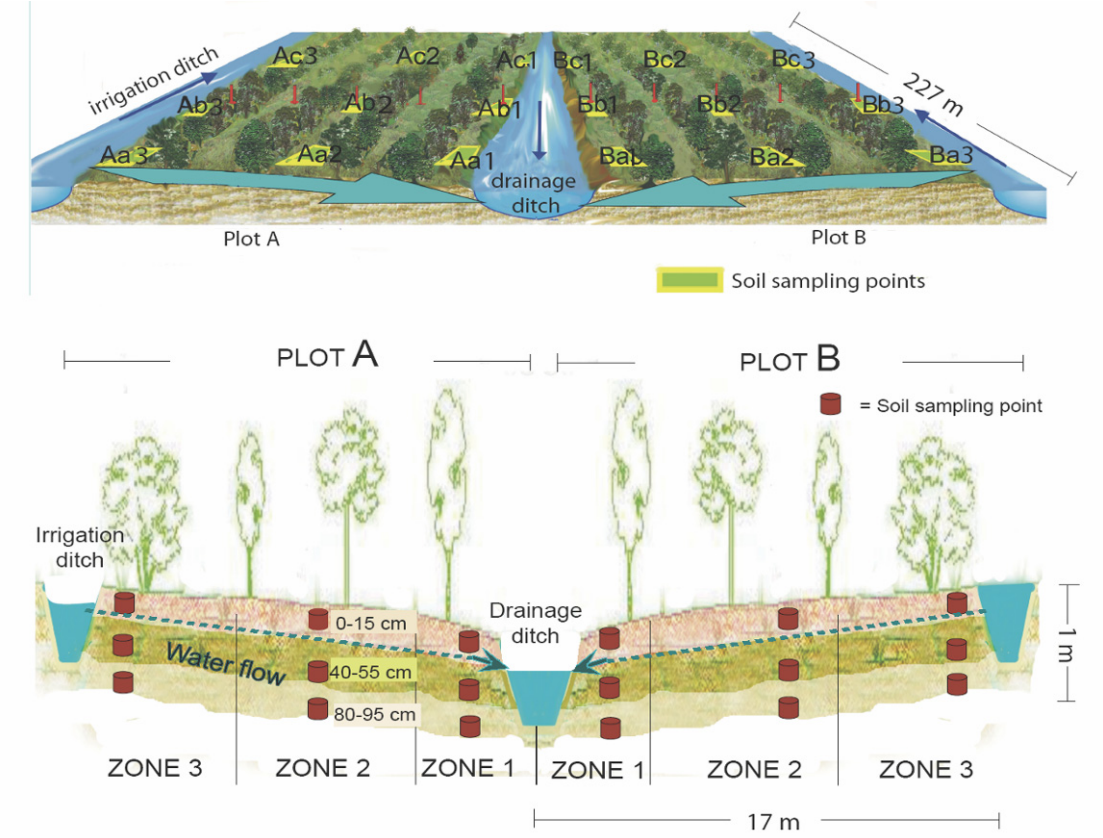


Fig. 5.1 - Perspective (above) and section (below) of the experimental site: each of the 2 plots are watered through an irrigation ditch carrying water from the Zero River. There is a difference in elevation between the irrigation ditches and the drainage ditch, resulting in a sub-surface flow of water running through the wooded buffer strips. Soil sampling in each plot was located at nine points, three for each zone. Zone 3 is close to the irrigation ditch, zone 2 is at mid distance between the two ditches and zone 1 is near the drainage ditch. For each sampling point the soil is sampled at three layers at different depths (S: 0-15 cm, M: 40-55 cm, D: 80-95 cm below the soil surface).

Fig. 5.2 – Soil sampling using a manual drill.

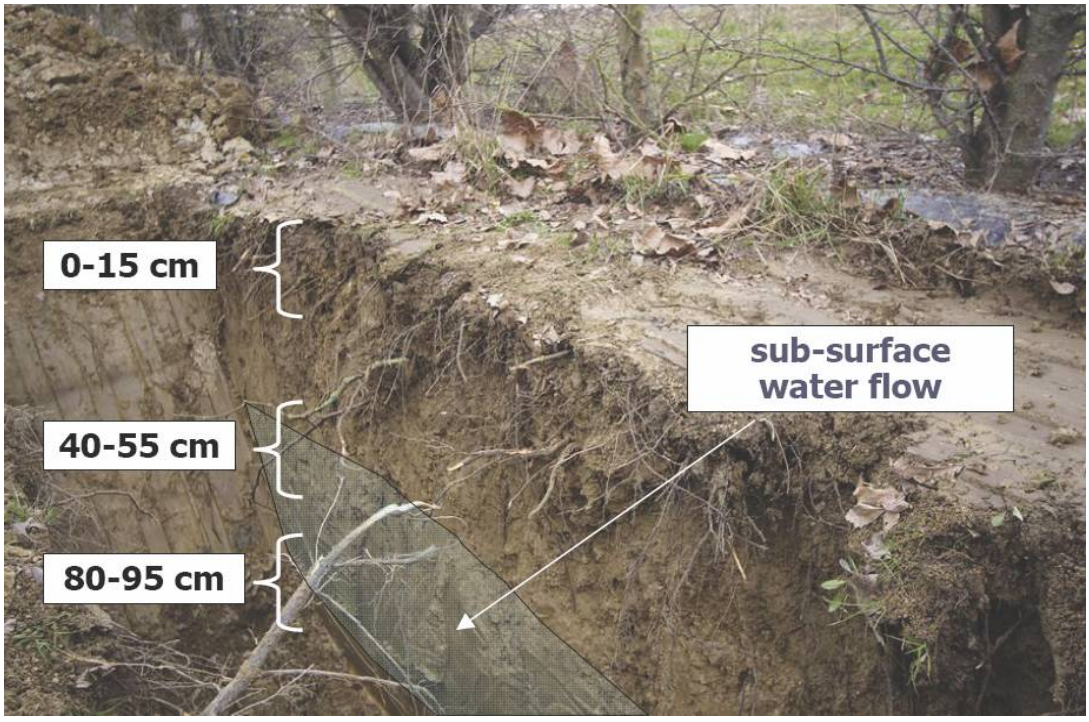


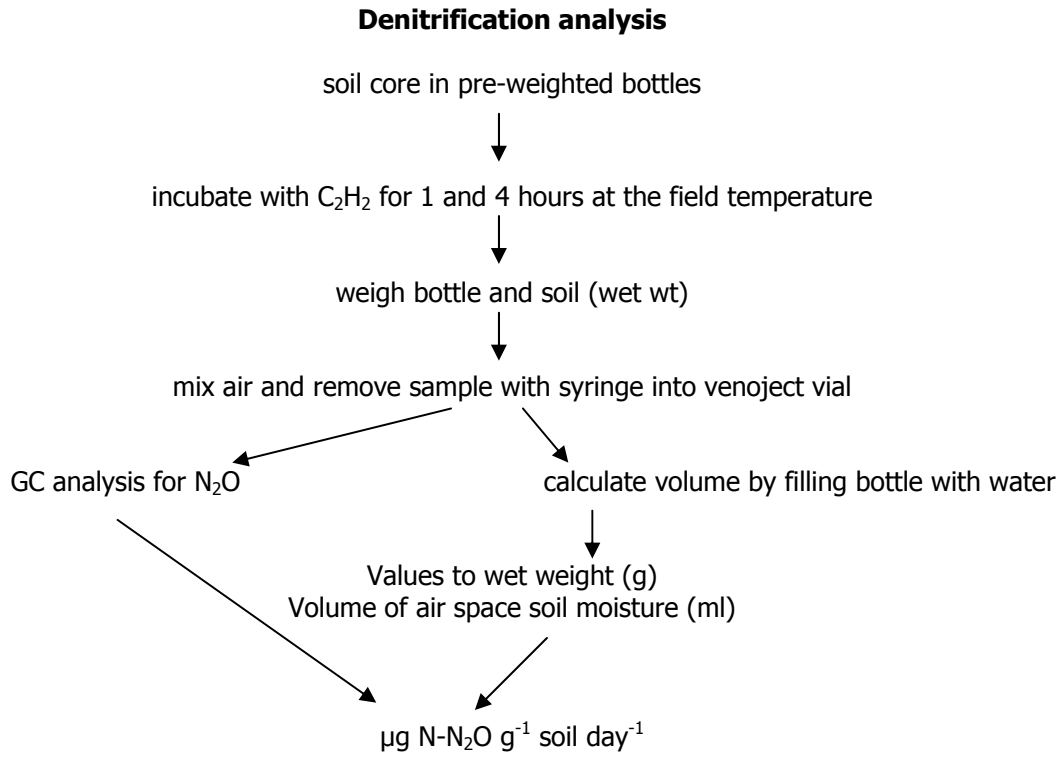
Fig. 5.3 –The three different layers are indicated in the soil profile. While the surface soil layer was subjected to the normal seasonal cycle and rich in organic matter, the medium and depth layers were often saturated because of the presence of the sub-surface water flow.

Fig. 5.4 – The agricultural area, left uncultivated but frequently tilled, bordering the woody buffer zone. The soil of this field has been sampled during 2009 – 2010 to measure denitrification activity.

5.2.2 In situ denitrification (DNT)

In situ denitrification (DNT) was assayed by the static core acetylene inhibition method (Yoshinari and Knowles, 1976). One hundred grams of fresh soil were weighed into glass screw top jars (250 ml) capped with rubber serum stoppers and then amended with acetone-free acetylene to bring soil atmosphere concentration to 10 KPa (10 % V/V) acetylene and 90 KPa air. Samples were incubated at field temperature, and denitrification rates were calculated as the rate of nitrous oxide ($N\text{-}N_2O$) accumulation in the head space between 1 and 4 h. Head space samples were removed from all cores and stored in 10 ml evacuated collection tubes (Venoject, Terumo Europe N.V., Leuven, Belgium). Gas samples were analysed via gas chromatography (Trace GC 2000, Thermo Fisher Scientific Inc.), equipped with an electron capture detector (ECD ^{63}Ni) and a VARIAN CP7554 poraPLOT Q (VARIAN Inc.) column (27.5 m x 0.53 mm, film 20 µm).

The main steps of methodology are summarized in the following scheme:



5.2.2.1 Remarks and comments on the method

Denitrification is the microbial reduction of nitrogenous oxides to gaseous nitrogen (Tiedje, 1982):

NO_3^-	→	NO_2^-	→	[NO]	→	N_2O	→	N_2
nitrate		nitrite		nitric oxide		nitrous oxide		dinitrogen

Though nitrogen is the ultimate end-product, in soils the process is often partially inhibited before complete reduction has occurred, resulting in release of nitrous oxide to the atmosphere. Addition of acetylene to the soil, however, can result in a complete inhibition of nitrous oxide reduction to dinitrogen (Yoshinari and Knowles, 1976). Therefore the production rate of nitrous oxide in the presence of acetylene is commonly used as a measure of denitrification activity in soils.

Although the basic technique is simple to perform, an appreciation for the errors inherent in the technique is fundamental to deriving meaningful results.

The Annexes (Soil analysis by Pinay, G., Dowrick, D., Clément, J.C. and Troccaz, O.) of the monitoring protocol of NICOLAS project (see Burt, 2002), contains some important remarks and comments on the method.

For instance, it's important to consider that acetylene from a cylinder can contain contaminants (see Tiedje, 1982), such as acetone and carbon monoxide, which should be scrubbed from the gas before use by passing it through a solution of cupric chloride in concentrated hydrochloric acid, and then water (Walter *et. al.,* 1979). An alternative is to produce acetylene from the action of water on calcium carbide, which does not produce significant quantities of either substrates for denitrifiers or denitrification inhibitors (from Tiedje *et. al.,* 1989; see Hyman and Arp, 1987).

Moreover, during acetylene addition the chamber should be vented to maintain atmospheric pressure.

In soils where nitrous oxide is primarily a product of denitrification, emissions should increase linearly until acetylene addition (Fig. 5.5). Following addition of acetylene (1), denitrification produces only nitrous oxide, so the rate of nitrous oxide production increases until inhibition is complete (2). Therefore determination of the nitrous oxide production rate following point (2) should give an accurate measure of denitrification activity in the soil. The rate of nitrous oxide emission can be determined simply by sequential sampling of gas from the chamber headspace through a septum using a syringe and needle. It is important to note that because inhibition following acetylene addition is not instantaneous (curve between 1 and 2), the rate of emission should be determined from the point when inhibition is complete. The nitrous oxide concentration immediately following acetylene addition is often used as the starting point. For long incubations this may make little difference, because inhibition may reach completion after only, for example, 15 minutes. For short-term incubations of, for example, a few hours, this lag may be critical. Because analysing gas samples is time consuming and expensive, the number of samples taken from the chamber headspace to determine linearity is usually kept to a minimum. Though at least three points are needed to demonstrate that emissions have reached linearity, knowledge of the technique and experience of the soils used may make it acceptable to take only two samples, provided that the experimenter is sure of the time taken for acetylene inhibition to

reach completion, etc. It should be noted that acetylene inhibition will reach completion at different times in different soils, dependent on the soil sample size, soil water content, etc.

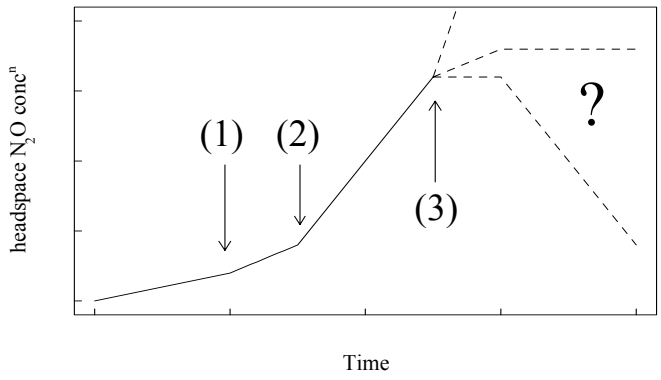


Fig. 5.5 - General pattern of nitrous oxide emission from a soil sample, before and following acetylene addition, when the major source of nitrous oxide is denitrification.

Following complete inhibition linearity will not continue for ever (3). Incubation for longer than a few days may result in the biodegradation of acetylene (Terry and Duxbury, 1985), or the exhaustion of the nitrate source for denitrification, because acetylene inhibits nitrification too (Hynes and Knowles, 1978; Walter *et. al.,* 1979). This can be a serious problem in soils where nitrification and denitrification are 'coupled', that is, any nitrate which is produced is immediately denitrified. In these cases, acetylene addition will immediately decrease nitrous oxide emission because of an inhibition of nitrate production by nitrification. This may be a problem in some waterlogged soils, such as lake sediments (Knowles, 1979) and salt marshes (Van Raalte and Patriquin, 1979). Homogenisation of soil samples prior to the determination of denitrification activity can also increase carbon availability or change the soil oxygen status, which are important controls on denitrification (see Tiedje *et. al.,* 1989).

Acetylene can also decrease nitrous oxide emissions from agricultural soils where a substantial amount of the nitrous oxide produced may be a by-product of nitrification (Lipschultz *et. al.,* 1981), an important source of nitrous oxide from drier soils. This is only a problem if the initial rate of production is to be related to denitrification activity, for example, to work out the ratio of denitrification products (N_2:N_2O). If the nitrous oxide

production rate from denitrification prior to acetylene addition is required, the rate of nitrous oxide production from nitrification can also be determined using a modification to the acetylene inhibition technique (see Davidson *et. al.,* 1986).

So, longer incubation periods are not necessarily better than shorter periods. A gas chromatograph fitted with an electron capture detector should be able to easily detect all but the lowest rates of nitrous oxide production over incubation periods of a few hours or even less. In fact, longer incubation times may not reflect denitrification activity because of other measurement effects. Though denitrification activity in a soil sample may be constant over a period of time, sealing the sample in a chamber may not result in a constant rate of nitrous oxide emission over that period (Fig. 5.6). This may be caused by a decreased concentration gradient between the air and the soil decreasing the rate of gas diffusion from the soil surface to the atmosphere (Livingston and Hutchinson, 1995), though decreases in carbon dioxide and methane emissions from soils over the longer-term have also been attributed to a release of inhibitory substances into the chamber headspace (see Magnusson, 1993). Chambers with a small volume: soil surface area (or soil volume) ratio are more susceptible to this error. Therefore it is important to fully determine the impact of a chamber design on trace gas flux from any soil types used in a study.

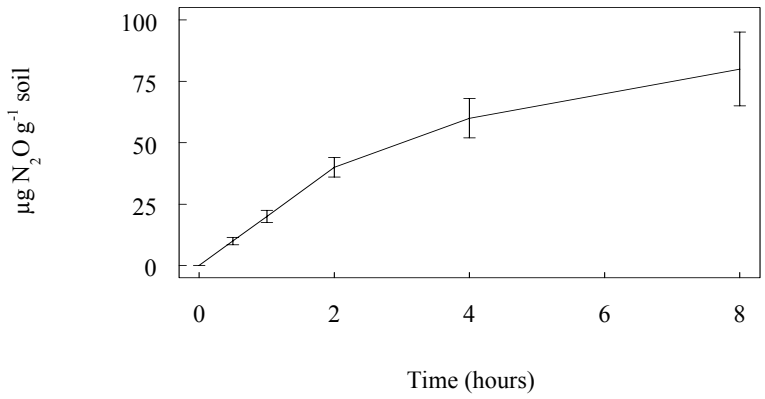


Fig. 5.6 - Nitrous oxide emissions from soil incubated inside a sealed chamber over an eight hour period.

To sum up, in studies on soil microbial denitrification it is important to consider the time taken for acetylene to diffuse into the soil and inhibit N_2O reduction when determining rates of denitrification activity.

Incubate soils in the presence of acetylene for short time periods (up to a few hours) rather than long periods (many hours or days). This will minimise both the effects of long-term acetylene exposure on the soil microorganisms and any decrease in the rate of trace gas emission caused by sealing soil samples in chambers. If samples need to be incubated for, say, a day or two prior to determination of denitrification activity, bottles can be incubated uncapped, then capped, acetylene added and the rate determined within a few hours.

These are not strict rules- it is often possible to incubate soils in the presence of acetylene for a number of days with little ill-effect. However, an increased awareness of the problems associated with determining soil denitrification activity will not only give better results, but also minimise the amount of work required to perform the assay.

5.2.3 Denitrification enzymatic activity (DEA)

Denitrification enzymatic activity (DEA) was measured for each soil sample using Smith and Tiedje's procedure (1979) (see also Groffman *et al.,* 1999). In the laboratory, four sets from each soil sample (30 g of fresh soil) were transferred into glass screw top jars (250 ml) capped with rubber serum stoppers, flushed with N_2 and incubated for 8 h with acetone-free acetylene to bring soil atmosphere concentration to 10 KPa (10 % V/V) acetylene and 90 KPa N_2. One set was amended only with nitrate (10 μg $N\text{-}NO_3$ g^{-1} fresh soil) and referred to as DEA+N; a second set was amended with organic carbon (4 mg C-glucose g^{-1} fresh soil) referred to as DEA+C; a third set was amended with both organic carbon and nitrate (10 μg $N\text{-}NO_3$ g^{1} and 4 mg C-glucose g^{-1}), referred to as DEA+N+C. The last set was incubated with only acetylene under N_2 atmosphere and referred to as DEA. All the samples were under water-saturated conditions obtained by adding 1 ml of distilled water for each gram of soil. Denitrification rates (μg $N\text{-}N_2O$ g^{-1} soil day^{-1}) were calculated as the rate of nitrous oxide ($N\text{-}N_2O$) accumulation in the head space between 4 and 8 h.

5.2.4 Additional soil analysis

To better interpret the denitrification activity, each soil sample was further analyzed for soil moisture, organic C, total N, $N\text{-}NO_3$, $N\text{-}NO_2$ and $N\text{-}NH_4$. Soil moisture was determined gravimetrically after drying subsamples at 104 °C for 24 h and by dividing the difference between wet and dry masses by the mass of the dry sample. The organic C content was determined by oxidizing the organic matter with acid dichromate reagent. The excess of chromate left after C oxidation was analyzed by spectrophotometric measurements (Nelson and Sommers, 1982). For determining the $N\text{-}NO_3$, $N\text{-}NO_2$ and $N\text{-}NH_4$ contents, subsamples were extracted with 2M KCl and quantified by the Griess-Illosvay method and spectrophotometric measurement (APHA AWWA WEF, 2005a and 2005b). Dissolved total N was determined with 2M KCl extraction followed by the persulphate oxidation method (Valderrama, 1981).

N microbial immobilization was estimated using a simplified version of the Jenkinson and Poulson (1976) fumigation procedure. This method is based on chloroform fumigation, followed by immediate extraction with K_2SO_4 (35 g l^{-1}) and measurement of total N released by chloroform in the soil extract (Brookes *et al.* 1985). The concentration of total N released is measured by using the above mentioned persulphate oxidation method. N microbial immobilization is obtained by the difference between total N measured with and without the fumigation procedure.

5.2.5 Statistical analysis

All the results were statistically analyzed by ANOVA (variance analysis). To analyze the effects of seasons, depth, years and zones (distance from the irrigation ditch) on DNT and on potential denitrification rates, both three-way and two way factorial ANOVA with interaction terms were used. To analyse effects of different sites (riparian buffer and agricultural area) and depth on potential denitrification two-way factorial ANOVA was run. To analyze both DNT and potential denitrification differences depending on zones in the medium layer one-way ANOVA was applied. During 1999 – 2002, only the second and third years were considered because of the lack of some data from the first year; for 2008-2010 all the data were considered.

The paired t test was used to examine differences between DNT and DEA under water-saturated conditions during 2008 – 2010.

All the analyses were conducted using the software "R", a free software environment for statistical computing and graphics.

5.3 Results

5.3.1 *In situ* denitrification (DNT)

The *in situ* denitrification process has been analysed in term of differences between seasons, years, soil layers and zones (see the experimental design at Fig. 5.1). Differences between the two periods 2000 – 2002 and 2008 – 2010 are also described.

5.3.1.1 Differences between seasons

During 2000-2002 the *in situ* denitrification process had a significant variability in the 0-15 cm and 40-55 cm layers, with highest peaks recorded during summer (Fig. 5.8a, Fig. 5.7a). In this season the combination of high temperature, a reduced competition with plant uptake and saturated conditions due to high water table levels, could give the better conditions for denitrification (Gumiero *et al.*, 2011); this concerns in particular the medium layer.

In 2008-2010 seasonal differences in DNT process are still significant (Tab. 5.3), but a clear reduction of denitrification activity during summer and autumn was observed in all the layers (Fig. 5.8b, Fig. 5.7b). This trend was not observed in zone 3, which is located near the irrigation ditch (Fig. 5.9).

This suggests that the inhibition of DNT during the warm seasons is mostly linked to the lower level of the perched aquifer measured during 2008-2010 in zone 2 and zone 1 (Fig. 5.10), probably due to the increase of evapotranspiration in the grown woody buffer system.

Indeed, differently that during 2000-2002 (Fig. 5.10a), in 2008-2010 (Fig. 5.10b) the medium layer in zone 2 and in zone 1 is usually not saturated (except than in Winter 2009). The zone 3 is not affected so much from the increase of evapotranspiration and the medium layer is mostly still saturated.

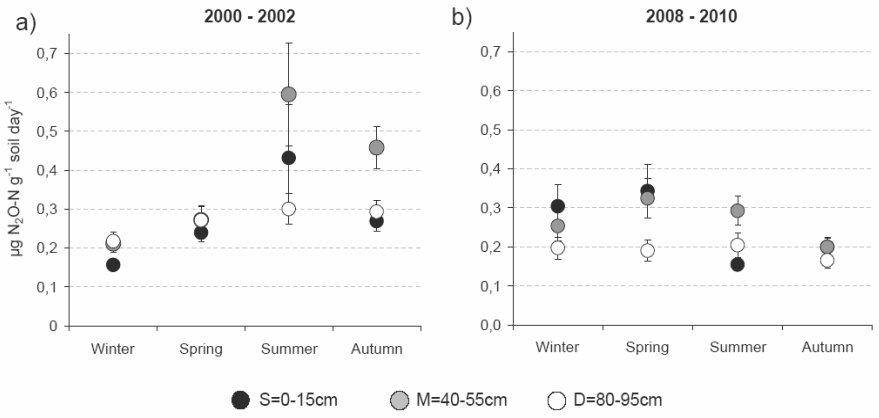


Fig. 5.7 - Denitrification rates, calculated as µg $N\text{-}N_2O$ g^{-1} day^{-1}, for each season and each layer at different depths. The values are the means of all the samples collected during 2000 - 2002 (a) and 2008 - 2010 (b). The vertical bars represent standard errors.

