

Effects of Cultivation Practices on Denitrification and its Product Ratios

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Abstract

Cultivated soils are the greatest anthropogenic source of nitrous oxide (N_2O), a potent greenhouse gas and stratospheric pollutant. Denitrification is believed to be the major process responsible for N_2O production in soils. To determine how selected long-term cultivation practices affects basic denitrification properties (potential rates and product ratios) and deduce potential mitigation strategies, incubation studies with soil samples from two long-term (46 and 20 year) agricultural field experiments were carried out. Soil from a field trial in Hungary (Nyírlugos) comparing rates of mineral fertilization (ammonium nitrate) and liming (pulverized limestone) on a poor sandy, acidic soil, and soil from a field trial in Austria (Fuchsenbigl) studying the effects of tillage practices (minimum, reduced, and conventional) on a rich loamy, neutral soil were used. Soil samples (0-10 cm) were incubated (up to 60 hours) under oxic and anoxic conditions using acetylene (C_2H_2) blockage. Denitrification potentials were measured with ample amounts of NO_3^- and the gaseous composition of denitrification products ($\text{N}_2\text{O}/(\text{N}_2\text{O}+\text{N}_2)$) was determined 10 and 40 hours after the onset of denitrification. The soils' chemical properties (pH and organic carbon content) were compared to biological activities (O_2 consumption, CO_2 production, denitrification potential) as well as to denitrification product ratios (percent N_2O produced). Contrasts in biological activities could be attributed to management practices. A clear inverse relationship between the denitrification product ratio ($\text{N}_2\text{O}/(\text{N}_2\text{O}+\text{N}_2)$) and pH was found for the Hungarian soils ($R^2=0.86$, $p<0.0001$), whereas the denitrification product ratio was negatively correlated with the soil organic carbon content (in 0-10 cm) as affected by tillage practice in the Austrian soils ($R^2=0.57$, $p<0.05$), which showed only small variations in pH across treatments. While increasing levels of mineral fertilization appeared to have variable effects on pH and consequently on the denitrification product ratio, liming clearly increased pH and reduced the percentage of N_2O produced. No differences in potential denitrification activity were detected for these sandy soils, meaning that the specific product ratio of the denitrifier communities is an important factor for the amount of nitrous oxide potentially emitted from these soils. In contrast, minimum tillage in the Austrian field trial clearly increased denitrification potential in the topsoil, probably due to the gradual build-up of soil organic matter commonly observed under minimum tillage. Interestingly, denitrifier communities in soil under minimum tillage efficiently reduced nitrate all the way to N_2 , suggesting that potential N_2O emissions are in the same order of magnitude or lower than in the conventional or reduced tillage systems. It is concluded that long-term liming may mitigate denitrification derived N_2O emissions from acidic agricultural soils. However, comparison of denitrification properties across the experimental sites also suggests that management strategies improving soil organic matter content and soil fertility may be equally beneficial. Therefore, other amendments such as compost, charcoal or other industrial byproducts should be studied with respect to their combined effects on improving the soil's buffering capacity by soil organic matter buildup and to support efficient soil denitrifier communities. Denitrification is an indispensable soil function for returning the ever increasing amounts of anthropogenically fixed nitrogen as inert nitrogen gas (N_2) to the atmosphere. Strategies mitigating N_2O emitted during this process are needed, and should be developed in the context of sustainable agriculture.

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1. Introduction

1.1 Consequences of greenhouse gas emissions: focus on Nitrous Oxide

We are in the midst of an open-ended test of the compatibility of our civilization and