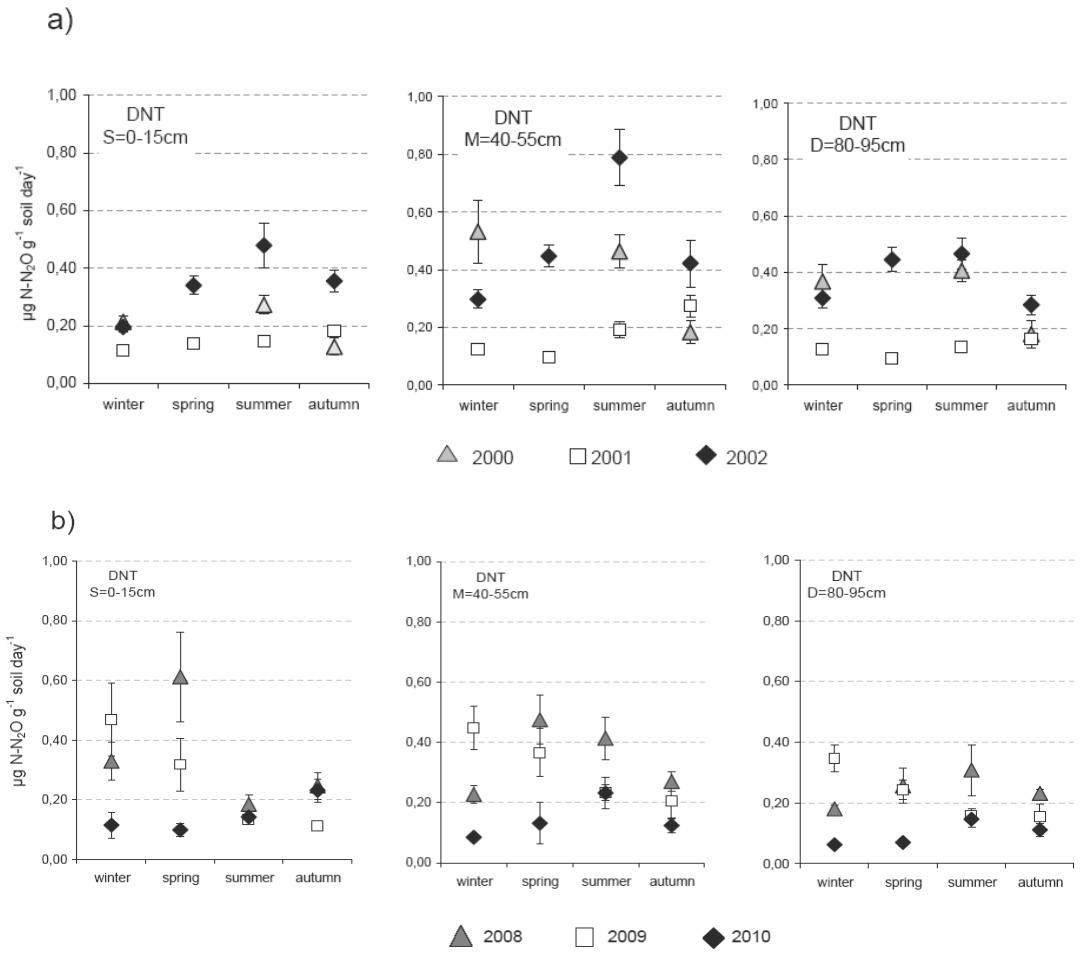


Fig. 5.8 - Denitrification rates, as µg $N\text{-}N_2O$ g^{-1} day^{-1}, for each year, season and layer at different depths during 2000 – 2002 (a) and 2008 – 2010 (b). The values are the means of 18 measurements (nine in each plot) during 2000 – 2002 and only 9 during 2008 – 2010. Bars represent standard errors.

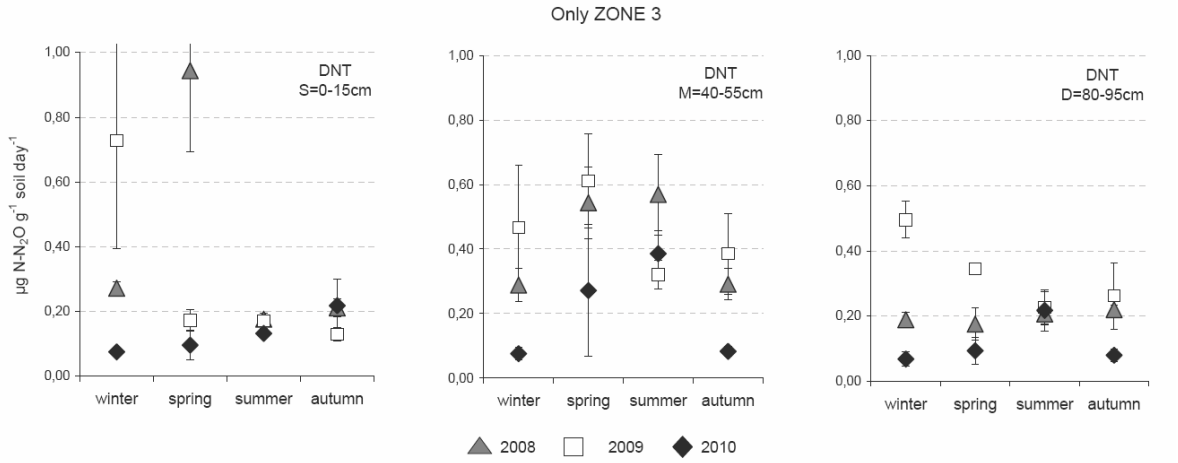


Fig. 5.9 – Denitrification rates, as µg $N\text{-}N_2O$ g^{-1} day^{-1}, for each year, season and layer at different depths during 2008 – 2010. **Only zone 3** (located near the irrigation ditch) is considered. The values are the means of 3 measurements. Bars represent standard errors.

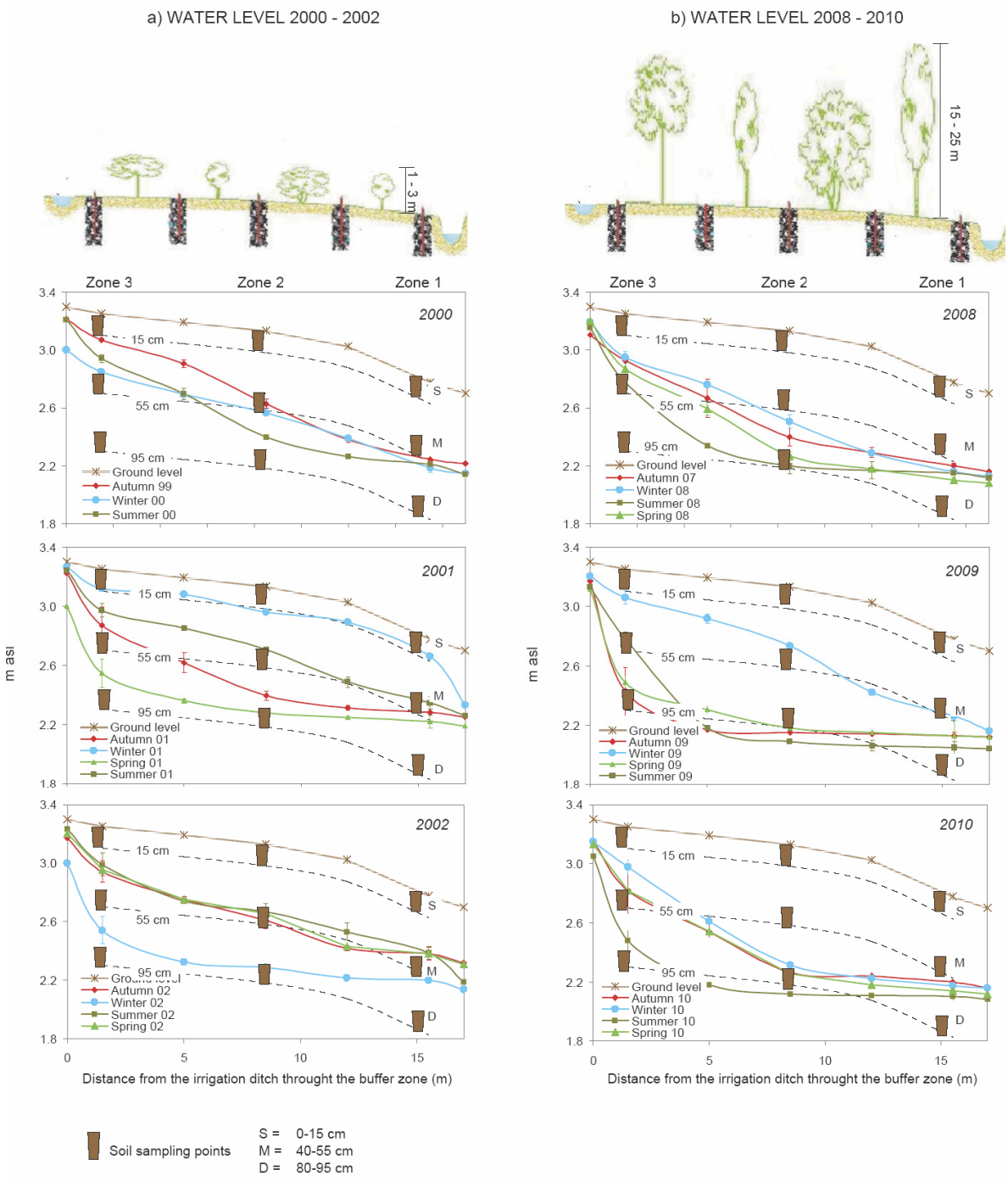


Fig. 5.10 – Ground level and water table elevation measured in plot A (measures in ditches and piezometers) during the seasonal samplings in the 6 monitored years (a = 2000 - 2002 and b = 2008 – 2010). Values for plot B, in 2000 - 2002 were very similar. Bars represent standard error. Soil sampling points are also represented. They are located at nine points, three for each zone. Zone 3 is close to the irrigation ditch, zone 2 is at mid distance between the two ditches and zone 1 is near the drainage ditch. For each sampling point the soil was collected at three different depths layers (S: 0-15 cm, M: 40-55 cm, D: 80-95 cm below the soil surface).

5.3.1.2 Differences between years

If we consider the initial period (2000 – 2002), in all layers the *in situ* denitrification activity was significantly lower during the second year (2001) but increased in the third year (Fig. 5.11). At the start of the experiment, a considerable amount of residual combined nitrogen and organic carbon would have been present in soil (Tab. 5.1), derived from previous agricultural activities.

During the first year, they would have been reduced by leaching, microbial activity and plant uptake. This may account for the limited denitrification activity detected during the second year as compared to the first. This trend may also be due to (i) the higher nitrogen uptake by the plants (a mean of 104 g m^{-2} herbaceous vegetation biomass for the first year as compared to 298 g m^{-2} for the second), which started to grow quickly, thus reducing the amount of inorganic nitrogen available to the denitrifying bacteria and (ii) to the still limited organic carbon released by the young vegetation. Plant colonization and growth stabilized in the third year, hence denitrification activity could take place effectively.

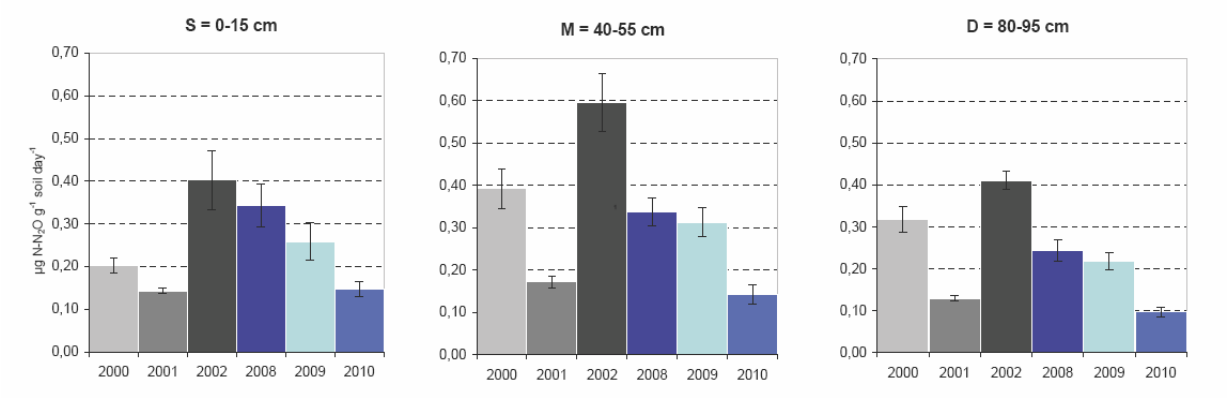


Fig. 5.11 – Overall comparison of annual average of *in situ* denitrification rates (DNT) in the three different layers. The values are the means of 64 measurements for each year during 2000-2002 and 32 during 2008 – 2010. The vertical bars represent standard errors.

Tab. 5.1 - Soil nitrogen (mg N Kg^{-1} soil) and organic carbon (%) content in the three layers of the buffer zone, for the three monitored years (± standard error).

Year	Layer	Inorganic N *mg N Kg^{-1} soil*	Organic C *%*
2000	S (0-15 cm)	4.97 ± 0.56	1.03 ± 0.05
	M (40-55 cm)	5.71 ± 0.68	0.78 ± 0.05
	D (80-95 cm)	4.08 ± 0.45	0.71 ± 0.06
2001	S (0-15 cm)	1.91 ± 0.10	0.87 ± 0.03
	M (40-55 cm)	2.71 ± 0.13	0.55 ± 0.02
	D (80-95 cm)	1.86 ± 0.14	0.31 ± 0.02
2002	S (0-15 cm)	1.97 ± 0.12	0.82 ± 0.02
	M (40-55 cm)	2.40 ± 0.10	0.50 ± 0.02
	D (80-95 cm)	1.86 ± 0.10	0.31 ± 0.02
2008	S (0-15 cm)	2.78 ± 0.29	1.21 ± 0.05
	M (40-55 cm)	2.28 ± 0.17	0.63 ± 0.02
	D (80-95 cm)	1.59 ± 0.13	0.45 ± 0.03
2009	S (0-15 cm)	6.19 ± 0.38	1.35 ± 0.06
	M (40-55 cm)	2.05 ± 0.15	0.57 ± 0.03
	D (80-95 cm)	1.12 ± 0.12	0.41 ± 0.04
2010	S (0-15 cm)	7.91 ± 0.85	1.50 ± 0.09
	M (40-55 cm)	1.92 ± 0.23	0.60 ± 0.04
	D (80-95 cm)	0.60 ± 0.22	0.36 ± 0.03

In the last three years monitoring (2008 – 2010), although with clear annual differences (see Fig. 5.11 and Tab 5.5), a substantial decline in the denitrification process in the intermediate and deep layers was observed. As described in the previous paragraph 5.3.1.1, the main reason for this decrease is the lowering of the level of saturated soil, probably due to increased evapotranspiration. On the other hand, in the surface layer, subject to changes induced by normal climate variations, the decrease of denitrification activity was not evident. This indicates that DNT is mainly influenced by the climatic and hydrological situation occurring at the time of each seasonal sampling.

These general considerations are not able to fully explain the significant reduction in DNT process registered in 2010. Indeed, this seems also to depend from some particular reasons:

(i) during the Winter temperature was very low (Tab. 5.2) and the bacterial activity rather reduced; as a consequence no nitrogen removal in water crossing the woody buffer strip was measured (see Fig. 4.3); (ii) during the Autumn the temperature was also lower than usual (Tab. 5.2) due to the late sampling date (November instead of early October); iii) at the end of November 2009 the first row of trees (containing about the 50% of total biomass, see Fig. 3.1) was cropped; this may have led to greater competition between denitrification process and vegetation uptake from the following spring 2010.

Tab. 5.2 – Sampling dates and corresponding rainfall (total amount in the five days before) and temperature, during the period 2008 – 2010. In bold some important data to consider.

Period		*Date day/month/year*	*Total rainfall 5 days before sampling (mm)*	*External temperature during sampling °C*
2007/08				
	Autumn	16/10/2007	0.0	15
	Winter	10/03/2008	10.0	13
	Spring	28/04/2008	**41.4**	25
	Summer	01/07/2008	0.6	25
2009				
	Winter	30/03/2009	**78.6**	10
	Spring	18/05/2009	0.2	27
	Summer	03/08/2009	0.0	30
	Autumn	05/10/2009	0.8	23
2010				
	Winter	08/03/2010	13.0	**3**
	Spring	17/05/2010	**59.0**	20
	Summer	19/07/2010	6.0	35
	Autumn	15/11/2010	15.0	**9**

5.3.1.3 Differences between layers

In 2000-2002 denitrification rates differed significantly ($P<0.05$) (Tab. 5.3 and Fig. 5.8a) among soil layers; the highest denitrification rates took place in the 40-55 cm soil depth layer (Fig. 5.12a) that is placed on the border between the saturated and unsaturated zones. In 2008 – 2010 the situation was very similar if we consider only the zone 3 (Fig. 5.12c), while there were no significant differences between surface and medium layer if the whole system is considered (Fig. 5.12b). This is another interesting confirmation of the effects of

groundwater level decrease observed during 2008 - 2010 and of the dependence of denitrification process from water-table elevation. The ANOVA analyses also suggested that although there are high intra-factors differences among the three factors they do not affects each other because no significance interactions was found (Tab. 5.4).

In addition, during 2008 – 2010 the surface layer appeared to be more suitable for denitrification, with high peaks registered after heavy rainfall; see for example the peaks registered in Winter 2009 and Spring 2008 (Fig. 5.8b) and the corresponding rainfall (Tab. 5.2). The increased availability of organic carbon and inorganic nitrogen due to vegetation supply (Tab. 5.1), if anoxic conditions occur, supports high peaks of denitrification. The wide standard error suggests that this is not representing a general condition of surface layer but it could be peculiar of only particular places (hotspots).

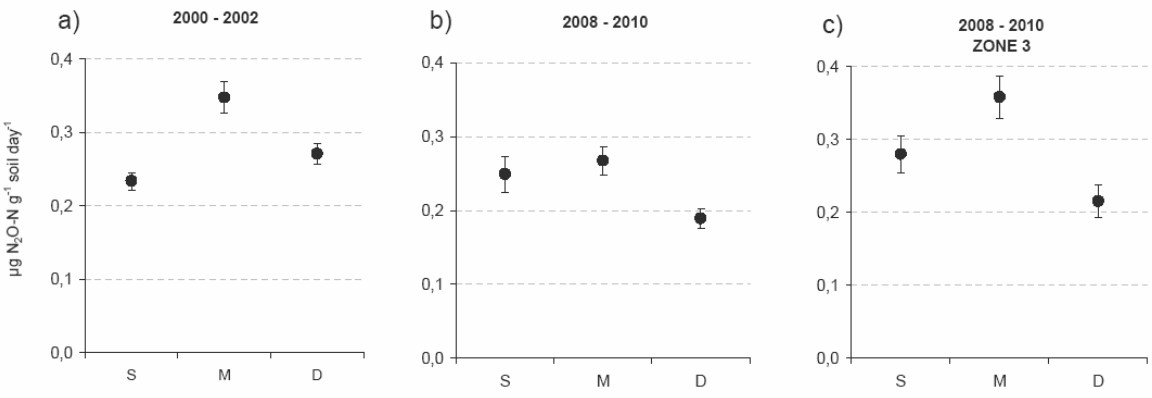


Fig. 5.12 - Denitrification rates calculated for the three layers at different depths (S: 0-15 cm, M: 40-55 cm, D: 80-95 cm below the soil surface). The values are the means of all the samples collected during 2001 and 2002 (a); during 2008 – 2010 (b); during 2008 – 2010 but **only in zone 3** (c). The vertical bars represent standard errors.

Tab. 5.3 - Three-way ANOVA exploring the differences in denitrification rate during 2001 - 2002; the factors include seasons, soil depth (layers) and distance from irrigation ditch (zones). Significant relationships and the level of significance are indicated by 0.05=* and 0.001 = **.

Three-way ANOVA 2000 - 2002		*df*	*mean square*	*F*	*P*
MAIN EFFECTS	*(combined)*				
	Seasons **	3	1.225	8.846	0.000
	Layers *	2	0.600	4.334	0.014
	Zones	2	0.346	2.497	0.084
2-way interaction	*(combined)*				
	Seasons x Layers	6	0.206	1.485	0.182
	Seasons x Zones *	6	0.322	2.324	0.032
	Layer x Zones	4	0.110	0.796	0.529
3-way interaction					
	Seasons x Layers x Zones	12	0.067	0.486	0.923

Tab. 5.4 - Three-way ANOVA exploring the differences in denitrification rate during 2008 - 2010; the factors include seasons, soil depth (layers) and distance from irrigation ditch (zones). Significant relationships and the level of significance are indicated by 0.000=***; 0.001 = **; 0.01=*.

Three-way ANOVA DNT - 2008-2010		*df*	*mean square*	*F*	*P*
MAIN EFFECTS	*(combined)*				
	Seasons *	3	0.148	3.825	0.010
	Layers **	2	0.184	4.766	0.009
	Zones **	2	0.200	5.181	0.006
2-way interaction	*(combined)*				
	Seasons x Layers	6	0.076	1.967	0.070
	Seasons x Zones	6	0.015	0.402	0.877
	Layers x Zones	4	0.044	1.136	0.340
3-way interaction					
	Seasons x Layers x Zones	122	0.028	0.715	0.737

Tab. 5.5 - Three-way ANOVA exploring the differences in denitrification rate during 2008 - 2010; the factors include years, soil depth (layers) and distance from irrigation ditch (zones). Significant relationships and the level of significance are indicated by 0.000=***; 0.001 = **; 0.01=*.

Three-way ANOVA DNT - 2008-2010		*df*	*mean square*	*F*	*P*
MAIN EFFECTS	*(combined)*				
	Years ***	2	0.958	28.159	0.000
	Layers **	2	0.184	5.422	0.005
	Zones **	2	0.200	5.893	0.003
2-way interaction	*(combined)*				
	Years x Layers	6	0.019	0.554	0.696
	Years x Zones	6	0.051	1.501	0.202
	Layers x Zones	4	0.044	1.292	0.273
3-way interaction					
	Years x Layers x Zones	82	0.021	0.630	0.752

5.3.1.4 Differences between zones

Denitrification activity in soil samples coming from the three different zones (see Fig. 5.1) was evaluated and compared. Unlike the first three years, in 2008-2010 there is, overall, a significant ($p<0.006$) difference between the three zones (Tab. 5.4 and Tab. 5.5). This can not be attributed to the superficial and deep layers, where no clear differences between zones were observed (Fig. 5.13). On the contrary, as regards the middle layer, although some differences between the most active seasons (summer and autumn) in zone 3 and zone 1 and 2 had already been observed during the first three years (Fig. 5.14), it was much more evident (Fig. 5.15a) over the last three years during all the seasons. In addition to reasons related to the different level of the water table already discussed above, by looking at Fig. 5.15 (b) it's possible to appreciate how the higher DNT activity in zone 3 may also depend on the greater availability of organic carbon. Considering that during 2000 – 2002 no significant differences in carbon distribution between zones were observed, this is linked to the presence of species with more rapid growth in row 1 (Fig. 3.11) and to a greater transport capacity of carbon to the deeper soil layers.

The different availability of nitrate in zone 3 and 2 if compared with zone 1, could also play a role (Fig. 5.15b). On the contrary the quantitative distribution of microbial community is not different in the 3 zones (Fig. 5.15d), even if a qualitative difference can't be excluded.

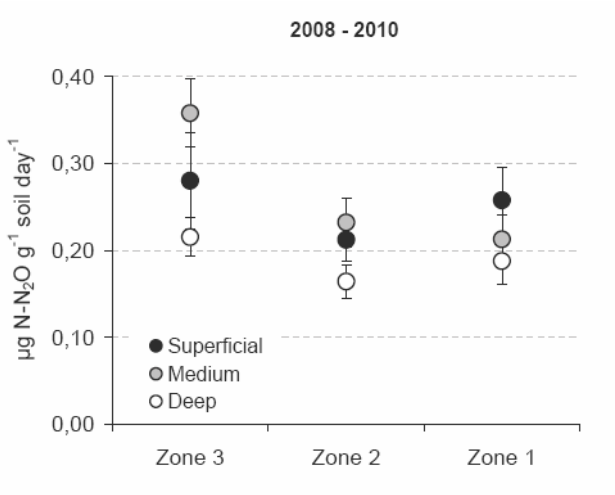


Fig. 5.13 - Comparison of the denitrification rates, as µg $N\text{-}N_2O$ g^{-1} day^{-1}, of the three Zones in the three different layers during 2008 and 2010. The vertical bars represent standard errors.

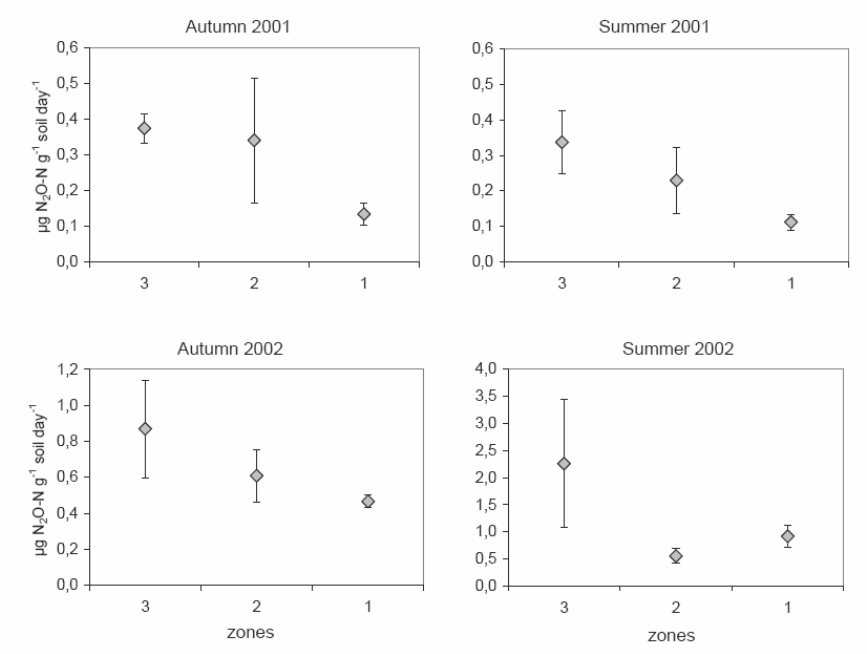


Fig. 5.14 - Comparison of the denitrification rates, as µg $N\text{-}N_2O$ g^{-1} day^{-1}, of the three Zones during Autumn and Summer of 2001 and 2002 years. The vertical bars represent standard errors.

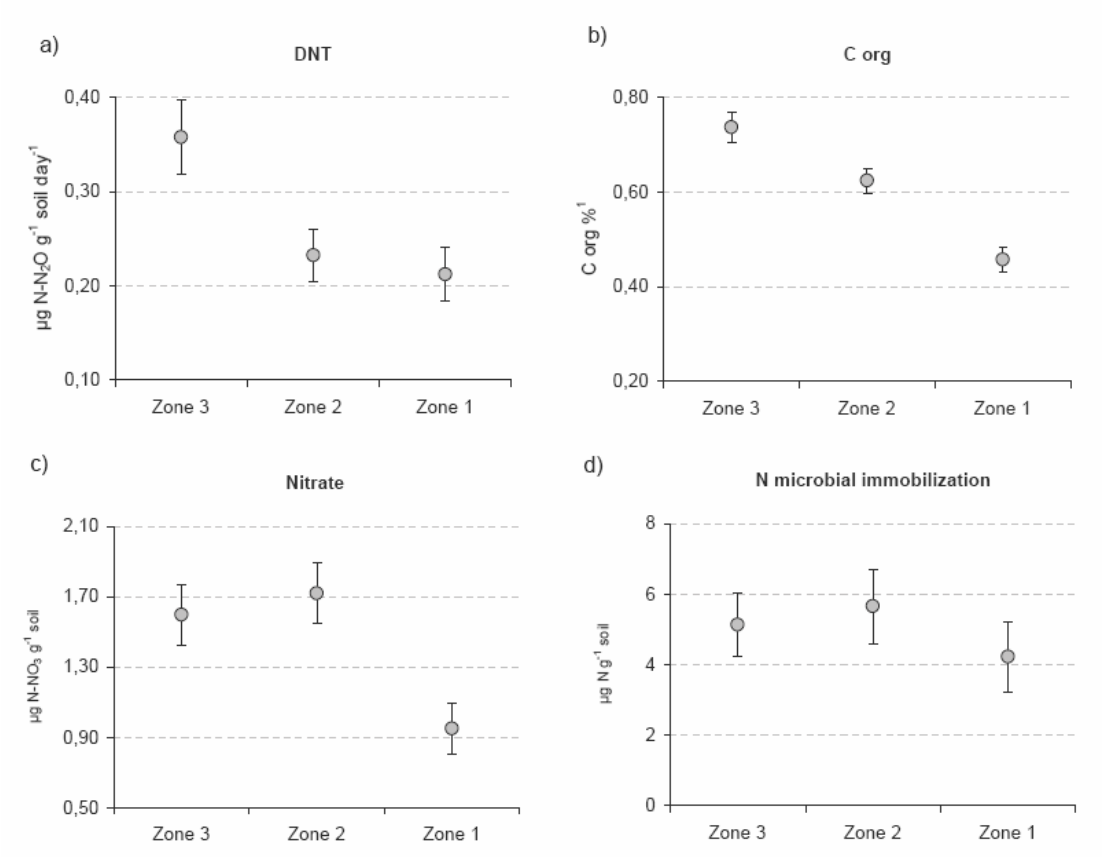


Fig. 5.15 - Comparison of the *in situ* denitrification rates a), organic carbon content b), nitrate availability c) and N microbial content d) in the three different zones during 2008 – 2010 in the medium (40-55 cm) layer. The vertical bars represent standard errors.

5.3.2 Denitrification enzymatic activity

5.3.2.1 Comparison between DNT and Denitrification enzymatic activity

The set incubated under anaerobiosis (referred to as DEA) was compared with *in situ* denitrification rates (DNT) for each year in the three different layers (Fig. 5.16d and Fig. 5.17d). During 2000 – 2002 there was no appreciable increase in denitrification activity; during 2008 – 2010 significant ($t = -12.5351$, $df = 323$ and p-value $< 2.2e^{-16}$) differences were observed in all the layers (Fig. 5.17d). In surface layer these differences have to be attributed mostly to the increase in carbon and nitrogen availability (Tab. 5.1, Fig. 5.21 and Fig. 5.22). In medium and deep layers they are depending more from the reduction of DNT (see Fig. 5.11) than from the slight increase of DEA.

For each year in the three different layers DEA was also compared with sample under anaerobiosis and amended with both organic carbon and nitrate (DEA+N+C in Fig. 5.16a and Fig. 5.17a), only with nitrate (DEA+N in Fig. 5.16b and Fig. 5.17b) and only with organic carbon (DEA+C in Fig. 5.16c and Fig. 5.17c).

During 2000 – 2002 (Fig. 5.18), the 0-15 cm soil layer had the maximum potential for denitrification through enzymatic activity (DEA+N+C) (Fig. 5.16a). Similar results were observed also during 2008 – 2010 (Fig. 5.17a and Tab. 5.6). This potential dramatically decreases going from surface to deeper soil layers and it's mostly related to the distribution of microbial population living in the soil (Fig. 5.19 and Fig. 5.20). Also note that this potential, in surface and medium layers, has gone increasing starting from the first two years (Fig. 5.18a and b) until the third year, while, during 2008 – 2010, no further significant increase was observed.

The addition of nitrate alone (DEA+N) to the samples (Fig. 5.16b) resulted in increased denitrification in the 0-15 cm soil layer only. During 2008 – 2009 this trend results still more evident (Fig. 5.17b and Tab. 5.7); this confirms that the limiting factor for the 0-15cm is nitrate and that DEA+N is correlated to carbon availability on soil (Fig. 5.23).

Adding only glucose (DEA+C) to the soil resulted in a significant increase in denitrification rates in all the layers during the first year but this effect was limited to the medium layer for the second and third years (Fig. 5.16c). The same response was also recorded in the

analysis carried out during 2008 – 2010 (Fig. 5.17c and Tab. 5.8) where the limiting factor for the 40 – 55 cm layer is still the availability of organic carbon.

In summary, i) the limiting factor for the 0-15 cm soil layer, appears to be the availability of nitrate (Fig. 5.16b and Fig. 5.17b); ii) the limiting factor for the 40-55 cm layer is the availability of organic carbon (Fig. 5.16c and Fig. 5.17c); iii) microbial activity typically decreases toward the deep layer (80-95 cm) with no clear limiting factors (Fig. 5.16a, b, c and Fig. 5.17a, b, c).

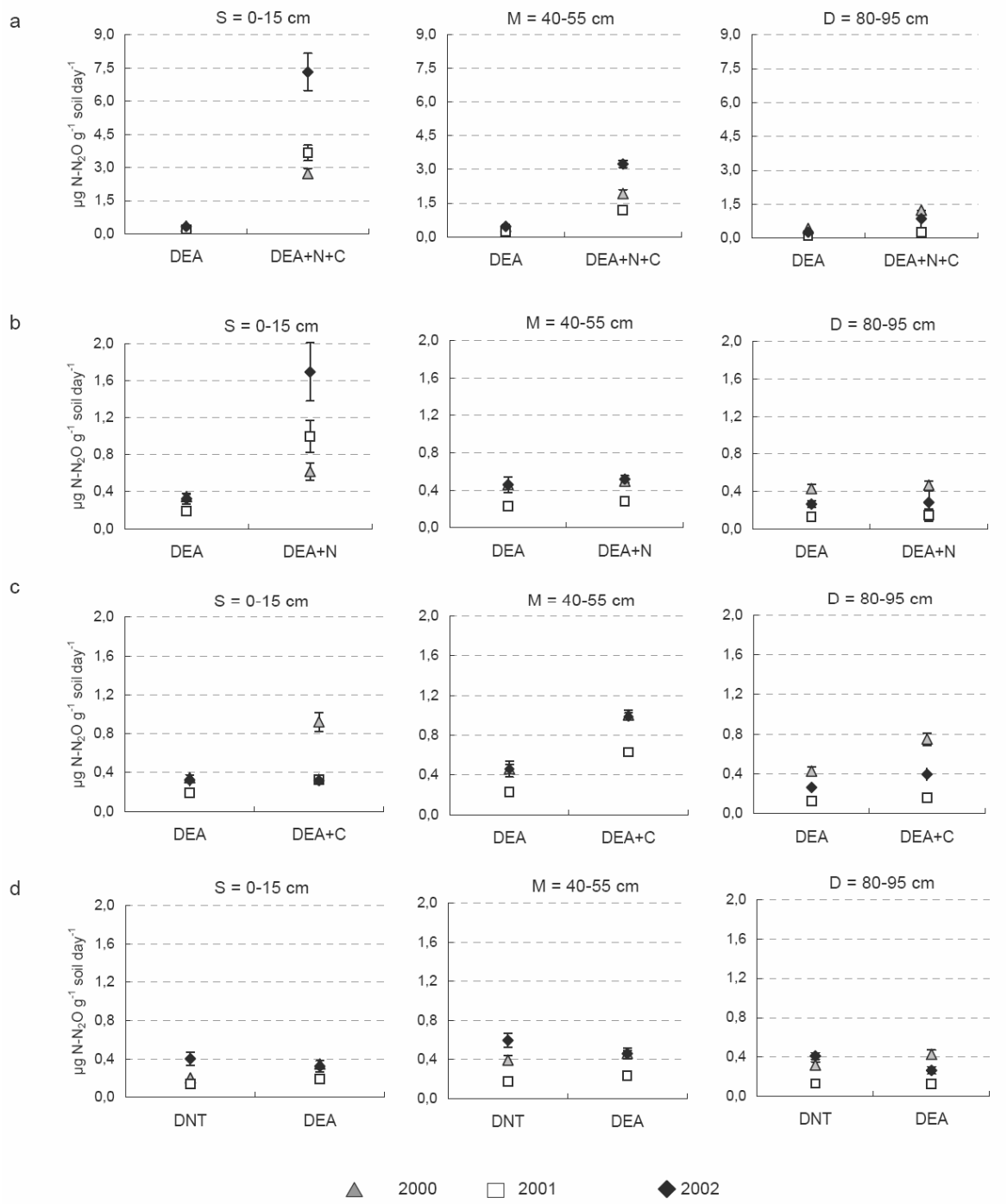


Fig. 5.16 - Comparison of annual average (during 2000 – 2002) in the three different layers between potential denitrification obtained after incubation under anaerobic conditions without any addition (DEA) and potential denitrification with the addition of (a) both nitrate and organic carbon (DEA+N+C), (b) only nitrate (DEA+N), (c) only organic carbon (DEA+C) and (d) *in situ* denitrification rates (DNT).

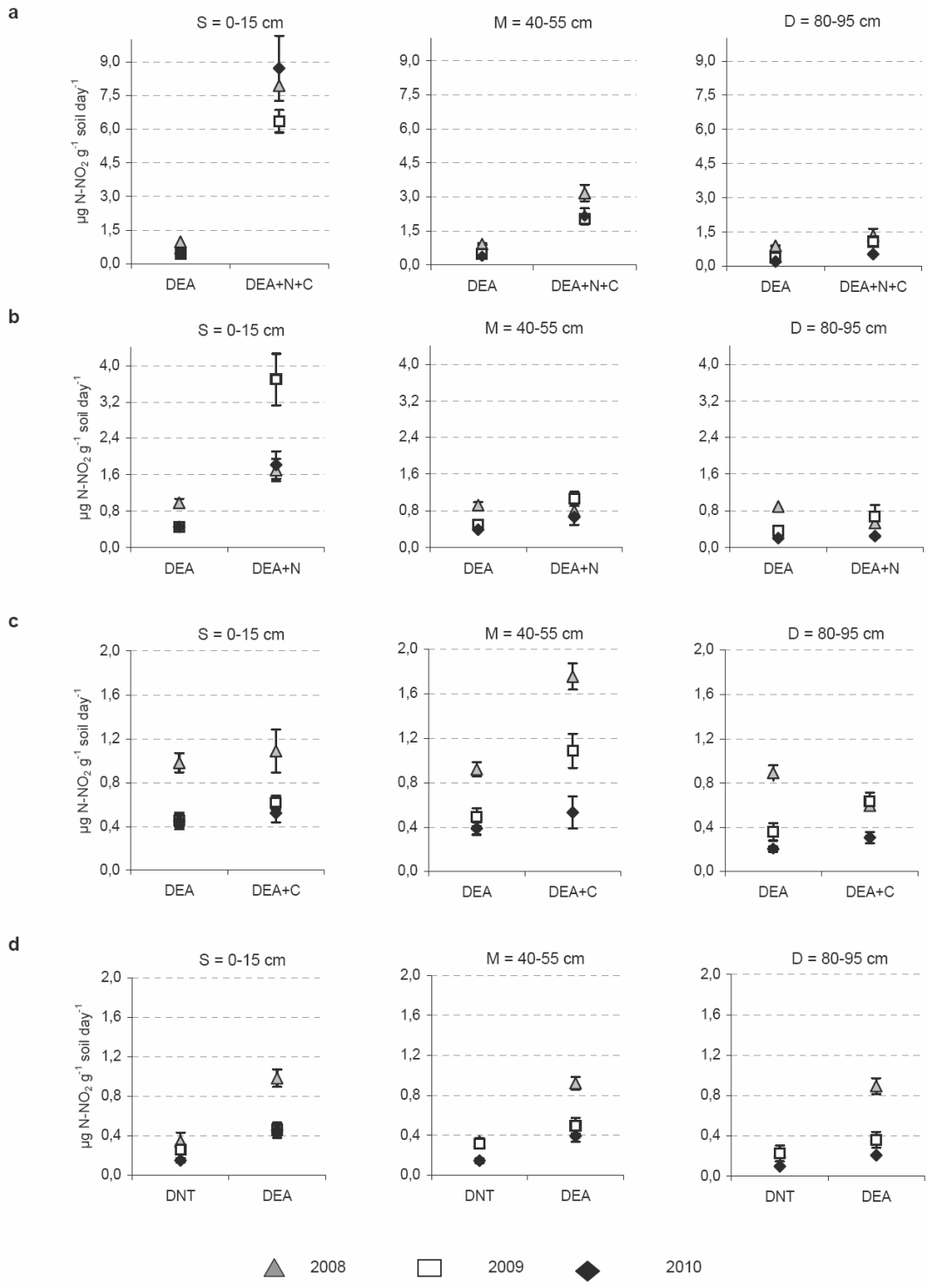


Fig. 5.17 - Comparison of annual average (during 2008 – 2010) in the three different layers between potential denitrification obtained after incubation under anaerobic conditions without any addition (DEA) and potential denitrification with the addition of (a) both nitrate and organic carbon (DEA+N+C), (b) only nitrate (DEA+N), (c) only organic carbon (DEA+C) and (d) *in situ* denitrification rates (DNT).

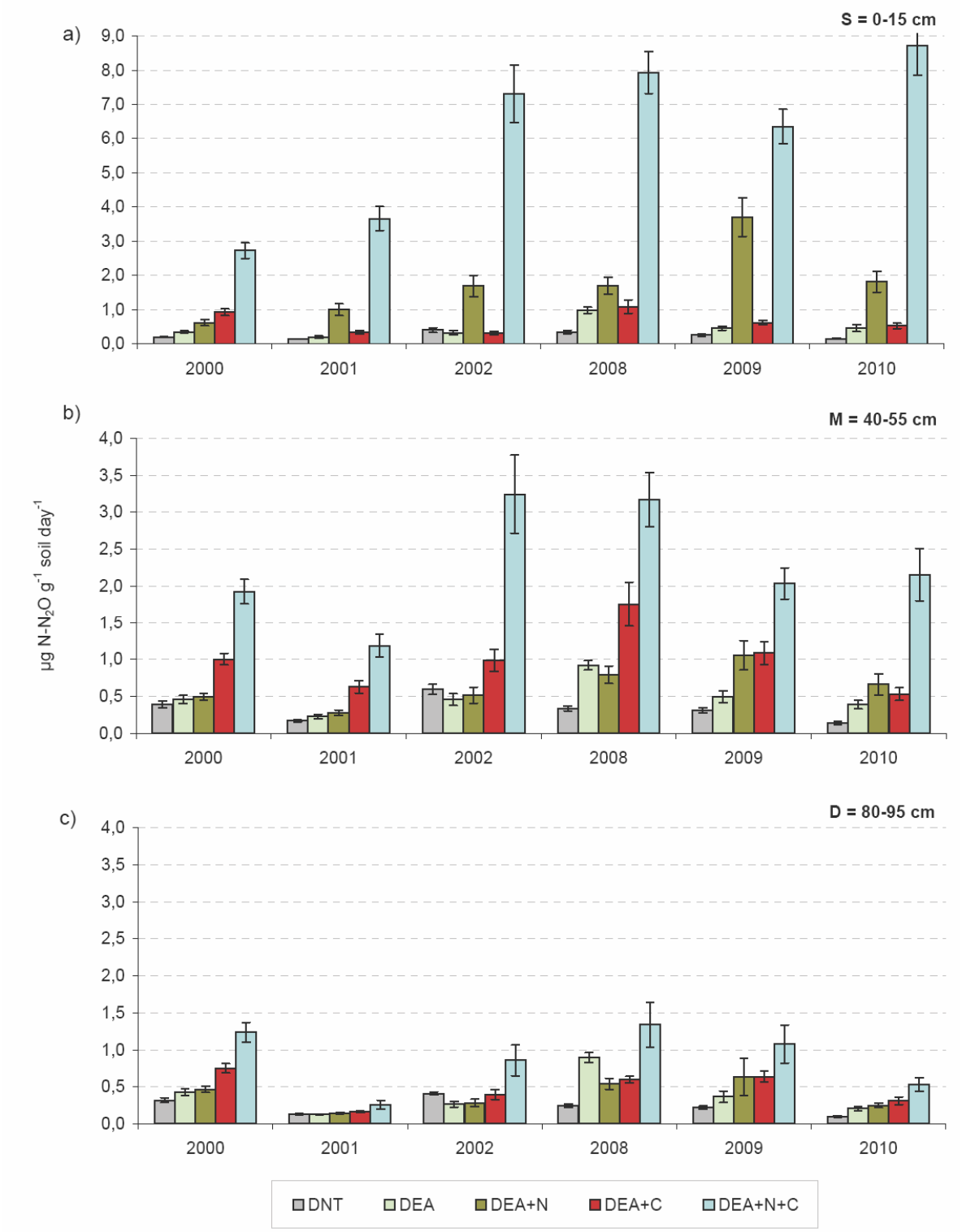


Fig. 5.18 - Comparison of annual average, for all the monitored years, in the three different layers between *in situ* denitrification rates (DNT) and potential denitrification obtained after incubation under anaerobic conditions without any addition (DEA), potential denitrification with the addition of only nitrate (DEA+N), only carbon (DEA+C) and both nitrate and organic carbon (DEA+N+C).

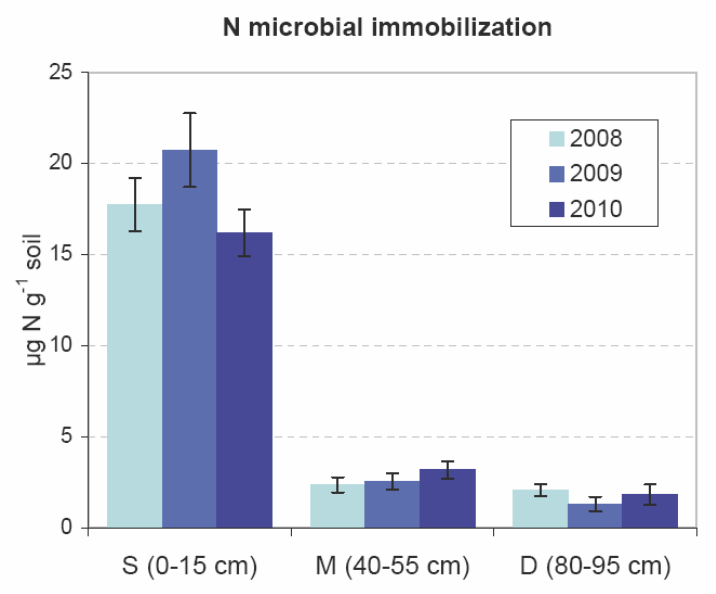


Fig. 5.19 – Values of N microbial immobilization (expressed as µg N g^{-1} soil) in the 3 different layers measured in 2008, 2009 and 2010.

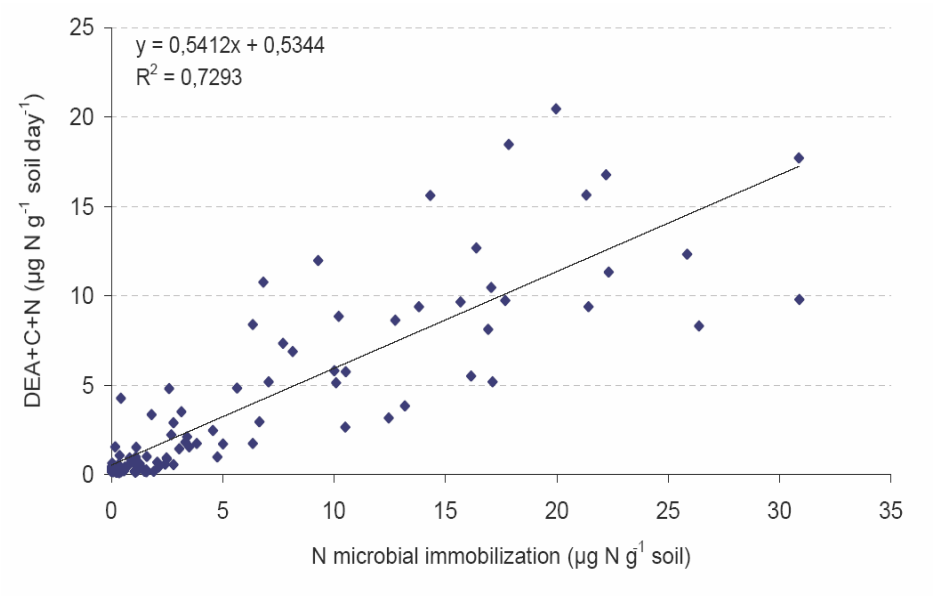


Fig. 5.20 – Correlation between N microbial immobilization and DEA+C+N observed during 2010.

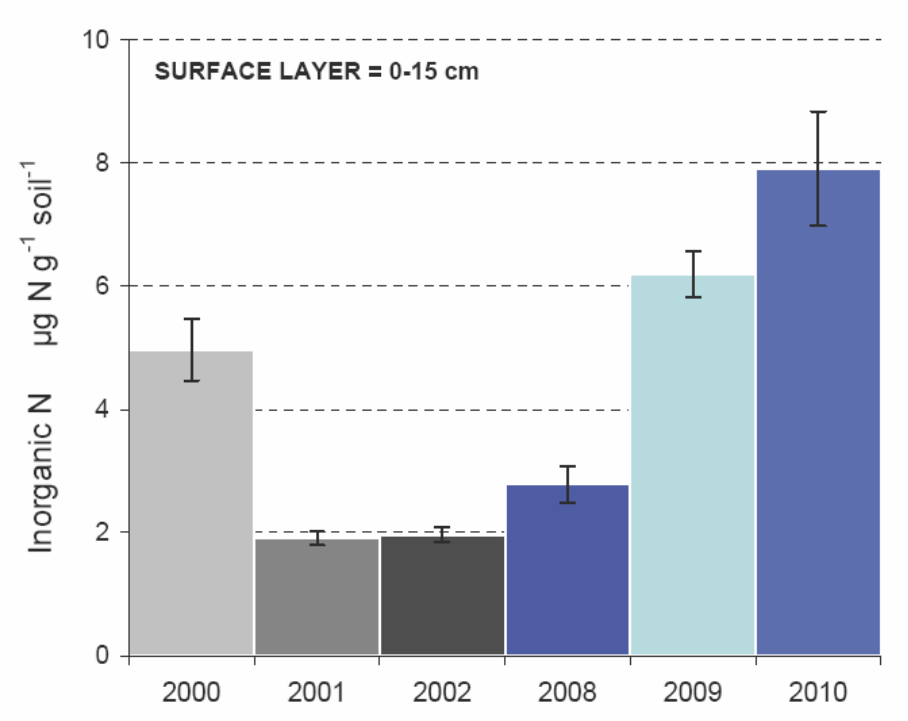


Fig. 5.21 – Inorganic Nitrogen content, expressed as µg N g^{-1} $soil^{-1}$, in different years in the surface layer.

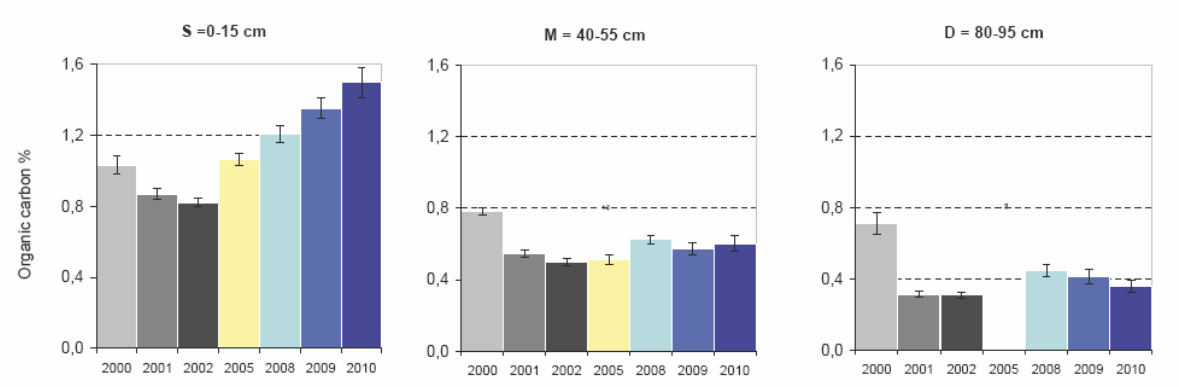


Fig. 5.22 - Organic carbon content, expressed as %, in different years in the three different layers.

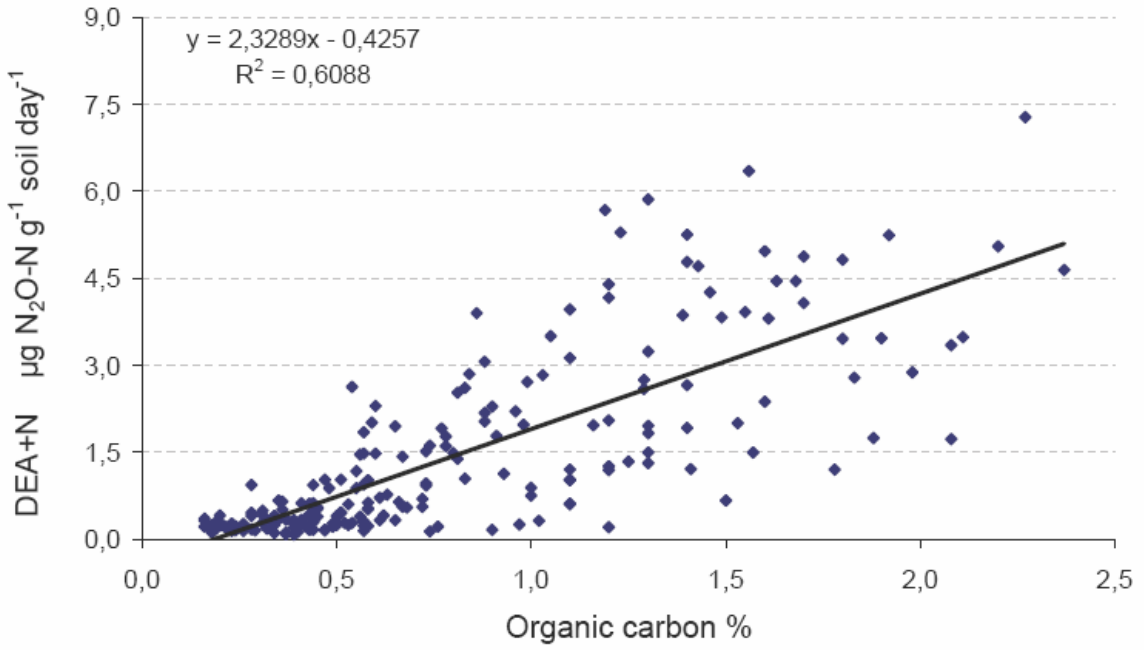


Fig. 5.23 - Correlation between Organic carbon content (%) and DEA+N observed during 2008 – 2010.

Tab. 5.6 - Two-way ANOVA exploring the differences in DEA+N+C rates during 2008 - 2010; the factors include soil depth (layers) and distance from irrigation ditch (zones). Significant relationships and the level of significance are indicated by 0.000=***; 0.001 = **; 0.01=*.

Two-way ANOVA DEA+N+C (2008-2010)		*df*	*mean square*	*F*	*P*
MAIN EFFECTS	*(combined)*				
	Layers ***	2	0.184	173.61	0.000
	Zones	2	0.200	0.966	0.382
2-way interaction	*(combined)*				
	Layers x Zones	4	11.670	1.525	0.195

Tab. 5.7 - Two-way ANOVA exploring the differences in DEA+N rates during 2008 - 2010; the factors include soil depth (layers) and distance from irrigation ditch (zones). Significant relationships and the level of significance are indicated by 0.000=***; 0.001 = **; 0.01=*.

Two-way ANOVA DEA+N (2008-2010)		*df*	*mean square*	*F*	*P*
MAIN EFFECTS	*(combined)*				
	Layers ***	2	112.339	42.544	0.000
	Zones**	2	14.462	5.477	0.004
2-way interaction	*(combined)*				
	Layers x Zones	4	1.427	0.540	0.706

Tab. 5.8 - Two-way ANOVA exploring the differences in DEA+C rates during 2008 - 2010; the factors include soil depth (layers) and distance from irrigation ditch (zones). Significant relationships and the level of significance are indicated by 0.000=***; 0.001 = **; 0.01=*.

Two-way ANOVA DEA+C (2008-2010)		*df*	*mean square*	*F*	*P*
MAIN EFFECTS	*(combined)*				
	Layers ***	2	10.833	13.296	0.000
	Zones	2	1.409	1.729	0.179
2-way interaction	*(combined)*				
	Layers x Zones	4	1.142	1.401	0.233

5.3.2.2 Differences between zones

Additional informations can be drawn from the comparison of the potential denitrification between different zones in different layers. While in the surface and deeper layers no significant differences were observed, again, in the medium layer zone 3 (located near the irrigation ditch) had a higher denitrification potential (Fig. 5.24 a, b and Tab. 5.9). In particular, it notes that:

- DEA+N (Fig. 5.24a and Fig. 5.25) was clearly higher in zone 3 according with the greater availability of organic carbon C (Fig. 5.24d);
- even DEA+C+N (Fig. 5.24b and Fig. 5.25) resulted more active in the zone 3; this may be related to a qualitative differentiation of bacterial communities in this area;

- DEA+C (Fig. 5.24c and Fig. 5.25) was not clearly distinct into the three zones even if the performance of each zone seems related to nitrate availability (Fig. 5.24e).

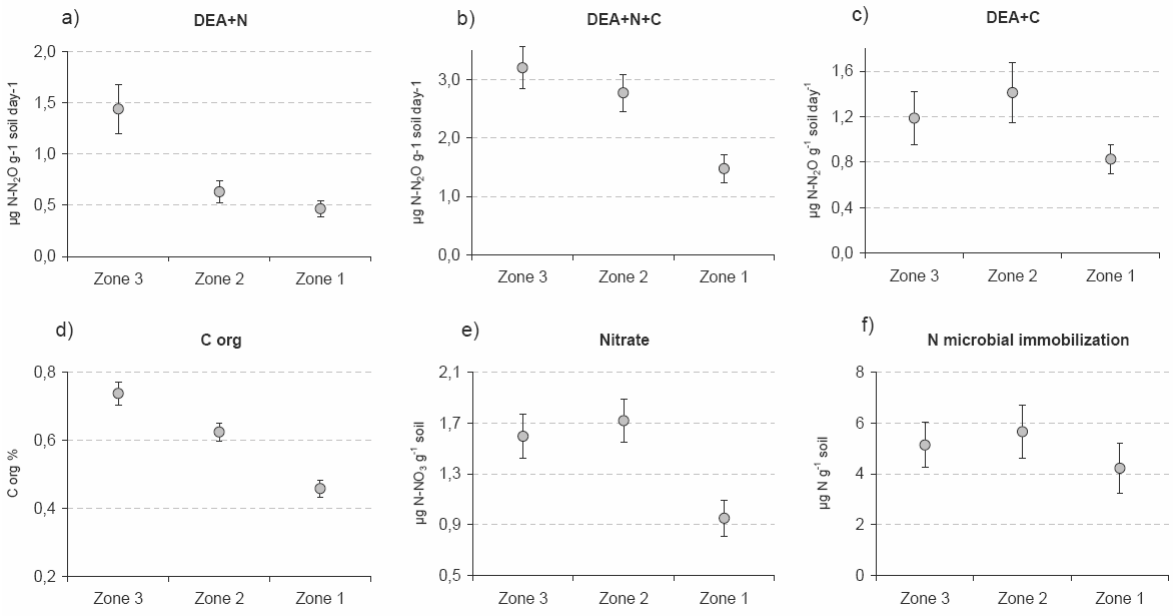


Fig. 5.24 – Comparison of a) potential denitrification with the addition of only nitrate (DEA+N), b) with the addition of both nitrate and organic carbon (DEA+N+C), c) with the addition of only carbon (DEA+C), d) organic carbon content, e) nitrate and N microbial immobilization between the 3 zones in the 40 – 55 cm layer.

Tab. 5.9 - One-way ANOVA exploring the differences in DEA, DEA+C, DEA+N, DEA+N+C rates during 2008 – 2010 between the three different zones in the medium layer (40-55 cm). Significant relationships and the level of significance are indicated by 0.000=***; 0.001 = **; 0.01=*.

One-way ANOVA **Only medium layer (2008-2010)**		*df*	*mean square*	*F*	*P*
DEA+N+C	Zones ***	2	29.186	8.620	0.000
DEA+N	Zones ***	2	9.804	13.653	0.000
DEA+C	Zones	2	3.140	1.886	0.156
DEA	Zones	2	0.129	0.586	0.558

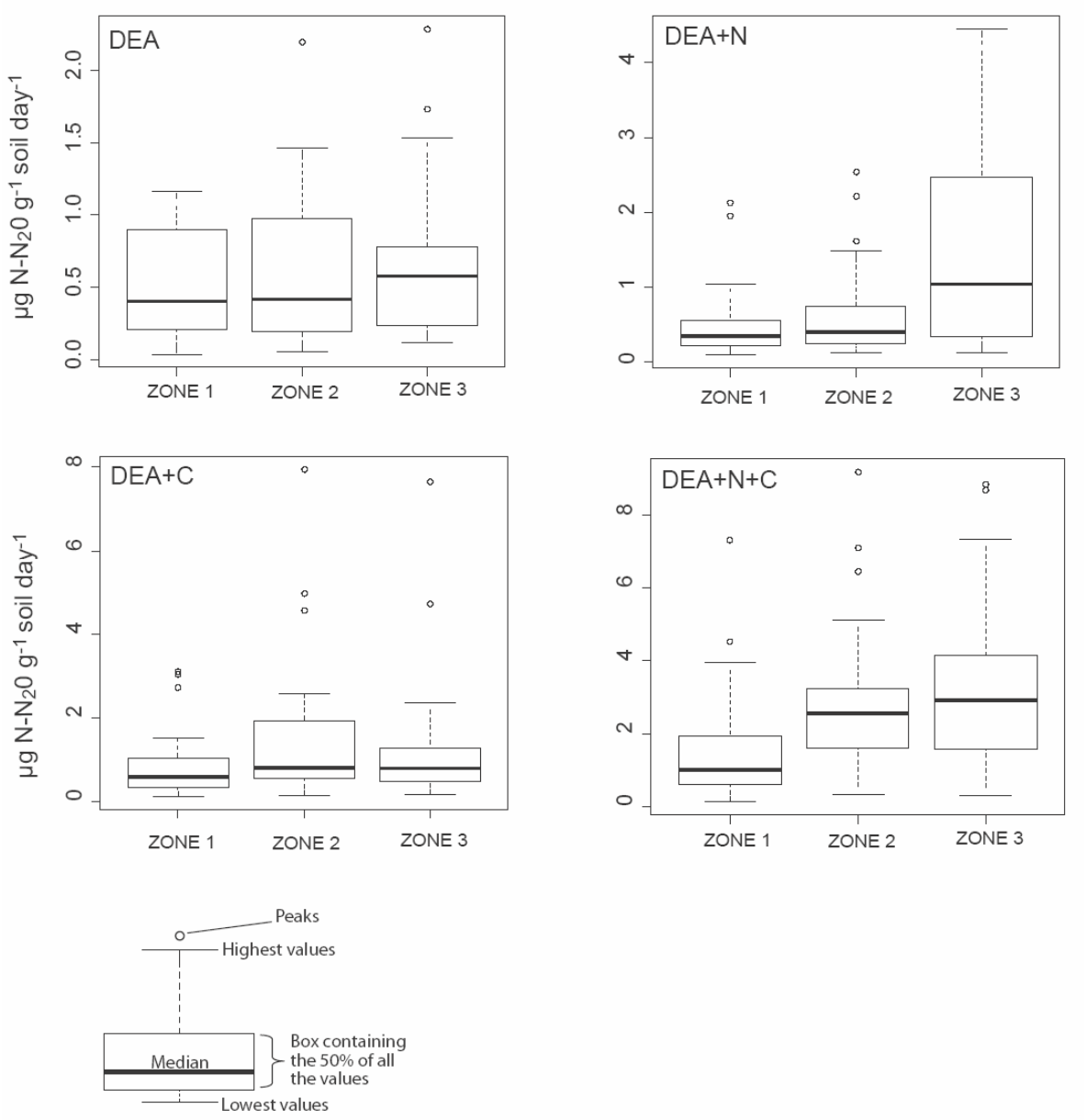


Fig. 5.25 – Differences in DEA, DEA+N, DEA+C and DEA+N+C rates during 2008 – 2010 between the three different zones in the medium layer (40-55 cm).

5.3.2.3 Comparison between seasons

Denitrification potential (DEA+C+N) shows significant seasonal differences (Fig. 5.26), with highest values in summer. Even if temperature could be an important factor to limit bacterial metabolic activity and to explain this variability, it seems possible that denitrifying community dynamics (seasonal variations of its composition) have an impact on denitrification potential.

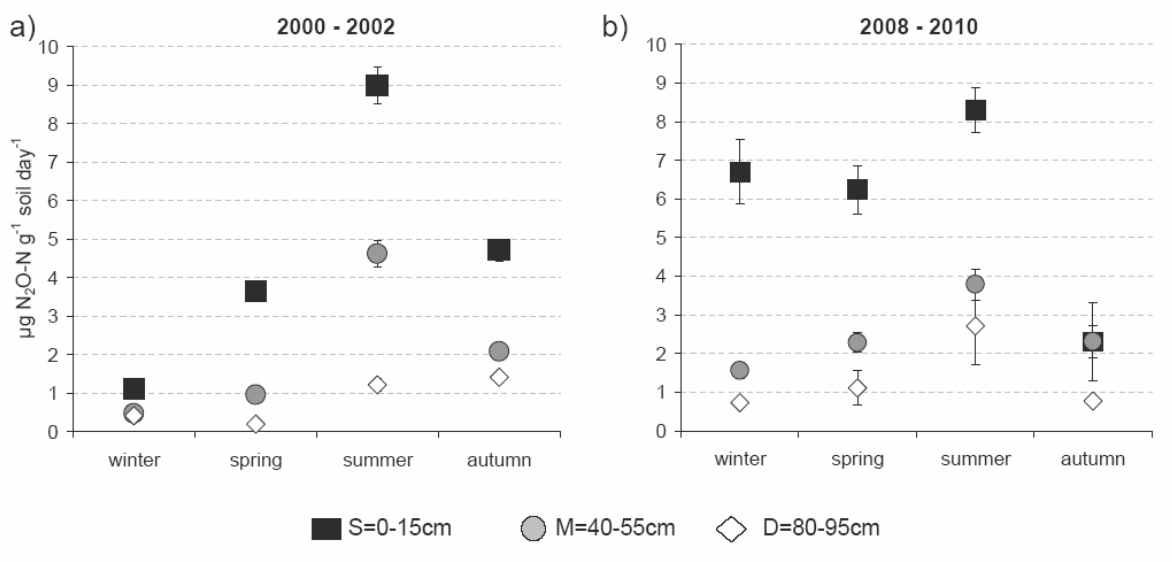


Fig. 5.26 – Denitrification potential rates (DEA+N+C), calculated as µg N-N_2O g^{-1} day^{-1}, for each season and each layer at different depths. The values are the means of all the samples collected during 2000 - 2002 (a) and 2008 - 2010 (b). The vertical bars represent standard errors.

5.3.2.4 Comparison between internal and external site

Starting from October 2009 and for the whole 2010 a comparison between DNT, DEA+N and DEA+N+C activity inside the buffer zone and in an agricultural area outside was made. The measured rates of *in situ* denitrification were not significantly different (Fig. 5.27).

DEA+N resulted significantly higher inside the woody riparian buffer both in surface and medium layers (Fig. 5.27, Fig. 5.28 and Tab. 5.10) while DEA+C+N was higher only in surface layer (Fig. 5.27, Fig. 5.28 and Tab 5.11). The difference between DEA+C+N inside and outside the buffer area resulted comparable with that recorded between the soil of the newly afforested buffer zone (2000 and 2001) and the values measured during the following years when plant colonization and growth stabilized (Fig. 5.18a). This suggests the hypothesis that differences in terms of potential denitrification between forested and agricultural areas are mostly depending from qualitative transformation of soil microbial communities.

In fact, in quantitative terms, significant differences between these areas were not observed (Fig. 5.29).

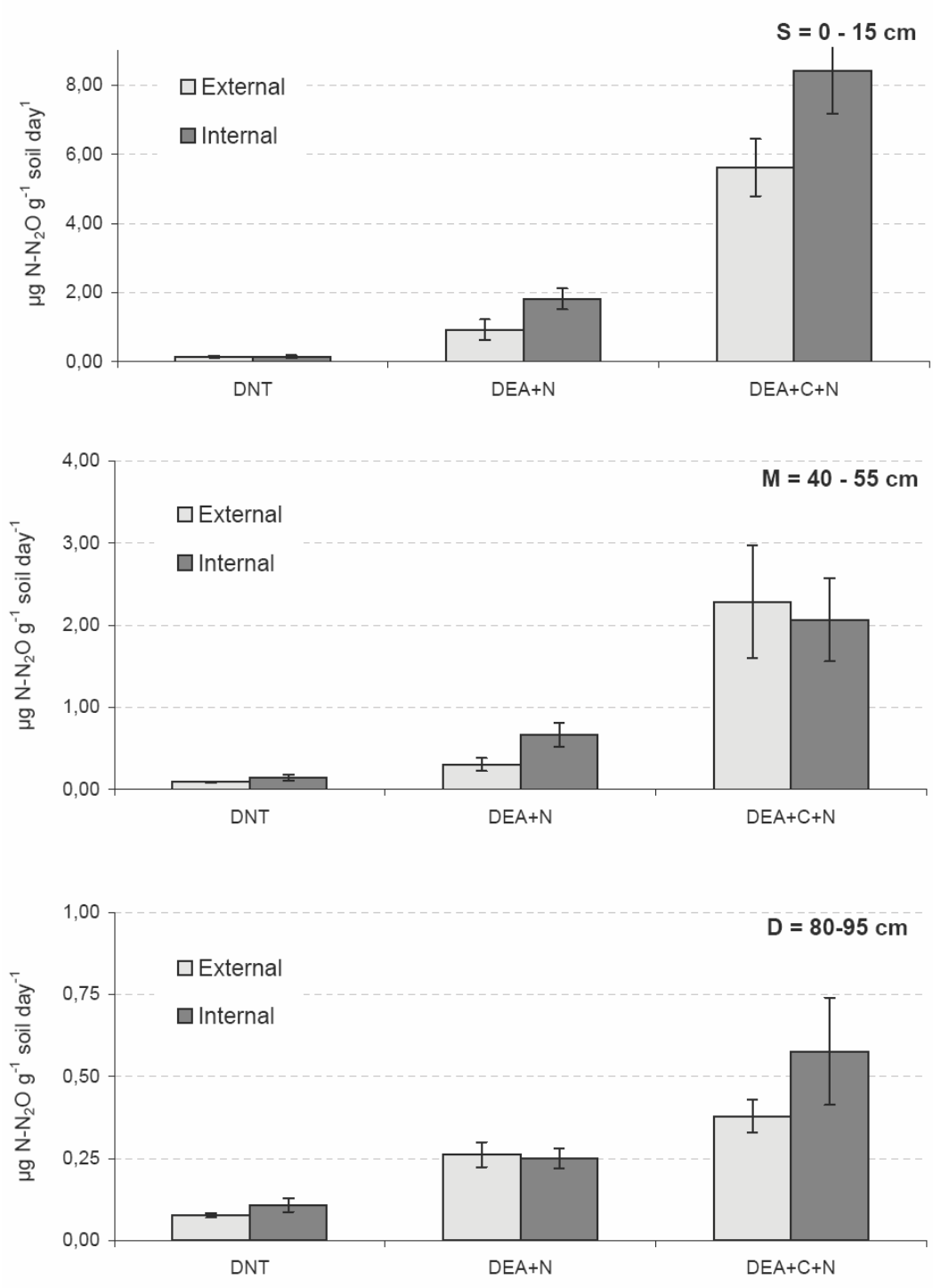


Fig. 5.27 - Comparison of *in situ* denitrification (DNT), potential denitrification with the addition of only nitrate (DEA+N) and with the addition of both nitrate and organic carbon (DEA+N+C), between internal and external sites during October 2009 and March, May, July and November 2010.

Tab. 5.10 - Two-way ANOVA exploring the differences in DEA+N rates during 2008 - 2010; the factors include site (inside and outside the riparian buffer) and soil depth (layers). Significant relationships and the level of significance are indicated by 0.000=***; 0.001 = **; 0.01=*.

Two-way ANOVA DEA+N (2008-2010)		*df*	*mean square*	*F*	*P*
MAIN EFFECTS	*(combined)*				
	in.out *	1	5.505	5.695	0.019
	Layers***	2	7.471	7.728	0.000

Tab. 5.11 - Two-way ANOVA exploring the differences in DEA+N+C rates during 2008 - 2010; the factors include site (inside and outside the riparian buffer) and soil depth (layers). Significant relationships and the level of significance are indicated by 0.000=***; 0.001 = **; 0.01=*.

Two-way ANOVA DEA+N+C (2008-2010)		*df*	*mean square*	*F*	*P*
MAIN EFFECTS	*(combined)*				
	in.out *	1	43.99	6.549	0.012
	Layers***	2	351.80	52.378	0.000

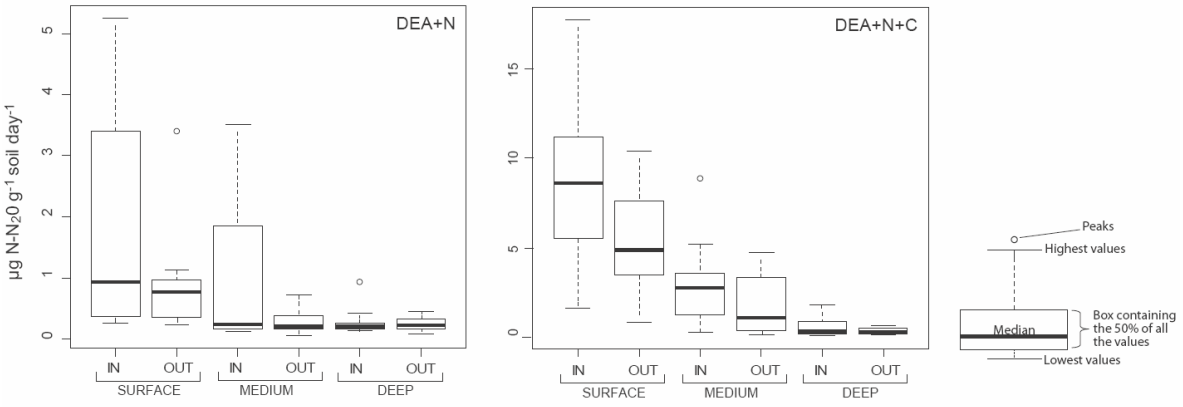


Fig. 5.28 - Comparison of potential denitrification with the addition of only nitrate (DEA+N) and with the addition of both nitrate and organic carbon (DEA+N+C), between internal and external sites in the three different layers during October 2009 and March, May, July and November 2010.

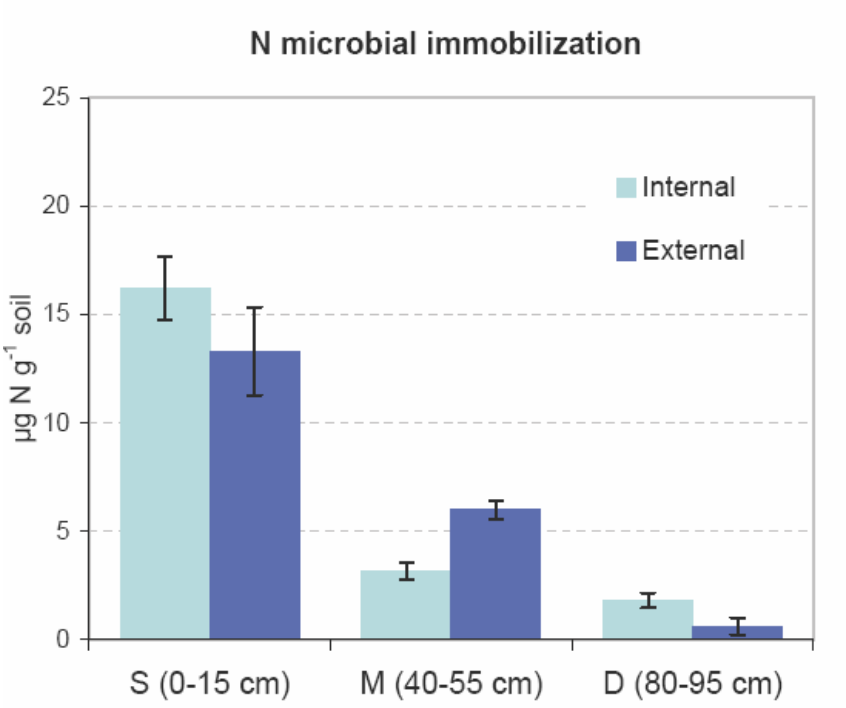


Fig. 5.29 - Comparison of N microbial immobilization (expressed as µg N g-1 soil) in the 3 different layers between internal and external site during October 2009 and March, May, July and November 2010.

5.4 Discussion

The aim of this study was to explore the possibility of reducing the level of nitrogen in rivers by forcing water to circulate through afforested buffers. Nitrogen reduction can be achieved by creating semi-natural floodplains where water flows could be efficiently managed to support high nitrogen removal by microbial denitrification. An overall and detailed appraisal of the nitrogen retention by the system is given, even if the relative contribution of the different processes involved (i.e. plant/microbial uptake and denitrification) were not determined individually.

Influence of subsurface flow regulation on denitrification activity. Subsurface flow through both soil and deeper sediments of a riparian zone is known to be of crucial importance to denitrification and other nitrogen cycle processes (Mitsch *et al.*, 1977; LaBough, 1986; Chescheir *et al.,* 1988; Correll and Weller, 1989; Dosskey and Bertsch, 1994; Pinay *et al.,* 2000). As recent studies indicated that climate has little impact on the overall N removal in riparian zones (Sabater *et al.,* 2003), water table elevations are among the prime determining factors for N dynamics (Hefting *et al.* 2004). Indeed, since denitrification potential increases significantly towards the soil surface, water table elevation can control the degree to which nitrate reduction by denitrification is optimised. Burt *et al*. (2002), reporting results from a pan-European experiment (NICOLAS), showed that denitrification process will be more effective within a riparian zone where topographic and soil conditions are conducive to a high water table for as long as possible during the year. These conditions usually occur when permeable soil overlays impermeable bedrock and the land surface slope is low (5-10°) (Pinay and Burt, 2001). Our results demonstrate that, even in the fine textured soil of the present experimental site, the anoxic conditions required for denitrification can be obtained by creating semi-natural floodplains where water flows can be suitably managed, i.e. by maintaining a slope of 4%. Under these hydrologic conditions the higher denitrification rates were reached in the soil layer often saturated by the perched aquifer. In particular the highest rates of denitrification were measured in the medium layer (40 – 55 cm deep), where, due to its location on the border between the unsaturated and saturated zone, aerobic processes (like nitrification) and anaerobic denitrification can occur in close proximity. On the other hand, the low values recorded in all soil layers in winter

and spring indicate that the experimental design was unable to overcome other key limiting factors for denitrification such as low winter temperatures and plant competition in spring, even though a constant water flow was maintained. In addition, the water table regulation seems not to be the key factor to control denitrification in the deeper soil layer (around 1 m below soil surface); indeed, the measures on potential denitrification demonstrated that in this case the most important limiting factor is the lower bacterial population living deep in the soil.

The long-term monitoring has also shown that once the riparian wood has grown, it can affect very significantly on the water table elevation especially during the dry seasons. When evapotranspiration is high, there is a significant lowering of the subsurface flow level resulting in inhibition of the denitrification process. In these terms, the competition between plants and bacteria is not limited to the use of nitrates, but also to the capability of woody plants to influence soil hydrology and the bacterial processes depending on it.

Finally, in our system, the dynamics of the superficial layer are not affected by the regulation of subsurface flow level and demonstrate a pattern strictly dependent on weather events. It is well recognized that precipitation events are important triggers of denitrification activity being stimulated by the restricted O_2-diffusion into wetted soil (Ambus and Zechmeinster-Boltenstern, 2007). In according to this, high peaks of denitrification were recorded in coincidence of sampling done during rain events. Considering that these peaks were spatially scattered, and that their frequency increased once the riparian wood has grown, denitrification activity in soil surface seems to be associated with the so-called "hotspots", e.g. the proximal vicinity of degrading organic matter (litter) where O_2-free zones have developed due to high respiratory activity simultaneously with relatively high abundance of NO_3 and degradable C-sources.

The conversion of the site in an efficient buffer zone. The area was rapidly converted from agricultural land to a tree-covered buffer even though there was no vegetation before the appropriate tree species were planted. Our results indicate that a buffer zone set up for nitrate removal from river water starts to be effective, in terms of nitrogen removal in water, during the second year (see Fig. 4.3), when high nitrogen uptake by the plants (a

mean of 104 g m^2 herbaceous vegetation biomass for the first year as compared to 298 g m^2 for the second), was registered.

Despite this, our results demonstrates that high denitrification potential was reached in the third year, hence plant colonization and growth stabilized; no further significant variations of this potential were registered during following years. During the first two years the denitrification potential was still comparable to the one registered in an agricultural area outside the riparian buffer. This suggests the hypothesis that differences in terms of potential denitrification between forested and agricultural areas are mostly depending from qualitative transformation (higher biodiversity) of soil microbial communities. This study, and other recent ones (Wallenstein *et al.,* 2006; McGill *et al.*, 2010), shows that denitrification potential changes with time and site, so it is clear that denitrifier community dynamics do have an impact on denitrification potential.

The results obtained by another linked doctoral research project (Rahman, 2011), specifically based on comparison between composition, biomass and distribution of bacterial community inside and ouside the buffer system, supported this hypothesis.

This aspect is not usually considered in the literature where typically the microbial process of denitrification is viewed simply a function of environmental factors (e.g. C, N, and redox status) (Schimel, 2001). As previously suggested by McGill et al. (2010), the results of this study indicate that there is a need to complement microbial community functioning data with microbial community composition data to improve our understanding of how microbial diversity and ecosystem functioning are related.

Denitrification potential. As expected, although DNT was highest in the 40-55 cm soil layer, denitrification potential (measured as DEA+C+N) was highest in the top soil layer. Lower values of denitrification potential in the deeper soil layer (80-95 cm) are generally attributed to the lower microbial populations living deeper in the soil. This is in agreement with a number of studies where potential denitrification values are reported to generally decrease with soil depth (Hunt *et al.*, 2007; Hunt *et al.*, 2004). For example, Ambus and Lowrance (1991) reported that denitrification potential was mainly concentrated in the top 2 cm of soil in two riparian forest soils. A synthesis of research projects on different soils in the Netherlands demonstrates higher potential in 0-20 cm layer, a lower but non-negligible

potential in 20-40 cm layer and a drastic decrease in deeper layers (Munch and Velthof, 2007).

The denitrification potential in addition of nitrate (DEA+N) on surface soil samples increased over time. It means that after 10 years from the conversion in a forested riparian soil, nitrate still represents the main limiting factor in the upper soil layer; the increase of organic carbon in the soil surface is able to guarantee the removal of higher quantities of inorganic nitrogen resulting from the process of mineralization. It's well known that deciduous tree species, like the ones planted in this riparian buffer, might generally increase nutrient cycling (Menyailo *et al.*, 2002) and influencing the carbon content in soil. According to Jobbagy and Jackson (2000), the percentage of organic carbon in the top 20 cm (relative to the first meter) averaged 33%, 42%, and 50% for shrublands, grasslands, and forests, respectively.

If an increase in carbon content in 0 – 15 cm layer was observed, in general no corresponding changes in medium and deeper layer were measured. For this reason the low carbon availability strongly affects the denitrification activity at 40-55 cm depth.

Starting from 2008 an increase of organic carbon availability was observed in the 40-55 cm layer but only in the zone 3, the one closer to the irrigation ditch and characterized by the presence of fast-growing species (*Salix alba* in particular). This confirms the role of plant functional types and hydrology on affecting the vertical distribution of organic carbon (Jobbagy and Jackson, 2000).

In deeper soil layers even the addition of both nitrate and organic carbon is not always able to promote high denitrification potential. Indeed, the strong differences recorded between the seasons and the low DEA+C+N values in the deepest soil layer underline once again the importance of other key limiting factors for denitrification, such as the temperature and the distribution of the microbial population.

Critical factors and optimization of the buffer capacity. Our results confirm that a suitable irrigation system and an appropriate soil arrangement are crucial for optimizing the nitrogen removal potential of an afforested buffer site. Particular attention must be paid to maintenance of the shallow perched water table as close as possible to surface soil layers. About this aspect, with the development of the tree line and the strong growth of their roots

a substantial decrease of the subsurface flow level was observed. If, as is realistic to assume, the presence of the no structured and characterized by very low permeability layer (90-150 cm depth), won't stop this trend, the denitrification process in the coming years will be further reduced.

Once the flow of nitrate enriched water was established through the shallow perched water table, the key factor affecting the level of denitrification was carbon availability. In our experiment the C supply now, and more and more in the coming years, will be ensured by litter fall and root production of several plant species expressly planted in the buffer zone. The selection of different fast-growing plant species may represent another critical factor in system design. McGill *et al.* (2010) indicated that plant communities clearly influence microbial activity and processes, and that the diversity of plant communities can positively affect the stability of microbial processes, including denitrification.

6 DENITRIFYING POPULATION DYNAMIC

6.1 Introduction

Denitrifiers are commonly found in many natural environments such as soil, marine and freshwater sediment, as well as in waste water treatment systems (Hallin *et al.*, 2007). In one of the first and most comprehensive studies exploring soil denitrifiers communities, Gamble *et al.,* (1977) isolated 1,500 bacteria and 146 of those were capable of complete denitrification. However, cultivation is known to be highly selective for certain organisms and the lack of appropriate tools to study this bacteria in the environment have limited our knowledge of denitrifier ecology. Today molecular tools are being developed or refined to asses both diversity and numbers of denitrifying populations in different ecosystems.

Considering this opportunity and the key-role of denitrification process in nitrogen removal in the studied riparian buffer, it has proved necessary to conduct studies aimed at the identification of specific microbial groups involved in soil enzymatic activities of particular interest. According to this, a specific analysis on the denitrifying microbial communities was conducted. The purpose of this study is to compare if the particular hydraulic management, the suspension of farming practices and the development of the woody and herbaceous vegetation have caused a change in terms of denitrifying microbial community composition compared to that found in a neighbouring agricultural area. As already explained (see the discussion in Chapter 5), the variations in potential denitrification in soils collected in different areas and seasons may be related to the different composition of denitrifying community.

In particular, this investigation was focused on *nirK*. This gene codes for the two types of nitrite reductase, an enzyme essential for the conversion of nitrite to nitric oxide, during the process of denitrification (Fig. 6.1). This could be considered the key step in the denitrification process, because once the nitrites are converted to nitric oxide the process became irreversible even if not necessarily reach completion (a fraction can also be directly released into the atmosphere such as nitrous oxide).

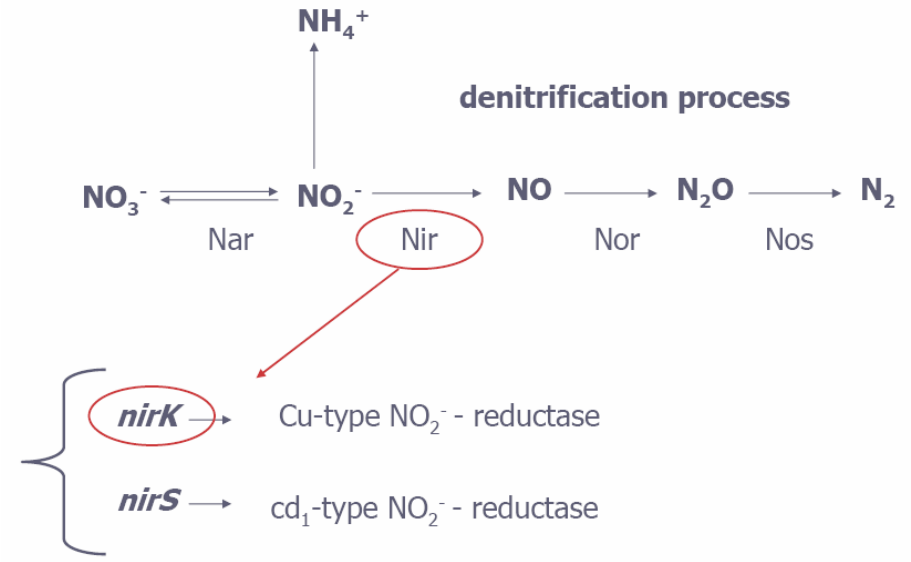


Fig. 6.1 – *NirK* gene encodes for Cu-type NO_2^-- nitrite reductase; this enzyme enables the conversion of nitrite to nitric oxide during the denitrification process.

6.2 Materials and methods

6.2.1 Sampling activity

For this study the same soil samples used to measure denitrification activity were analysed, even if a smaller number was considered. In detail, to analyse denitrifiers community dynamics inside the riparian buffer, the soils were sampled in 3 different points along the line bordering the piezometers net in plot A and indicated as Ab3, Ab2 and Ab1 in the following scheme (Fig. 6.2). As usual, for each point, the soil samples were taken at three different depths (S: 0-15 cm, M: 40-55 cm, D: 80-95 cm below the soil surface).

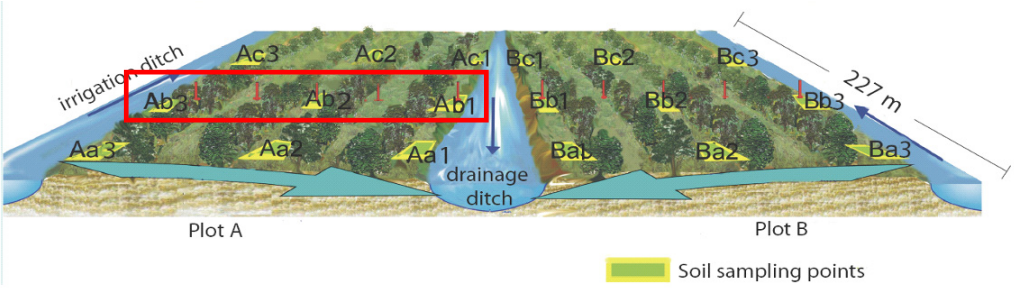


Fig. 6.2 – Soil sampling points inside the experimental site (evidenced by the red rectangle).

External samples were collected also from three different places (three replications) at three different depths (surface (S), 0-15 cm; medium (M) 40-55 cm; and deep (D) 80-95 cm) from an agricultural area, regularly tilled but left uncultivated during the last 2 year and bordering the woody buffer zone. Two different sampling periods were considered: October 2009 and March 2010 (see Tab. 6.1 for details).
Observe that during March 2010 temperature was very low and also denitrification activity resulted inhibited (Fig. 5.8 in chapter 5).

Tab. 6.1 – Sampling dates and corresponding rainfall (total amount in the five days before) and temperature.

Period		*Date day/month/year*	*Total rainfall 5 days before sampling mm*	*External temperature during sampling °C*
2009				
	Autumn	05/10/2009	0.8	23
2010				
	Winter	08/03/2010	13.0	**3**

6.2.2 Total DNA extraction from dry soil

DNA from soil was extracted using the Power Soil TM DNA Isolation Kit (Mo Bio Laboratories Inc., USA). DNA isolation was performed according to the manufacturers' instructions, modified as follows: extra glass beads (0.15 – 0.30 g, bead size 0.1 mm) were added to the soil samples and the cells were disrupted by bead beating (mini-bead beater™, Bio Spec products, USA), two times at 30 sec. Final purification of the extracted DNA was performed using the Wizard® DNA clean-up system (Promega, USA).

6.2.3 PCR amplification of denitrification genes encoding nitrite reductase (*nirK*)

PCR was used to amplify denitrification genes encoding nitrite reductase (*nirK*). Primers for *nirK* genes were PCR-amplified using the *nirK* primers Copper 583F (5′-TCATGGTGCTGCCGCGKGACGG3′) and Copper 909R (5′-GAACTTGCCGGTPGCCCAGAC 3′) (Liu *et al.*, 2003). The primer pairs amplifying gene

fragments of *nirK* were run with an initial denaturation of the DNA at 94 °C for 2 min, followed by 35 cycles of 30 s at 94 °C, 1 min at 51 °C and 1 min at 72 °C. The reaction was completed after 10 min at 72 °C. The reaction mixtures were placed in a minicycler (Bio Rad ICycler 170-8740). PCR amplification was performed in a total volume of 25 µl containing 2.5 µl of 10X PCR buffer (500 mM KCl, 15 mM $MgCl_2$ and 100 mM Tris–HCl, pH 9.0, at room temperature), 200 µM of each deoxynucleotide triphosphate, 1.25 U of Taq polymerase (Amersham Biosciences, NY, USA), 1.0 mM of each primer and 10– 100 ng DNA. The detection and the size of the amplicons were determined by agarose (0.9%) gel electrophoresis and UV translumination after ethidium bromide staining.

6.2.4 Denaturing gradient gel electrophoresis (DGGE) analysis

Community structure of denitrifying bacteria was characterized by denaturing gradient gel electrophoresis (DGGE) analysis of *nirK*. In DGGE pattern of amplified *nirK* gene, each DNA band at different locations and its relative concentration (brightness), may represent a particular microbial species and its relative abundance/richness within the microbial community (Muyzer *et al*., 1993, Wenhui *et al*., 2007). Because the PCR template is the total soil DNA, which included the DNA of culturable and unculturable microorganisms, PCR-DGGE can reflect more microbial species than culturable microorganisms.

NirK gene fragments were amplified by nested PCR, using the primers of Liu et al., (2003) for the first PCR step and the same primers, modified by addition of a *GC* clamp (5′-CGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGGAACTTGCCGGTKGCC CAGAC- 3′*)* to the 5′ end of the reverse primer, in the second PCR step (Wertz *et al.*, 2006). The final reagent concentrations for PCR were 1 µM primers, 200 µM of each dNTP, 1.75 U of Taq (Qbiogene, Carlsbad, USA), in 50 µl of 10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100, 1.5 mM MgCl2, pH 9. Thermocycling conditions were: 5 min at 94°C followed by a touchdown between 72°C and 67°C (one cycle of 94°C for 30 s, 72°C for 1 min and 72°C for 1 min) and four subsequent cycles in which the annealing temperature was decreased by 1°C/ cycle to reach 68°C. Touchdown was followed by 25 cycles (30 s at 94°C, 1 min at 67°C and 1 min at 72°C) followed by 7 min extension at 72°C. Denaturing gradient gel electrophoresis analysis of PCR products employed the D-

Code Universal Mutation Detection System (Bio-Rad) with a 6% polyacrylamide gel containing a gradient of 35–65% denaturant, 100% denaturing solution being defined as 7 M urea and 40% formamide. Gels were run for 4.5 h at 150 V in 1× TAE buffer at 60°C. The gels were silver stained, dried at 37 °C and scanned. At least two different DGGE runs were carried out for all samples and for both loading orders of the samples on gel, in order to estimate the reproducibility of DGGE profiles generated with different loading schemes of samples. As marker PCR-amplified fragments of *nirK* sequences of cultured denitrifier strains (*HCNT1, Ensifer meliloti 2011, Ensifer meliloti 41* and *Bradhyrhizobium japonicom)* were used.

6.2.5 DGGE cluster analysis

DGGE bands were identified by visually inspecting gel images in BioNumerics version 4.5 software program through band intensity. Brightness and contrast were adjusted for each image to facilitate band identification. Similarities between microbial communities of DGGE profiles generated by Dice similarity index were based on UPGAMA (Unweighted Pair Group Method using Arithmetic Averages) analysis using the BioNumerics version 4.5 software (www.applied-maths.com).

6.2.6 Sequencing of excised *nirK* DGGE bands

Individual light DGGE bands of PCR products were excised from the gel with a sterile scalpel. Each piece was transferred into 10 mL of sterile water and incubated overnight at 4°C to allow diffusion of the DNA. Eluted DNA was amplified with same primers. The re-amplified DNAs were run on a DGGE gel (as described above) and compared with the original sample. If a single band matched with the position that had been obtained, it was excised from the gel and re-amplified by using unclamped primers. If multiple bands were formed, the individual bands were also excised from the gel, purified, and amplified by using unclamped primers. For DNA sequencing reactions, 100 ng of re-amplified PCR products of DGGE band and 5 pmol of unclamped primer were used. The 16S rDNA sequence was analyzed using Chromas LITE (Version 2.01); the most similar bacterial species was found in the GenBank by using BLAST search (http://www.ncbi.nlm.nih.gov).

6.2.7 Principal component analysis (PCA)

Principal components analysis (PCA) was performed by XLSTAT 2007 software to determine the possible relationships between microbial communities on the basis of the DGGE banding patterns.

6.2.8 Denitrification enzymatic activity and soil chemical parameters

Denitrification enzymatic activity (DEA) was measured using Smith and Tiedje's procedure (1979) as described on paragraph 5.2.3. Organic carbon and nitrate content on soil were determined as explained in section 5.2.4.

6.3 Results

6.3.1 PCR amplification of *nirK* gene of soil denitrifying microbial communities

The amplification of *nirK* gene was successfully obtained in DNA extracted from superficial and medium depths soils both for internal and external samples (Fig. 6.3), while in the deep soil the amount of extracted DNA resulted generally not sufficient for amplification. Only one replicate, in October 2009 and one during March 2010, successfully amplified. For this reason, in the following DGGE analysis only superficial and medium layer samples were considered.

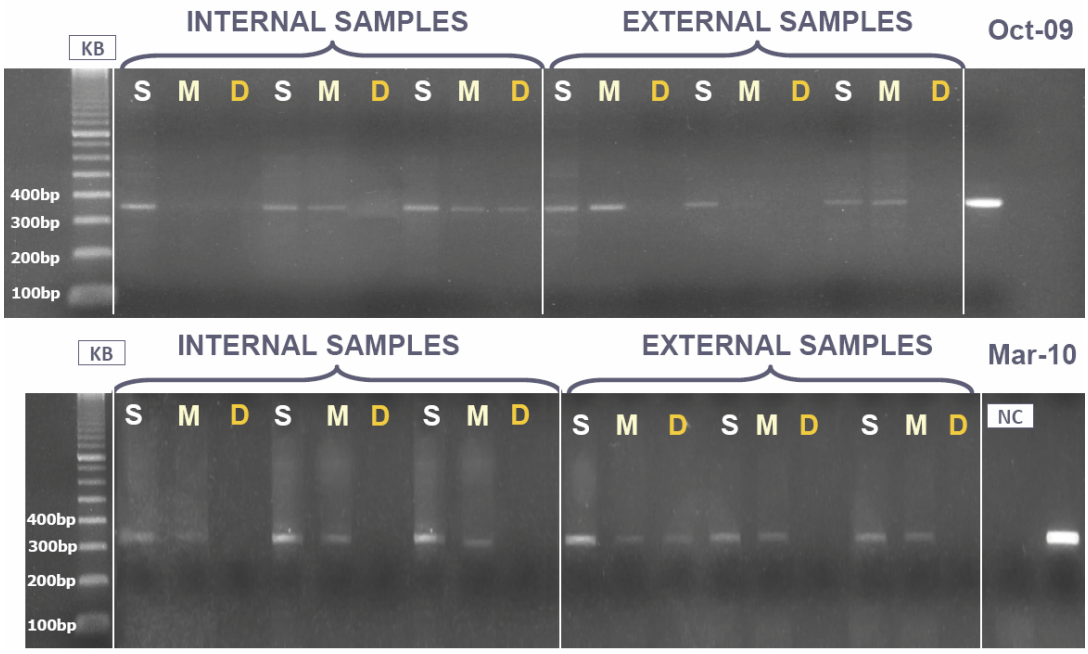


Fig. 6.3 – Amplification (PCR) of *nirK* gene in different layers obtained in October 2009 and March 2010 soil samples.

6.3.2 DGGE band analysis of denitrifying microbial communities of soil samples

In the current study, *nirK* gene PCR-DGGE based approaches were used to analyze a specific microbial population.

The *nirK* gene was successfully re-amplified (adding the GC-clamp) in all the considered soil samples. As example the gel of October 09 is reported in the following Fig. 6.4.

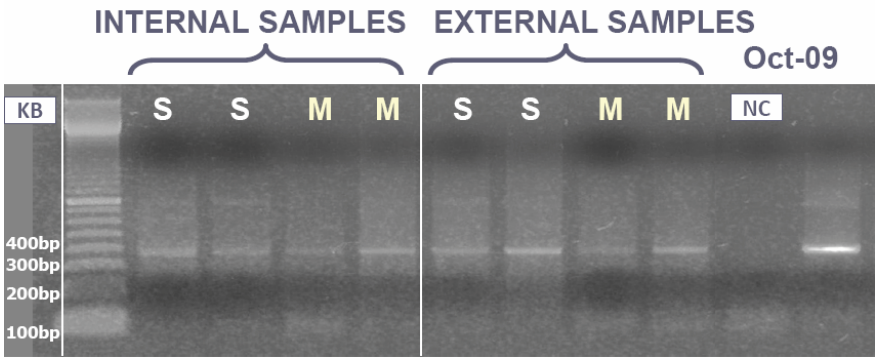


Fig. 6.4 - Amplification of the *nirK* gene for DGGE analysis in October 2009

Denaturing gradient gel electrophoresis analysis of PCR products obtained for October 09 and March 2010 are reported in the following Fig. 6.5 and Fig. 6.6 respectively.

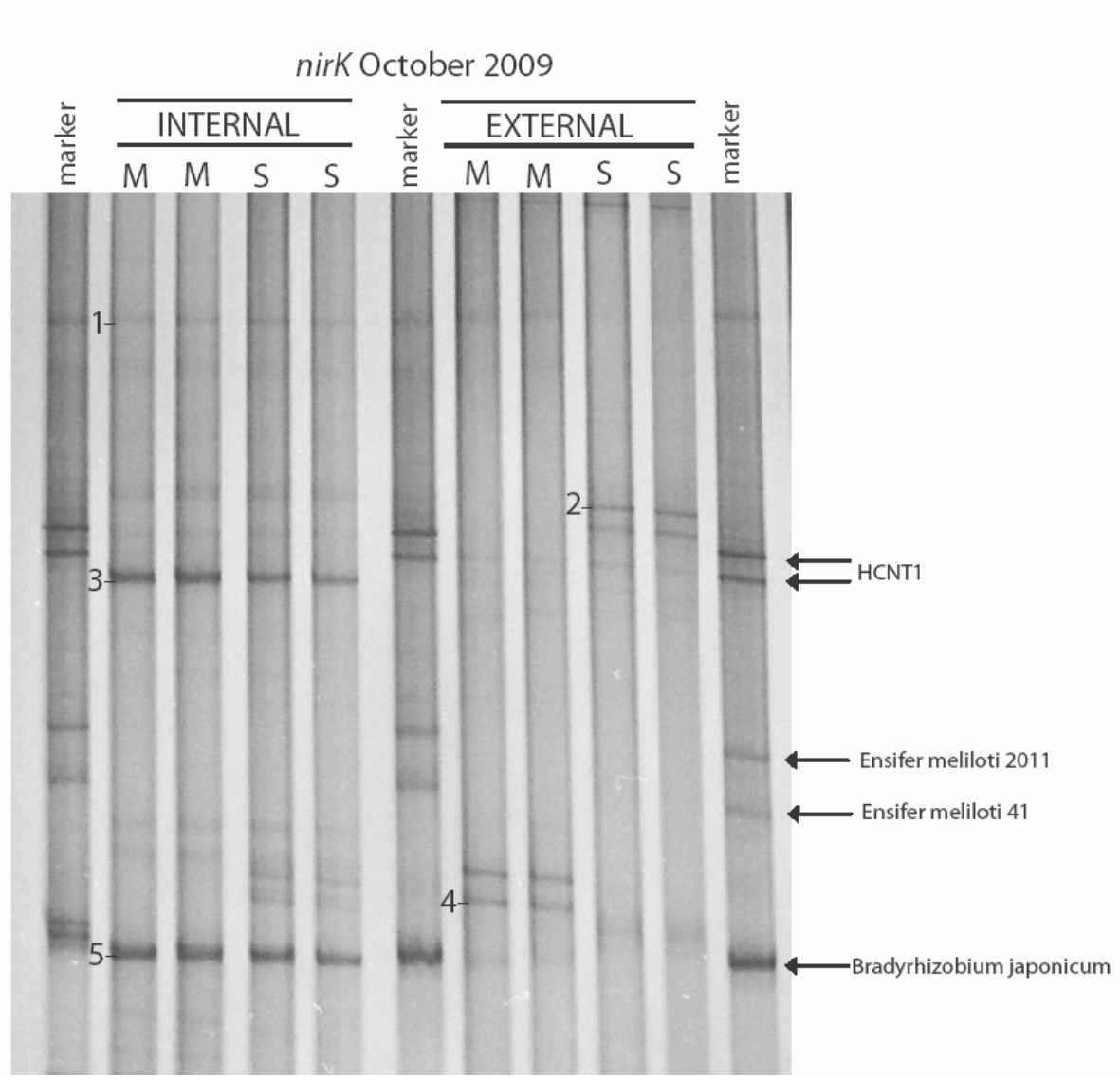


Fig. 6.5 – DGGE profile of *nirK* genes for medium (M) and surface (S) soil internal and external to the riparian buffer zone in October 2009. The markers consisted of *nirK* sequences of cultured denitrifier strains. The numbers indicate bands that were sequenced.

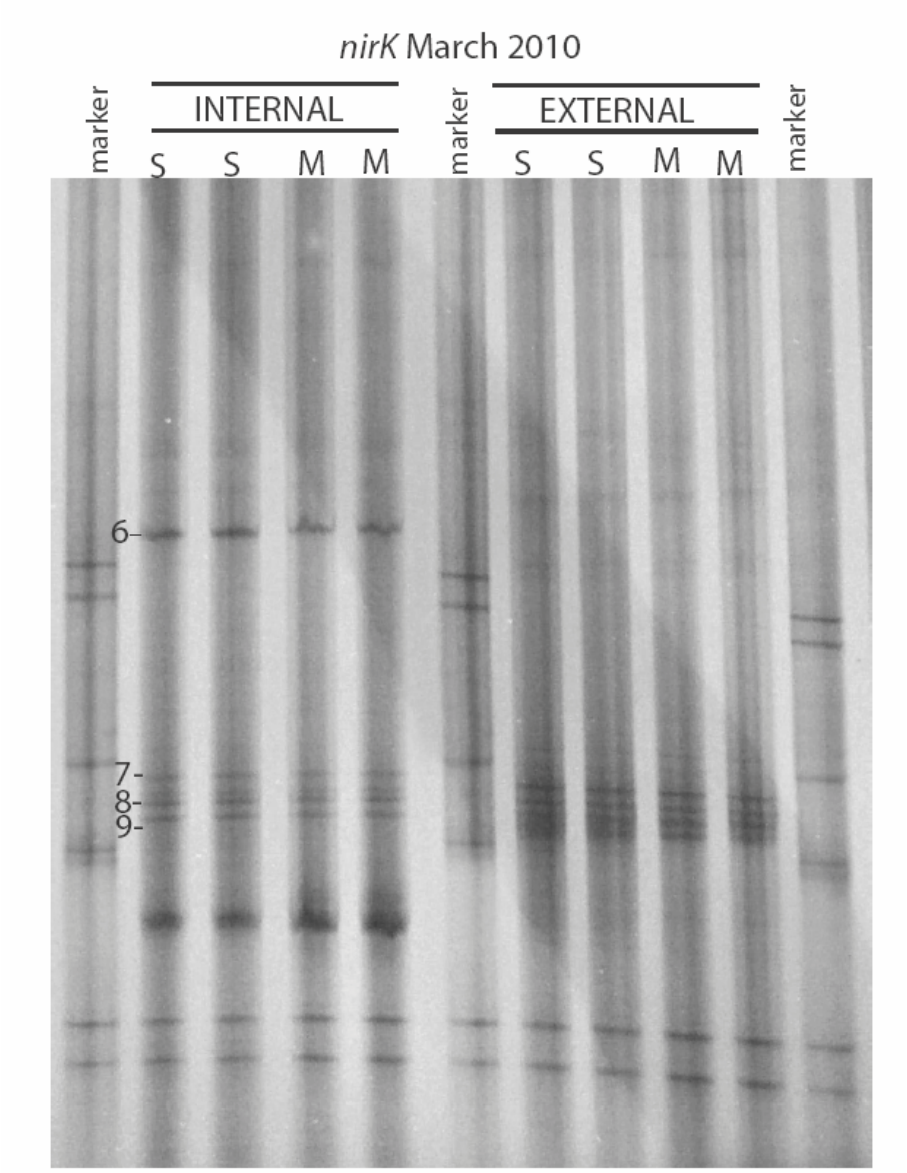


Fig. 6.6 - DGGE profile of *nirK* genes for medium (M) and surface (S) soil internal and external to the riparian buffer zone in March 2009. The numbers indicate bands that were sequenced.

The number of bands obtained (Tab. 6.2) are in accordance with previous researches on *nirK* in soils (Throbäck *et al.*, 2004; Wertz *et al.*, 2009)

Comparing external and internal sites higher numbers of bacterial species were found in the soil of the riparian buffer: 26-25 and 22-21 bands were counted for internal soil at two different soil depths, while only 9-15 and 14-16 bands were found for the external soil (Tab. 6.2). These results seem to indicate that the conversion of the agricultural area in a riparian

buffer zone kept under denitrifying conditions affects positively the soil microbial diversity even in term of *nirK* containing bacterial species.

Tab. 6.2 - Difference in band numbers obtained with DGGE analysis between internal and external soil samples

		Surface		Medium	
		Internal	**External**	**Internal**	**External**
2009	**October**	26	9	25	15
2010	**March**	22	14	21	16

6.3.3 Cluster analysis

The presence and absence of bands in the two PCR-DGGE profiles have been used to create a binary matrix for quantitative comparisons between two communities (Kropf *et al.*, 2004; Wilbur *et al.*, 2002).

The related results are shown in the following Fig. 6.7.

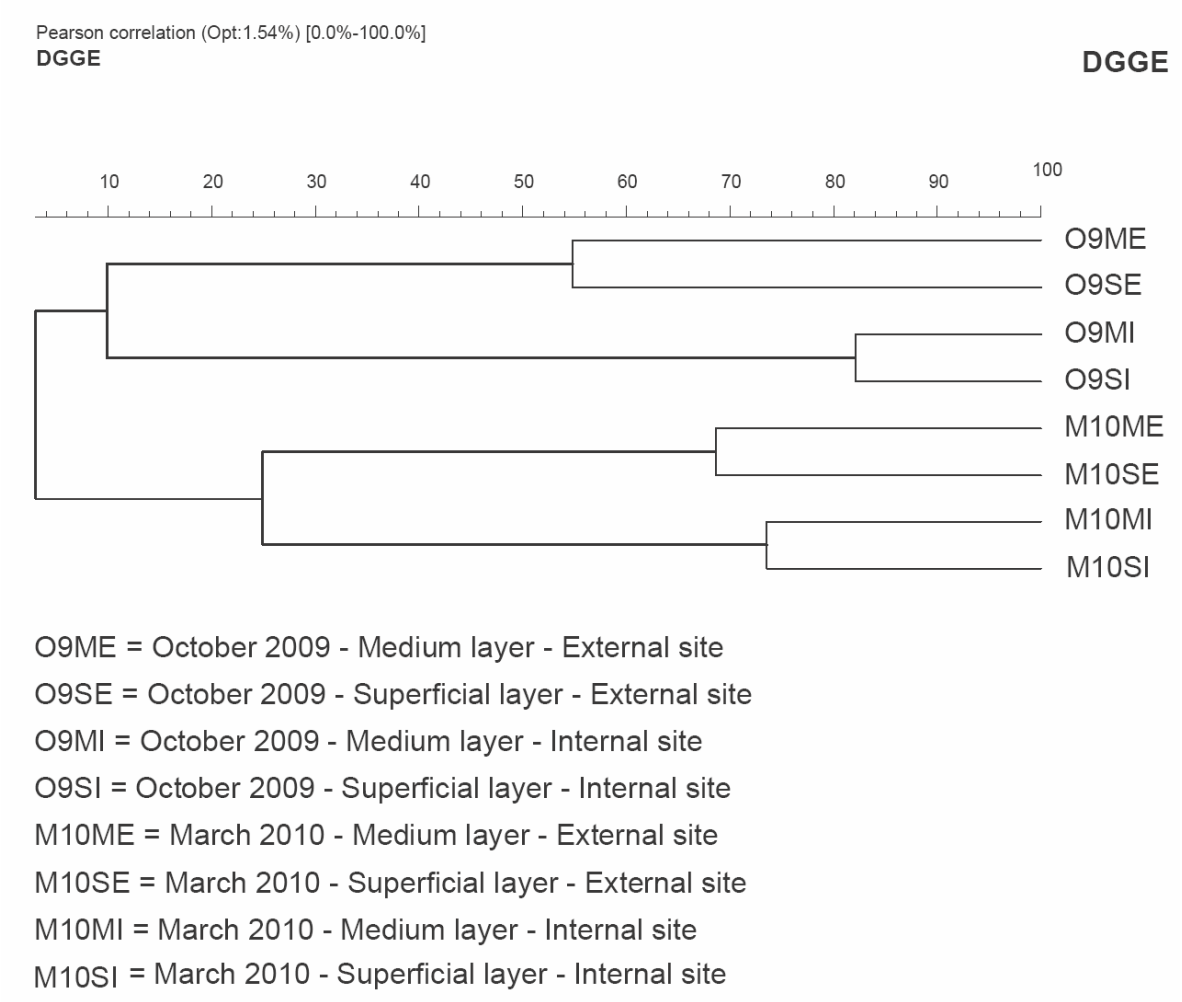


Fig. 6.7 - DGGE clusters analysis of microbial communities of internal and external soils for both October 2009 (indicated as O9) and March 2010 (M10). ME=medium layer external; SE= superficial layer external; MI=Medium layer internal; SI=superficial layer internal.

Cluster analysis of DGGE showed that distinct and separate cluster groups were obtained for the samples collected during October 2009 and March 2010, thus indicating that different seasonal conditions strongly affects the composition of denitrifying microbial community.

In addition, during each of the two seasons, a clear separation between the bacterial soil communities from inside and outside the buffer zone was evident.

On the other hand, the distinction between the surface and intermediate layers resulted less visible, both inside and outside the buffer area.

6.3.4 Principal component analysis (PCA) of the soil denitrifying microbial communities

Multivariate analysis methods, such as principal components analysis (PCA) (Pielou, 1969), have been used to analyze large data sets with greater sources of variation (Gremion *et al.,* 2004; Joynt *et al.,* 2006). PCA calculates and ranks the contribution of each variable in a profile, and the approach can be used to identify the main sources of variation observed between profiles (Wilbur *et al.*, 2002). For example, in DGGE profiles, the source (band) contributing to the greatest variability can be statistically determined, then the bands can be extracted from the gel, and their nucleotide sequences determined to identify specific components of the bacterial population.

The results obtained are shown in Fig. 6.8. Once again the profiles related to different seasons and internal and external soils remain as definitely separate. A small, but more evident separation between superficial and medium layers can be observed in the internal soil samples better than in the external ones.

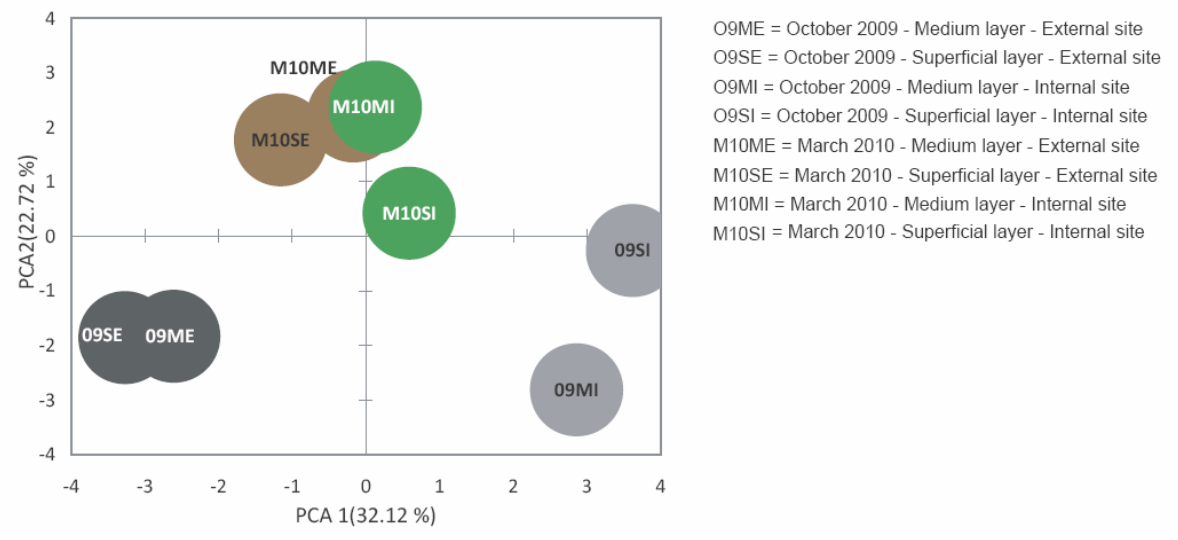


Fig. 6.8 - Principal component analysis based on DGGE band distribution.

6.3.5 Phylogenetic analyses of partial *nirK* sequences

Partial *nirK* sequences corresponding to 10 of DGGE band positions with different relative intensities of *nirK* DNA were subjected to phylogenetic analyses (Table 2).

To get phylogenetic affiliations of the bacterial community, individual DGGE bands were excised from the gel and sequenced to determine the diversity of the *nirK* gene fragments. The closest relatives derived from the sequenced fragments identified with the BLAST program were nearly all sequenced clones. The phylogenetic affiliation of the 9 selected bands revealed members of five different phylotypes with an overall prevalence of *Rhizobiales*. Also note the presence of some non-culturable and not yet classified species.

Tab. 6.3 - The phylogenetic affiliation of the selected bands

Source of band	Related species name	Division/ Family	Similarity
1	*Ensifer sp. R-32544*	Alphaproteobacteria	98%
2	*Pseudomonas fluorescens Pf0-1*	Gammaproteobacteria	85%
3	*Rhizobiales bacterium N21*	Alphaproteobacteria; Rhizobiales	93%
4	*Pseudomonas chlororaphis*	Gammaproteobacteria/Pseudomonadaceae	92%
5	*Bradyrhizobium japonicum*	Alphaproteobacteria/Bradyrhizobiaceae	97%
6	*uncultured bacterium*		93%
7	*uncultured bacterium*		92%
8	*Sinorhizobium sp. PD 12*	Alphaproteobacteria	93%
9	*no significant found*		

6.3.6 Potential denitrification related to analysis of denitrifiers community

A comparison of the potential denitrification (DEA+C+N) rates expressed by the same soils where molecular analysis was done is reported in the following Fig. 6.9 and Tab. 6.4. High differences between the two seasons were observed, both on the superficial and in the medium layers with significantly higher values in October 09. In both seasons, but only limited to the superficial layer, significant differences between internal and external soils were observed.

Higher values were always registered in the superficial layer. In summary, the same differences observed on denitrifying community composition were registered, except for the additional distinction between layers observed only on DEA+C+N.

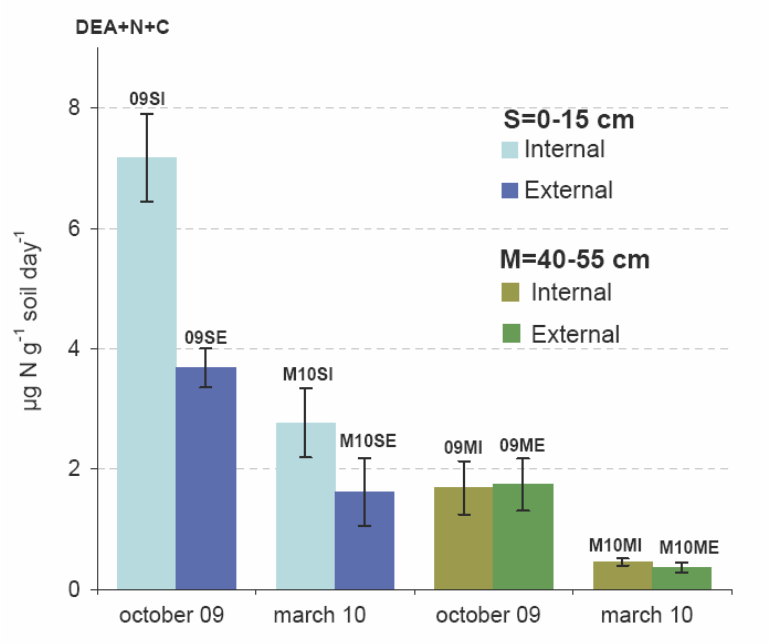


Fig. 6.9 – Potential denitrification (obtained in anoxic conditions and with the additions of Carbon and Nitrate) in the same soils where the analysis of denitrifiying community was done. October 2009 is indicated as O9, March 2010 as M10; ME=medium layer external; SE= superficial layer external; MI=Medium layer internal; SI=superficial layer internal.

Tab. 6.4 – DEA+N+C, nitrate and organic carbon content in the two layers of the buffer zone and of the agricultural area during the monitored seasons (± standard error).

Season	Site	Layer	N-NO_3 *mg N Kg^{-1} soil*	Organic C *%*	DEA+N+C *µg N-N_2O g^{-1} soil day^{-1}*
October 2009	riparian buffer	S (0-15 cm)	5.58 ± 0.47	1.70 ± 0.11	7.37 ± 0.72
		M (40-55 cm)	1.31 ± 0.34	0.54 ± 0.08	1.64 ± 0.44
	agricultural area	S (0-15 cm)	/	/	3.68 ± 0.32
		M (40-55 cm)	/	/	1.74 ± 0.43
March 2010	riparian buffer	S (0-15 cm)	2.25 ± 1.44	1.25 ± 0.31	2.84 ± 0.58
		M (40-55 cm)	1.00 ± 0.35	0.38 ± 0.06	0.45 ± 0.06
	agricultural area	S (0-15 cm)	0.60 ± 0.01	0.60 ± 0.01	1.61 ± 0.56
		M (40-55 cm)	1.41 ± 0.24	0.34 ± 0.07	0.37 ± 0.09

6.4 Discussion

There is growing interest in how soil management, plant communities and soil microbial community structure and functioning are linked. Of particular interest are the implications that this inter-relationship may have on buffer zones functioning, because microbial organisms are the crucial mediators of nitrogen removal. It was shown in several studies that individual plant species can influence microbial communities, in particular in their rhizosphere (Grayston *et al.*, 1998; Wardle *et al.*, 2003; Bremer *et al.*, 2007). Furthermore, the presence of different plants may play a significant role in controlling ecosystem processes and over all ecosystem functioning (Beierkuhnlein and Jentsch, 2005; Hector *et al.*, 2005). A major, but yet largely unresolved question in microbial ecology is whether microbial community structure and function are interlinked.

Our study, according to other recent researches (Baudoin *et al.*, 2003; Bremer *et al.*, 2009; Dandie *et al.*, 2011) investigated the link between *nirK*-type denitrifier community composition and denitrification enzyme activity.

Differences between riparian and agricultural soil. In our study the *nirK*-type denitrifier community significantly differs between riparian and agricultural soils in surface layer. This is in accordance with another recent study concerning this matter (Dandie *et al.,* 2011).

A major issue, not specifically investigated in our study, is to highlight which are the factors inducing this differentiation. Riparian and agricultural zones usually differ in soil properties, soil water content, plant presence and/or composition and nutrient concentration. A previous study (Bremer *et al.*, 2009) demonstrates that the presence as well as the combination of different plants affected the composition of the *nirK*-type denitrifier. According to this and other studies (Kuske *et al.*, 2002; Smalla *et al.*, 2001; Grayston *et al.*, 2001) the plants play an important role in supporting and developing microbial diversity in soil. Indeed, different amount, spectrum and composition of root exudates or decomposing root material coming from plants resulted in different denitrifying communities (Griffiths *et al.*, 1999; Jaeger *et al.*, 1999). Similarly, in several studies, the microbial communities of the rhizosphere were found to be distinct from those of the bulk

soil in the vicinity of plants (Costa *et al.*, 2006). These differences have been attributed to the effect of root exudates on microbial communities (Bais *et al.*, 2006).
In our sites, the undisturbed woody and herbaceous vegetation within the riparian buffer increased the amount of organic carbon available on soil (in particular in the superficial layer); on the contrary in the agricultural zone a depletion of organic Carbon due to soil erosion processes and disturbance (planting, tillage) activities was observed (Tab. 6.4). Considering that organic carbon would be predicted to be the most important factor selecting for distinct soil denitrifiers, because the majority of them are aerobic heterotrophs that may seldom use their denitrification capacity, a role of this element in affecting denitrifying community could be taken into account also in our case study.
On the same time, differences in water content, due to the particular irrigation water management adopted inside the riparian zone and to the vegetation cover, could be an additional and important regulating factor since it strongly affects oxygen availability.
Instead, according to previous researches (Dandie *et al.*, 2011), differences on $N\text{-}NO_3^-$ content, are in general less influencing the composition of denitrifying community.

*Seasonal effects***.** In our study, sampling time strongly influenced the *nirK*-containing community composition. Seasonal effects on soil microbial communities composition (Grayston *et al.*, 2001; Carney and Matson, 2006) as well as on denitrifiers (Wolsing and Prieme, 2004; Boyle *et al.*, 2006; Bremer *et al.*, 2009) have been reported previously. However, to our knowledge no other studies have observed denitrifying communities in any natural environment for more than one year. These findings highlight the need for long-term ecological studies that examine N transformation and community dynamics both seasonally and over several years.

Difference between layers. To our knowledge, differences between denitrifiers in function of soil depth were not investigated in previous studies.
In our research, no significant differences between superficial (0-15 cm) and medium (40 – 55 cm) layers on the community composition were observed. This result is not surprising if we consider the agricultural soil, where the tillage activity regularly mix the soil in the first 60-80 cm, while is quite surprising in the soil of the riparian buffer, where the soil

management is different in the two layers. Moreover, we have to consider that in 2009 and 2010, due to the described (see Fig. 5.10) lowering of groundwater, both layers are located in the unsaturated zone. For this reason, the main distinguishing factor between the two layers during 2000-2002 was not observed in the present period of investigation.

Interlink between community structure and function. There is still a limited understanding of the controls on denitrifier abundance and diversity in the environment and of the relationships between denitrification activity and denitrifying community structure and/or abundance (Philippot *et al.*, 2009).

Other authors suggest that the relationship between denitrifying communities and their functioning may be ecosystem specific (Rich and Myrold, 2004), and that the activity of denitrification enzymes may sometimes be coupled to community composition, while in other cases, it may be determined by environmental factors (Wallenstein *et al.*, 2006).

However there is a substantial agreement that as denitrification potential changes with time and site, denitrifying community dynamics must have an impact on denitrification potential (McGill *et al.*, 2010). It's also well known that plant presence, plant combination and time affect the denitrification activity (Boyle *et al.*, 2006; Bremer *et al.*, 2009; Dandie *et al.,* 2011).

In our study, potential denitrification (DEA+C+N) strongly differs between seasons, soil management (riparian buffer and agricultural site) and soil layers. According to our data, denitrifying community composition could affect, in combination with other environmental factors, either seasonal and sites (internal and external) differences on potential denitrification, but not the difference between surface and deeper soil layers. Different denitrification potentials between layers at different dephts could be better explained by considering the very high difference in term of microbial biomass abundance (see Fig. 5.19). In addition we have to consider the results obtained by Bremer *et al.*, 2009 that indicated a strong and direct linkage of denitrifying community composition and functioning, but also that plants had additional effects on denitrifier function that could not be solely explained by their effects on *nirK*-type denitrifying community composition.

7 CONCLUSIONS

During this work an impressive database on different components of a forested buffer system was produced, plus there are two remarkable aspects that may attract the interest for the results obtained: (i) the possibility to study the same riparian buffer system for a long term period (10 years), starting from its conversion from a previous agricultural area and following its development to the present situation; (ii) the peculiar structure of the system based on the integration of hydraulic works (drains, pumping system) within a natural system (the forest); this may give the possibility to directly control some parameters, such as water inflow and outflow, which in natural riparian systems cannot generally be managed.

This research focus mainly on the monitoring period 2007-2010, but in order to better understand the processes under investigation, data collected between 1999 and 2002 were also considered.

One of the aims of this study was to explore the possibility of reducing the level of nitrogen in rivers by forcing water to circulate through afforested riparian buffers.

The results obtained demonstrate that:

- a buffer strip 15 meters wide can remove an excess of nitrate, supplied in continuous by irrigation waters with concentrations typical of freshwater bodies (less than 5 mg/L $N\text{-}NO_3$), and that narrower buffer strips (e.g. 5 meters wide with only one row of trees) are likely to be adequate; this was more evident during the warm season (April/May to November), while in the winter period (from December to March) the system was less effective;
- this performances could be observed only one year after the arable land was converted to a wooded wetland; during the second year, the experimental buffer system started to efficiently remove nitrogen loads flowing through, reaching 80-85% of nitrate removal corresponding to about 73 Kg ha^{-1} $year^{-1}$ of $N\text{-}NO_3$; by a long term monitoring it has been possible to conclude that such effectiveness could be also maintained for the following years;
- an increasing capability of the system to even remove the organic nitrogen instead of leaching it, like it was found to happen during the first 3 years, was observed during 2008 – 2010, while an opposite trend was recorded for $N\text{-}NH_4$, with a considerable increase of the losses during the last three years. It may be due to an increasing

capability of the system to mineralize the organic nitrogen coupled to a not corresponding increase of the plant assimilation and bacterial nitrification activities;

- although the above opposite trend in removing different forms of nitrogen, high retention rates of total N were globally observed with values ranging between 55% to 64%, corresponding to a removal of 74 to 84 Kg ha^{-1} $year^{-1}$;
- further addition of nitrogen to the system during 2009 – 2010 gave conflicting results: while during 2009 the retention rates did not substantially change if compared to standard years and the system resulted able to remove higher amounts of nitrate (52% and 140 Kg ha^{-1} $year^{-1}$), during 2010 it resulted less effective (37% and 106 Kg ha^{-1} $year^{-1}$). To explain this result further studies are necessary even if some hypothesis were here reported (see pharagraph 4.3).

The other important objective of this work was to investigate the role of the microbial denitrification process in nitrogen removal and its relationship with the main environmental limiting factors (hydrology, soil, climate, vegetation and nutrients availability). The results obtained demonstrate that:

- even in the fine textured soil of the present experimental site, the anoxic conditions required for denitrification can be obtained by creating semi-natural floodplains where water flows can be suitably managed; under these hydrologic conditions, high denitrification rates were registered in the soil layer that is often saturated by the perched aquifer. In particular, the highest denitrification rates were measured in the soil layers located on the border between the unsaturated and saturated zone, where aerobic processes (like nitrification) and anaerobic denitrification can occur in close proximity;
- the long-term monitoring has also shown that the development of the riparian wood can very significantly affect the water table elevation, especially during the dry seasons; when evapotranspiration is high, there is a significant lowering of the subsurface flow level resulting in inhibition of the denitrification process; in these terms, the competition between plants and bacteria is not limited to the use of nitrates, but also to the capability of woody plants to influence soil hydrology and the connected bacterial processes;
- potential denitrification (DEA) strongly differs between seasons, soil depth and soil management (riparian buffer and agricultural site): warmer seasons have in general higher potential denitrification rates that, as expected, generally decrease with soil depth,

depending on the distribution of the microbial population; in addition, the surface layer of the riparian soil showed a greater denitrification potential as compared to the agricultural soil;

- in general, the organic carbon availability resulted the most limiting factor for denitrification; if an increase of the carbon content in 0 – 15 cm layer, due to vegetation and litter decay, was observed along the monitoring years, no corresponding changes in deeper soil layer (40-95 cm) were measured; for this reason the low carbon availability still strongly affects the denitrification activity in deeper soil layers.

A number of scientific papers report on the comparison between denitrification rates and environmental limiting factors (i.e. hydrology, soil, climate, vegetation). On the contrary, only few studies are trying to relate denitrification measures to the composition, biomass and distribution of the denitrifying bacterial community. For this reason this relationship has also been faced in this project through the investigation on *nirK*-type denitrifying community. More specifically, it was observed that:

- *nirK*-type denitrifying community composition significantly differs between riparian and agricultural soils and between different seasons;
- on the contrary, reduced difference in composition between different soil layers were observed both inside and outside the riparian buffer;
- comparing these results with the differences observed on denitrification potential it was concluded that the bacterial composition could affect, in combination with other environmental factors, either seasonal and sites (internal and external) differences of potential denitrification; however, the same clear effect was not revealed on the soil layers, suggesting that denitrification potentials at different soil depths may depend mostly upon the size of the bacterial population, rather than its specific composition.

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Appendix

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Shallow groundwater nitrogen and denitrification in a newly afforested, subirrigated riparian buffer

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Summary

1. The EU 'Nitrates Directive' (Directive 91/676/EEC) and the WFD (Water Framework Directive 2000/60/EEC) introduced a series of measures designed to reduce and prevent water pollution caused or induced by nitrates from agricultural sources. Therefore, there is an urgent requirement to control the nitrate concentration in freshwater. The objective of this paper was to verify the potential capacity of a specifically designed afforested riparian zone in removing the excess of nitrogen from river water.

2. A buffer zone was set with irrigation ditches, to produce a subsurface water flow carrying water from the study river through the buffer strip to drainage ditches. This experimental system enables the co-occurrence of two main processes: vegetation/microbial nitrogen uptake and denitrification. Both *in situ* denitrification and denitrification potential were measured at different soil depths, and nitrogen removal of water passing through the buffer system was measured.

3. After the first year, high removal rates (63–64%) of total nitrogen in water were recorded. The lowest rate of denitrification took place in the upper soil layer, while maximum denitrification occurred in the medium layer (40–55 cm). Denitrification occurred mainly in the first few metres of the irrigation ditches leading away from the river. The denitrification rates clearly increased from the second to the third year, with highest rates in summer and autumn. Denitrification potential indicated that carbon availability was the most limiting factor.

4. *Synthesis and applications.* This study has demonstrated that nitrogen levels can be reduced in rivers by forcing water to circulate through afforested buffers. Nitrogen was removed both by plants and by microbial denitrification. Such activity can be supported by promoting anoxic conditions through appropriate water flow management. This could be achieved by creating semi-natural floodplains where water flows can be efficiently managed as in a drained wetland.

Key-words: denitrification, denitrification enzyme activity, eutrophication, nitrate remediation, potential denitrification, riparian zone, water quality

Introduction

The contamination of surface and groundwater by nitrates is a major factor affecting estuarine eutrophication (Howarth & Marino 2006; Hakanson, Bryhn & Hytteborn 2007) and drinking water supplies in many European countries (EEA 2005). The control of water pollution, especially nitrates, was an important concern for the Nitrates Directive (91/676/EEC). The WFD (Water Framework Directive 2000/60/ECC) has the specific aim of enhancing the status of all European water systems. The WFD (Art. 10) confirms and reinforces the need to reduce nonpoint pollution using the same strategy and the same actions proposed by the Nitrates Directive.

Agriculture is a significant source of combined nitrogen release to the environment, because fertilizer inputs to crops are generally higher than the amount of nitrogen required to maximize plant productivity (Driscoll *et al.* 2003). According to recent studies, agricultural practices are typically responsible for 50–80% of the total nitrogen load to groundwater and freshwater (EEA 2005; JRC 2006). Nitrate concentration in a number of intensive agricultural areas exceeds the maximum value of 50 mg NO_3 L^{-1} for drinking water. Although surface water quality trends have generally stabilized during the last few years, more effort is required to achieve the objectives of the Nitrates Directive. Regional estimates of the application

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rate of nitrogen from manure exceed 170 kg ha^{-1} year at the local level in several European countries. Approximately 16·5 million tons of nitrogen was applied to European soils in 2003, with 7·6 million tons per year derived from animal husbandry (mainly cows, pigs, poultry and sheep) and 8·9 million tons from mineral fertilizers.

Riparian zones, located at the interface between terrestrial human activities and aquatic ecosystems, play a key role as a buffer system (Lowrance, Todd & Asmussen 1983; Lowrance *et al*. 1984; Peterjohn & Correll 1984; Hunter & Faulker 2001; Spruill 2004; Carline & Walsh 2007; Pinay *et al*. 2007). A buffer zone can be defined as a transition area from one ecosystem to another, in this case from an agro-ecosystem to an aquatic ecosystem.

There are several biological mechanisms through which excess nitrogen is removed in riparian buffer zones: some act as temporary sinks, for instance uptake and assimilation by plants and microbes, and other permanently remove nitrogen from the soil in a gaseous form, i.e. denitrification processes (Hedin *et al*. 1998; Hefting & de Klein 1998). The two processes, vegetational/microbial uptake of available nitrogen and denitrification, can work together to provide a buffer zone protecting aquatic ecosystems from excessive nitrogen loads (Lowrance *et al*. 1984, 1997; Peterjohn & Correll 1984; Pinay, Roques & Fabre 1993; Haycock *et al*. 1997; Pinay *et al*. 2000, 2007; Sabater *et al*. 2003).

The effective removal of nitrate within riparian zones is dependent upon the presence of conditions conducive to high denitrification rates as well as to the growth of vegetation. Denitrification capacity of the soil can be evaluated and possibly enhanced in order to increase nitrogen (N) removal. Exploitation of *in situ* denitrification (DNT) to reduce nitrate load depends largely on local conditions such as the reduction capacity of the soils, the redox potential, temperature, nitrate concentration and organic carbon availability (Pinay, Burt & Gumiero 2006). Denitrification is most active in soils rich in organic matter and having high moisture content and low oxygen (Pinay, Ruffinoni & Fabre 1995), all characteristics commonly found in riparian zones.

Low-order streams are considered the most suitable for controlling nitrogen fluxes because of their great interaction potential with both riparian and agricultural areas (Décamps *et al*. 2004; Pinay, Burt & Gumiero 2006). However, in this study, we explored the possibility of reducing the in-stream nitrate concentration of high-order water courses flowing into the Venice Lagoon by creating semi-natural floodplains where water flow can be managed. In this case, water management was applied to pump river water into irrigation ditches so causing the water to flow through the riparian buffer.

North-east Italy includes one of the major drained reclamation regions of the country, and a considerable portion of the Venice Lagoon catchment area is located within this region. Over the past decades, nutrient loads delivered to the Venice Lagoon have attracted considerable concern, resulting in the establishment of a series of nitrogen and phosphorus reduction targets by the local government (Regional Authority) in 1995. For Dese and Zero Rivers, two of the main rivers managed by the local drainage authority (Consorzio di Bonifica Acque Risorgive), a reduction of 150×10^3 kg per year of total N and 40×10^3 kg per year of total P was established.

Several actions were undertaken to achieve these objectives, one of which was the conversion of a cultivated area of about 30 ha to a forested buffer strip, irrigated with freshwater from the Zero River. Inside this afforested area, a pilot experimental scale system was established in order to find the most suitable conditions for enhancing denitrification activity. The experimental forest buffer received almost continuous subsurface water flow with the aim of enhancing nitrate removal through denitrification.

The efficiency of the buffering capacity of this afforested area on nonpoint pollution sources of nitrogen was evaluated through detailed measurements of weather, hydrology, water quality, soil chemical parameters and denitrification rates. In this paper, we focus mainly on DNT and denitrification enzyme activity (DEA) to determine the processes responsible for reducing nitrogen in the whole system.

Materials and methods

The experimental site is located 15 km from Venice, Italy. The climate is subcontinental with temperatures ranging from a daytime average of 1 °C in January to 23 °C in July and August. The mean value of rainfall is 900 mm per year, peaking in autumn and spring and with lower values in winter and summer. Between 0 and 90 cm below the surface, the soil texture is extremely homogeneous, categorized as 'silty clay loam' (according to textural classification USDA-SCS 1984). An unstructured and calcic layer, with high content of loam and clay, occurs at around 90–150 cm depth, and owing to its very low permeability, it prevents interactions between alluvial groundwater and the near surface soil (ARPAV 2004).

The experimental area occupies a total area of about 0·70 ha (227 m long and 30 m wide) and was designed to rigorously monitor the hydrological fluxes and to carefully characterize the hydrology of the buffer system. It was planned according to the NICOLAS project (Burt *et al*. 2002) to examine a three-zone buffer system. Zone 3 is close to the irrigation ditch, zone 2 is at mid-distance between the two ditches and zone 1 is close to the drainage ditch. The two replicate sides of the drainage ditch were designated A and B, and all the measurements were carried out on both plots (see Fig. 1). Ridges and furrows facilitate subsurface water flow throughout the field from the inlet point, represented by two irrigation ditches where water is pumped through, to the parallel drainage ditches located at lower elevation (Fig. 1). The average slope between irrigation and drainage ditches is 4% (Fig. 2). Several tree and shrub species (white willow *Salix alba* L., almond willow *Salix triandra*, black alder *Alnus glutinosa* (L.) Gaertner, pedunculate oak *Quercus robur* L., field maple *Acer campestre* L., common hazel *Corylus avellana* L., common hawthorn *Crataegus monogyna* Jacq., manna ash *Fraxinus ornus* L. and black dogwood *Frangula alnus* L.) were planted in spring 1999 and were arranged in four parallel rows for each plot as indicated in Fig. 1.

HYDROLOGIC MONITORING

Two 5 × 3 m grids of piezometers (1·5 m depth and 38 mm diameter each) were installed in each plot in September 1999 giving a total of 30 piezometers. These were used to determine water-table depths and to collect water samples in monthly sampling.

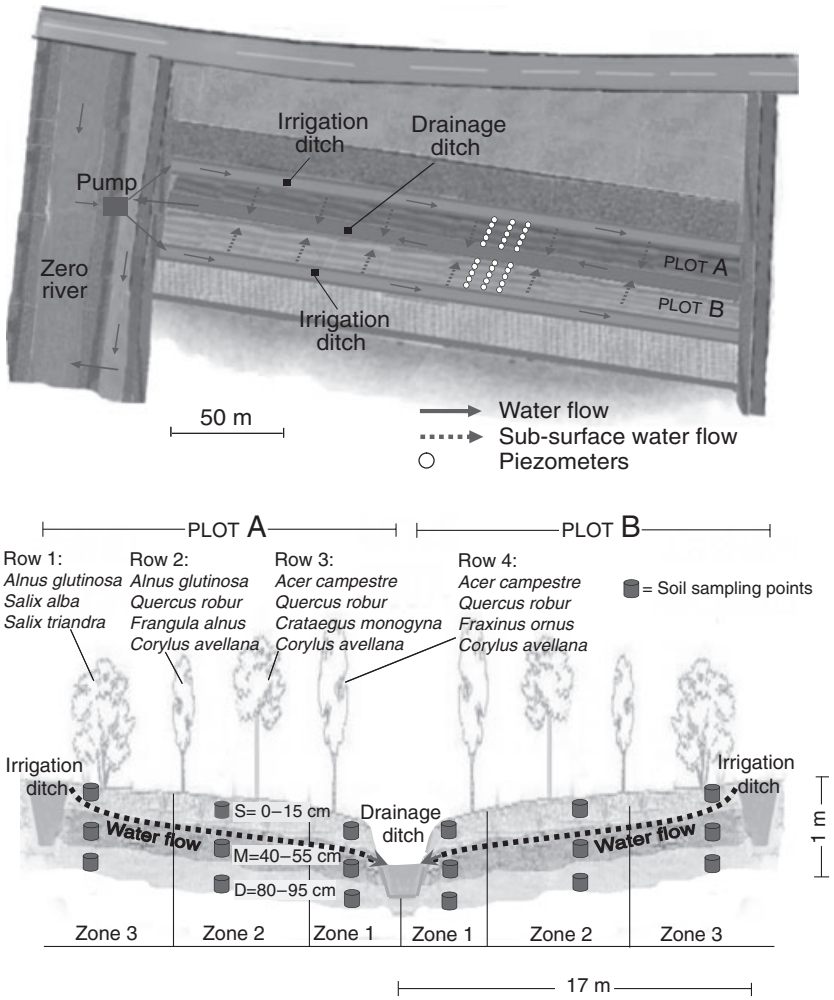


Fig. 1. Plan (above) and section (below) of the experimental site: each of the two plots is watered through an irrigation ditch carrying water from the Zero River. There is a difference in elevation between the irrigation ditches and the drainage ditch, resulting in a subsurface flow of water running through the wooded buffer strips. Soil sampling in each plot was located at nine points, three for each zone. Zone 3 is close to the irrigation ditch, zone 2 is at mid-distance between the two ditches and zone 1 is near the drainage ditch. For each sampling point, the soil is sampled at three layers at different depths (S: 0–15 cm, M: 40–55 cm, D: 80–95 cm below the soil surface).

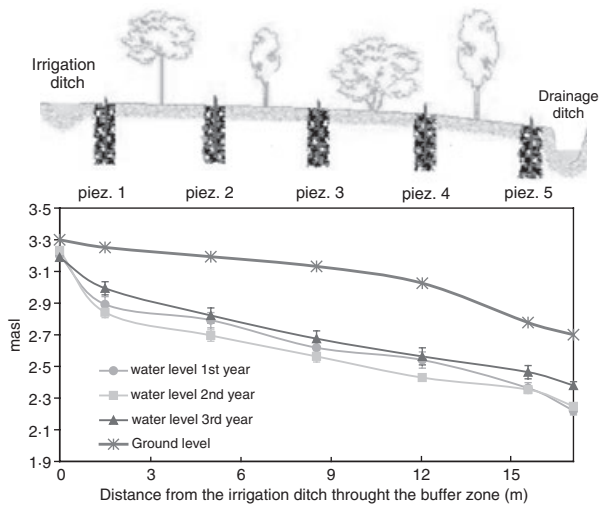


Fig. 2. Ground-level and mean annual water-table elevation measured in plot A (monthly measures in ditches and piezometers) during the three monitored years. Values for plot B were very similar. Bars represent standard error.

In each plot, two additional piezometers (one near the irrigation ditch and one near the drainage ditch) were equipped with a pressure transducer (Druck – PT-B1; GE Measurement & Control Solutions, Billerica, Massachusetts, USA) connected to a data logger (Smart-Reader 7 Plus; ACR Systems Inc., Surrey, BC, Canada) to record water-table elevations every 15 min. The data were collected from October 1999 to October 2002. Subsurface water depth was also measured monthly (using phreatimeters: Electric contact gauge KL 015; Ott Messtechnik GmbH & Co. KG, Kempten, Germany) in the 30 piezometers and during the entire period of study.

The volume of the introduced irrigation water was continuously measured by a flowmeter (Datawater WMPE; Maddalena S.p.A., Udine, Italy) inserted in the water supply line. Tracer experiments (Dierberg & DeBusk 2005) using the organic fluorescent tracer Rhodamine WT (RWT) were performed in 2007 to describe the hydraulic behaviour of the system. Two hundred grams of diluted (21·33%) RWT was injected into the irrigation ditch, and subsequently, 99 samples were collected every 60 min from the discharge ditch by an ISCO 6700 automatic sampler (Teledyne Isco Inc., Lincoln, NE, USA). All samples were filtered through a 0·45-μm fibreglass filter, and RWT concentration was determined by a fluorometer (SCUFA®; Turner Designs Inc., Sunnyvale, CA, USA).

The concentration of chloride, a biologically inert conservative tracer (Altman & Parizek 1995), was measured monthly to monitor dilution and dispersion (Sabater *et al.* 2003).

An automatic weather station near the experimental site recorded many climatic parameters (air temperature, rainfall, wind direction and velocity, air moisture, global radiation) and gave the opportunity to estimate the EPT potential by Penman–Monteith approach, using

a combination equation that combines the radiation and the aerodynamic terms (Allen *et al.* 1998).

The water balance of the afforested buffer area was estimated using the following formula

$$I + R - \mathrm{ET} = D,$$

where I = irrigation volume; R = rainfall; ET = evapotranspiration; D = drainage back to river.

WATER QUALITY

Water pumped from the Zero River into the irrigation ditches was sampled daily as a single discrete sample by using an automatic sampler (American Sigma – Portable sampler 900 standard, with 24 one-L bottles; Hach Company, Loveland, CO, USA). The irrigation and drainage ditches were sampled monthly by grab sampling of the general shallow flow. Piezometers were pumped using a hand pump (Kartell –MR 50 H *c*. 240 mL; Kartell S.p.A., Milan, Italy) first to remove two well volumes and then sampled after water had recharged the well. Field measurements were made of pH (pH meter handylab 1; Schott-Geräte GmbH, Mainz, Germany), temperature (°C) and electrical conductivity using a Schott-Geräte Conductivity meter handylab LF with integrated temperature sensor.

Water samples for analyses were filtered through a 0·45-μm PVDF filter in the laboratory and analysed within 24–36 h for N-NO_3, N-NO_2, N-NH_4, total nitrogen and chloride.

Dissolved anions (Cl, N-NO_3) were determined by ion liquid chromatography (Pfaff, Hautman & Munch 1997). Dissolved N-NO_2 was determined by the Griess-Illosvay method and spectrophotometric measurements (APHA AWWA WEF 2005a). Dissolved N-NH_4 was determined by the indophenol blue method and spectrophotometric measurements (APHA AWWA WEF 2005b). Dissolved total N was determined with the persulphate oxidation method (Valderrama 1981) followed by nitrate analysis. Nitrate was reduced to nitrite by cadmium reduction and determined as explained. Organic N was determined by calculation (Norg = Ntot−N-NH_3−N-NO_2−N-NO_3).

DENITRIFICATION AND CHEMICAL PARAMETERS OF SOIL

In each plot (A and B), soil samples were collected using a manual drill, from nine different places (three replications for each of the three zones) at three different depths [surface (S), 0–15 cm; medium (M) 40–55 cm and deep (D) 80–95 cm] (see Fig. 1). Samples were taken seasonally (every 3 months) for 3 years (October 1999–October 2002) with 54 soil samples per season and 12 total sample dates. Winter, spring, summer and autumn samples were taken in January, April, July and October, respectively. *In situ* DNT was assayed by the static core acetylene inhibition method (Yoshinari & Knowles 1976). One hundred grams of fresh soil was weighed into glass screw top jars (250 mL) capped with rubber serum stoppers and then amended with acetone-free acetylene to bring soil atmosphere concentration to 10 KPa (10% V/V) acetylene and 90 KPa air. Samples were incubated at field temperature, and denitrification rates were calculated as the rate of nitrous oxide (N-N_2O) accumulation in the head space between 1 and 4 h. Head space samples were removed from all cores and stored in 10-mL evacuated collection tubes (Venoject; Terumo Europe N.V., Leuven, Belgium). Gas samples were analysed via gas chromatography (Trace GC 2000; Thermo Fisher Scientific Inc. Waltham, Massachusett, USA), equipped with an electron capture detector (^{63}Ni) and a VARIAN CP7554 poraPLOT Q (VARIAN Inc.) column (27·5 m × 0·53 mm, film 20 μm).

Denitrification enzymatic activity was measured for each soil sample using Smith & Tiedje's procedure (1979) (see also Groffman *et al.* 1999). In the laboratory, four sets from each soil sample (30 g of fresh soil) were transferred into glass screw top jars (250 mL) capped with rubber serum stoppers, flushed with N_2 and incubated for 8 h with acetone-free acetylene to bring soil atmosphere concentration to 10 KPa (10% V/V) acetylene and 90 KPa N_2. One set was amended only with nitrate (10 μg N-NO_3 g^{-1} fresh soil); a second set was amended with organic carbon (4 mg C-glucose g^{-1} fresh soil); a third set was amended with both organic carbon and nitrate (10 μg N-NO_3 g^{-1} and 4 mg C-glucose g^{-1}), referred to as DEA. The last set was incubated with only acetylene under N_2 atmosphere. All the samples were under water-saturated conditions obtained by adding 1 mL of distilled water for each gram of soil. Denitrification rates (μg N-N_2O g^{-1} soil day^{-1}) were calculated as the rate of nitrous oxide (N-N_2O) accumulation in the head space between 4 and 8 h.

Each soil sample was further analysed for soil moisture, organic C and nitrate. Soil moisture was determined gravimetrically after drying subsamples at 104 °C for 24 h and by dividing the difference between wet and dry masses by the mass of the dry sample. The organic C content was determined by oxidizing the organic matter with acid dichromate reagent. The excess of chromate left after C oxidation was analysed by spectrophotometric measurements (Nelson & Sommers 1982). For determining the N-NO_3 contents, subsamples were extracted with 2 M KCl and quantified by the Griess-Illosvay method and spectrophotometric measurement (Keeney & Nelson 1982).

STATISTICAL ANALYSIS

All the results were statistically analysed by ANOVA (variance analysis).To analyse the effects of seasons, depth and zones (distance from irrigation ditch) on DNT rate, three-way factorial ANOVA with interaction terms was used. For this latter analysis only the second and third years were considered because of the lack of some data from the first year. For each of the three ANOVA factors, three soil replications in plot A and three in B were collected. Although the two plots of the experimental site (A and B) were designed as two exactly symmetrical sections that share the same drainage ditch and are very homogeneous in terms of soil and vegetation characteristics, the data should not be considered as completely independent. The analyses were conducted using STATSOFT ITALIA (StatSoft Italia S.r.l., Padova, Italy).

Results

HYDROLOGY

Irrigation volumes, rainfall, evapotranspiration and water balance for the 3 years are reported in Table 1. As a consequence

Table 1. Irrigation volumes, rainfall, evapotranspiration and water balance for the 3 years under study

Years	Irrigation volume (m-cu ha^{-1} per year)	Rainfall (m-cu ha^{-1} per year)	Evapotranspiration (m-cu ha^{-1} per year)	Drainage back to river (m-cu ha^{-1} per year)
1st	51917	7562	7274	52205
2nd	48060	8888	6963	49985
3rd	48600	11450	9611	50439

of the irrigation (an average of 17 500 m^3 $year^{-1}$ in each plot, about three times rainfall), a perched aquifer was created on the calcic layer located at around 90–150 cm depth. Thus, the water level in the experimental site was always between 25 and 60 cm below the soil surface (Fig. 2). While the 0–15 cm soil layer was subjected to the normal seasonal cycle, with water content (expressed as a percentage of the dry weight) of 13–24% to 21–31% in summer and winter, respectively, the 40 to 55cm and 80 to 95cm layers were often saturated.

The conservative tracer (chloride) concentrations measured in the water collected from different piezometers and ditches changed little through the buffer (from 10 to 15 mg Cl L^{-1}). Moreover, the mean value of chloride concentrations of regional groundwater is about 55 mg L^{-1} (Regional Environmental Agency, unpublished data). So, the lack of changes in the chloride concentration within the buffer and the large amount of water flowing into the shallow groundwater system makes the input of deeper groundwater to the shallow flow system unlikely. Therefore, it was assumed that dilution from existing groundwater was minor (see also Sabater *et al*. 2003).

The RWT injection, although carried out after the time period being reported, provides further evidence that the irrigation water was moving rapidly through the shallow perched aquifer and not seeping out into the alluvial aquifer. Indeed, by comparing mass balance in the irrigation and drainage ditches, a loss of only 9·6% of RWT was registered, with an average travel time through the shallow groundwater from the irrigation to the drainage ditch of 24·3 h. These results indicate that the deep seepage out of the shallow aquifer into the underlying alluvial aquifer is negligible.

NITROGEN RETENTION IN THE WATER

Figure 3 shows $N\text{-}NO_3$ concentration in the input water from the river Zero, through the 15 m of the buffer zone to the drainage ditch, for the three monitored years. It is evident that the systems did not remove nitrate during the first 6 months of monitoring. During the following months a considerable reduction in nitrate concentration was observed even at 3–4 m from the irrigation ditch. This performance was more evident during the warm season (April/May–November), while in the winter period (from December to March), the system was less effective.

The amount of the different chemical forms of combined nitrogen confirm that during the first year, the reduction in $N\text{-}NO_3$ remained below 40%, while in the second and third years, it reached and stabilized to more than 85% (Fig. 4). The same trend was evident for total nitrogen, with about 23% removed in the first year and more than 60% removed during the second and third years. Note that the leaching of organic nitrogen in the course of the first year (−152%) decreased considerably in the second and third years (−87% and −11%, respectively).

IN SITU DENITRIFICATION

The highest DNT rates took place in the 40–55 cm soil layer (Fig. 5). Denitrification rates in the different soil layers differed

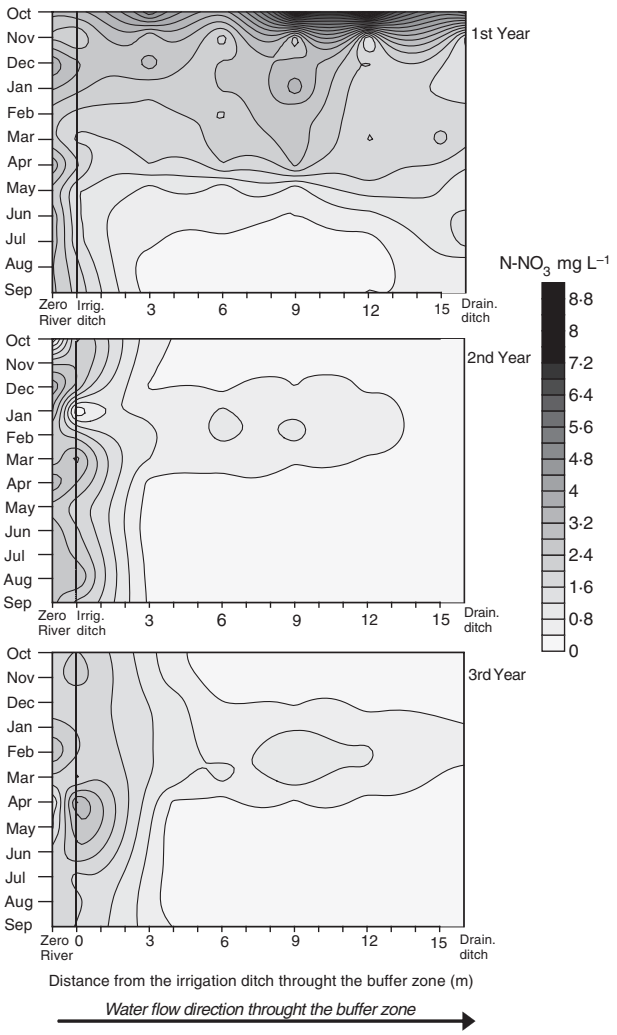
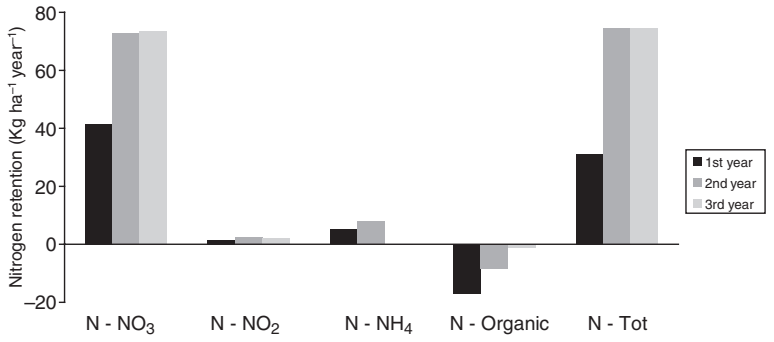


Fig. 3. $N\text{-}NO_3$ concentration in the input water from the river Zero, through the 15 m of the buffer zone to the drainage ditch, for the three monitored years. The grey scale on the right indicates the $N\text{-}NO_3$ concentration in mg L^{-1}. Data processing by the software 'Surfer® Version 8.01'. SSG-Surfer.com, a Division of Scientific Software Group, Sandy, Utah, USA.

significantly ($P < 0{\cdot}05$) during summer and to a lesser extent during autumn (Table 2, Fig. 5).

The *in situ* DNT activity was lowest during the second year but increased in the third year, with highest values recorded during the summer, demonstrating clear seasonal variations (Fig. 5 and Table 2). Overall, denitrification was higher in summer and autumn. This variability was significant for the 0 to 15cm and 40 to 55cm layers.

Denitrification activity in soil samples coming from the three different zones (see Fig. 1) was evaluated and compared. For the 40–55 cm soil layer, the highest rates of denitrification occur in zone 3 (located close to the irrigation ditch), causing a reduction in the amount of $N\text{-}NO_3$ moving through zone 1 (Fig. 6 and Table 2). The reduction in denitrification from zone 3 to zone 1 was especially pronounced during summer and autumn (Fig. 6), while in spring and winter, similar and very low denitrification activities were found through all the zones (data not shown).



	1st Year				2nd Year				3rd Year			
	Input	Output	Retention		Input	Output	Retention		Input	Output	Retention	
	kg ha^{-1} year^{-1}			%	kg ha^{-1} year^{-1}			%	kg ha^{-1} year^{-1}			%
N - NO_3	107·6	66·0	41·6	39%	85·0	12·3	72·7	86%	85·2	11·6	73·6	86%
N - NO_2	1·9	0·4	1·5	77%	2·6	0·3	2·3	88%	2·5	0·3	2·2	87%
N - NH_4	14·7	9·6	5·1	35%	18·7	10·8	7·9	42%	18·0	18·1	0·0	0%
N - Organic	11·2	28·2	–17·0	–152%	9·6	18·0	–8·4	–87%	12·0	13·3	–1·3	–11%
N - Tot	135·3	104·1	31·2	23%	115·9	41·4	74·5	64%	117·7	43·2	74·5	63%

Fig. 4. Combined nitrogen in terms of input, output and retention rates.

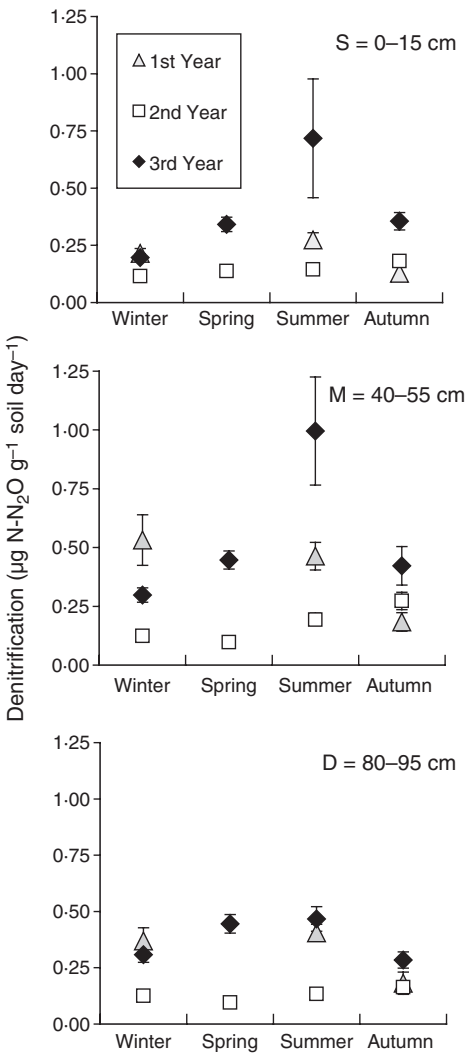


Fig. 5. Denitrification rates, as µg N-N_2O g^{-1} day^{-1}, for each year, season and layer at different depths. The values are the means of 18 measurements (nine in each plot) and bars represent standard errors.

Table 2. Three-way ANOVA exploring the differences in denitrification rate; the factors include seasons, soil depth (layers) and distance from irrigation ditch (zones)

Three-way ANOVA	d.f.	Mean square	*F*	*P*
Main effects (combined)				
Seasons**	3	1·225	8·846	0·000
Layers*	2	0·600	4·334	0·014
Zones	2	0·346	2·497	0·084
2-way interaction (combined)				
Seasons × layers	6	0·206	1·485	0·182
Seasons × zones*	6	0·322	2·324	0·032
Layer × zones	4	0·110	0·796	0·529
3-way interaction				
Seasons × layers × zones	12	0·067	0·486	0·923

Significant relationships and the level of significance are indicated by 0·05* and 0·001**.

DENITRIFICATION ENZYMATIC ACTIVITY AND THE EFFECTS OF CARBON AND NITROGEN

The 0–15 cm soil layer had the maximum potential for DEA (Fig. 7a). The addition of nitrate alone to the samples (Fig. 7b) resulted in increased denitrification in the 0–15 cm soil layer only. Adding only glucose to the soil resulted in a significant increase in denitrification rates in all the layers during the first year, but this effect was limited to the medium layer for the second and third years (Fig. 7c). There was no appreciable increase in denitrification activity in soil incubated without C and N addition but under anaerobiosis (Fig. 7d). In summary, (i) the limiting factor for the 0–15 cm soil layer, appears to be the availability of nitrate (Fig. 7b); (ii) the limiting factor for the 40- to 55-cm layer is the availability of organic carbon (Fig. 7c); (iii) microbial activity typically decreases toward the deep layer (80–95 cm) with no clear limiting factors (Fig. 7a–c).

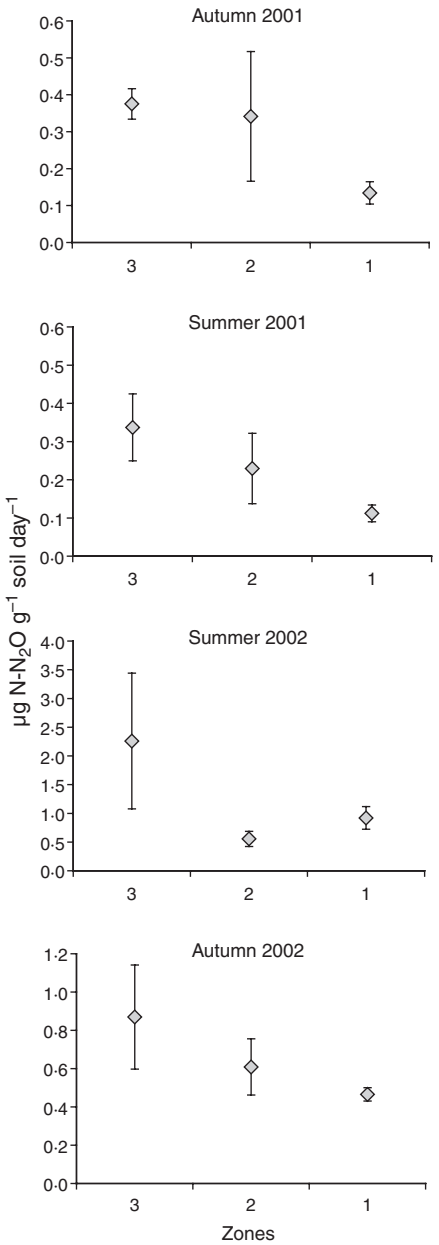


Fig. 6. Comparison of the denitrification rates, as μg $N\text{-}N_2O$ g^{-1} day^{-1}, of the three zones during autumn and summer of the second and third years. The vertical bars represent standard errors.

These effects become even more evident during the warmer seasons, although differences were smaller in winter, when temperature was an important limiting factor.

Discussion

The aim of this study was to explore the possibility of reducing the level of nitrogen in rivers by forcing water to circulate through afforested buffers. Nitrogen reduction can be achieved by creating semi-natural floodplains where water flows could be efficiently managed to support high nitrogen removal by microbial denitrification. An overall and detailed appraisal of the nitrogen retention by the system is given, even if the relative contribution of the different processes involved (i.e. plant/microbial uptake and denitrification) was not determined individually.

INFLUENCE OF SUBSURFACE FLOW REGULATION ON DENITRIFICATION ACTIVITY

Subsurface flow through both soil and deeper sediments of a riparian zone is known to be of crucial importance to denitrification and other nitrogen cycle processes (Mitsch, Dorge & Weimhoff 1977; LaBough 1986; Chescheir *et al.* 1988; Correll & Weller 1989; Dosskey & Bertsch 1994; Pinay *et al.* 2000). Because denitrification potential increases significantly towards the soil surface, water-table elevation can control the degree to which nitrate reduction by denitrification is optimized. Burt *et al.* (2002), reporting results from a pan-European experiment (NICOLAS), showed that denitrification process will be more effective within a riparian zone where topographic and soil conditions are conducive to a high water-table for as long as possible during the year. These conditions usually occur when permeable soil overlays impermeable bedrock and the land surface slope is low (5–10°) (Pinay & Burt 2001). Our results demonstrate that, even in the fine textured soil of the present experimental site, the anoxic conditions required for denitrification can be obtained by creating semi-natural floodplains where water flows can be suitably managed, i.e. by maintaining a slope of 4%. Under these hydrologic conditions, the higher denitrification rates were reached in the soil layer saturated by the perched aquifer. On the other hand, the lower values recorded in all soil layers in winter and spring indicate that the experimental design was unable to overcome other key limiting factors for denitrification such as low winter temperatures and plant competition in spring, even though a constant water flow was maintained.

THE CONVERSION OF THE SITE IN AN EFFICIENT BUFFER ZONE

Our results indicate that a buffer zone set-up for nitrate removal from river water starts to be effective during the second year (see Fig. 3). The area was rapidly converted from agricultural land to a tree-covered buffer even though there was no vegetation before the appropriate tree species were planted. At the start of the experiment, a considerable amount of residual combined nitrogen and organic carbon would have been present in soil (Table 3), derived from previous agricultural activities. During the first year, they would have been reduced by leaching, microbial activity and plant uptake. This may account for the limited denitrification activity detected in the study during the second year as compared with the first year. This trend may also be due to the higher nitrogen uptake by the plants (a mean of 104 g m^2 herbaceous vegetation biomass for the first year as compared with 298 g m^2 for the second), which started to grow quickly, thus reducing the amount of inorganic nitrogen available to the denitrifying bacteria and to the still limited organic carbon released by the young vegetation. Plant

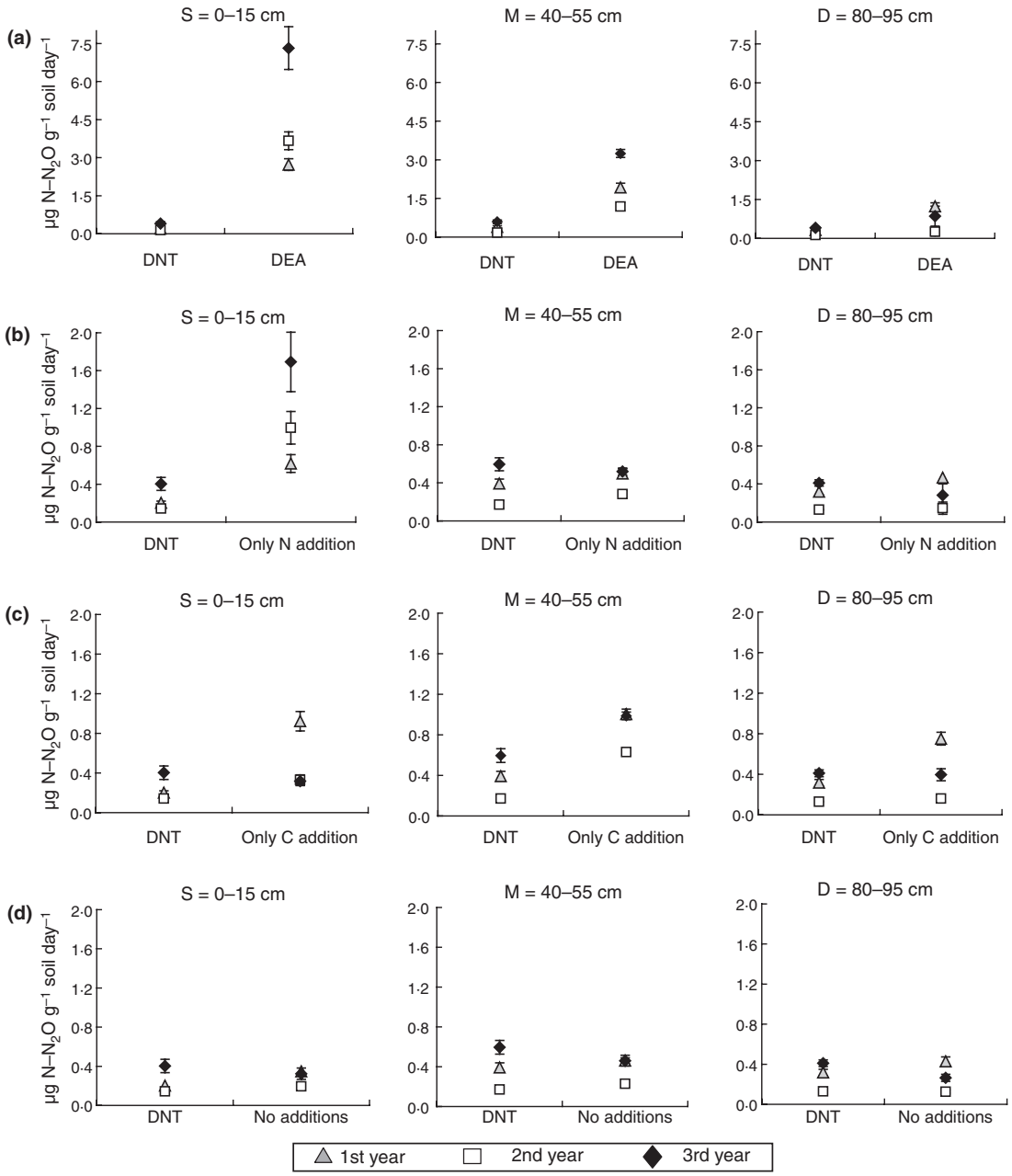


Fig. 7. Comparison of annual average in the three different layers between *in situ* denitrification rates (DNT) and potential denitrification obtained after incubation under anaerobic conditions with the addition of (a) both nitrate and organic carbon (denitrification enzyme activity), (b) only nitrate, (c) only organic carbon and (d) no additions. The vertical bars represent standard errors.

colonization and growth stabilized in the third year; hence, denitrification activity could take place effectively.

Our conclusions are further supported by the data reported in Fig. 4 where the reduction in $N\text{-}NO_3$ and total nitrogen during the first year was low, but reached and stabilized to significantly higher values in the second and third years, similar to values measured in other monitored European systems (see Pinay *et al.* 2007).

DENITRIFICATION POTENTIAL

As expected, although DNT was highest in the 40–55 cm soil layer, denitrification potential (measured as DEA) was highest in the top soil layer. Lower DEA in the deeper soil layer (80–95 cm) is generally attributed to the lower microbial populations living deeper in the soil. This is in agreement with a number of studies in natural forested riparian zones where DEA values are reported to generally decrease with soil depth (Hunt, Matheny & Stone 2004; Hunt, Matheny & Ro 2007). For example, Ambus & Lowrance (1991) reported that denitrification potential was mainly concentrated in the top 2 cm of soil in two riparian forest soils.

While nitrate represents the main limiting factor in the upper soil layer, organic carbon availability strongly affects denitrification activity at 40–55 cm depth. In deeper soil layers, even the addition of both nitrate and organic carbon is not always

Table 3. Soil nitrogen (mg N kg^{-1} soil) and organic carbon (%) content in the three layers of the buffer zone, for the three monitored years (± standard error)

Years	Layer	Inorganic N mg N kg^{-1} soil	Organic C %
1st	S (0–15 cm)	4·97 ± 0·56	1·03 ± 0·05
	M (40–55 cm)	5·71 ± 0·68	0·78 ± 0·05
	D (80–95 cm)	4·08 ± 0·45	0·71 ± 0·06
2nd	S (0–15 cm)	1·91 ± 0·10	0·87 ± 0·03
	M (40–55 cm)	2·71 ± 0·13	0·55 ± 0·02
	D (80–95 cm)	1·86 ± 0·14	0·31 ± 0·02
3rd	S (0–15 cm)	1·97 ± 0·12	0·82 ± 0·02
	M (40–55 cm)	2·40 ± 0·10	0·50 ± 0·02
	D (80–95 cm)	1·86 ± 0·10	0·31 ± 0·02

able to promote high denitrification potential. Indeed, the strong differences recorded between the seasons and the low DEA values in the deepest soil layer underline the importance of other key limiting factors for denitrification, such as the temperature and the distribution of the microbial population.

CRITICAL FACTORS AND OPTIMIZATION OF THE BUFFER CAPACITY

Our results confirm that a suitable irrigation system and an appropriate soil arrangement are crucial for optimizing the nitrogen removal potential of an afforested buffer site. Particular attention must be paid to maintenance of the shallow perched water-table as close as possible to surface soil layers.

Once the flow of nitrate enriched water was established through the shallow perched water-table, the key factor affecting the level of denitrification was carbon availability. In our experiment, the C supply in future will be ensured by litterfall and root production of several plant species expressly planted in the buffer zone. The selection of different fast-growing plant species may represent another critical factor in system design. McGill, Sutton-Grier & Wright (2010) indicated that plant communities clearly influence microbial activity and processes and that the diversity of plant communities can positively affect the stability of microbial processes, including DEA.

Finally, our results confirm that a maximum buffer strip width of 15 m can remove an excess of nitrate at concentrations typical of freshwater bodies (< 5 mg L^{-1} $N\text{-}NO_3$) and that narrower buffer strips (e.g. 5 m wide with only one row of trees) are likely to be adequate.

Acknowledgements

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Fig. S1. Ground level and mean annual water table depth from ground level measured in plot A (monthly measures in ditches and piezometers) during the three monitored years. Values for plot B were very similar. Bars represent standard error.

Fig. S2. Mean annual values of conservative tracer (chloride) concentrations measured monthly in the water collected from different piezometers and ditches.

Fig. S3. Denitrification rates for each season and layer at different depths. The values are the means of all the samples collected during 2001 and 2002. The vertical bars represent standard errors.

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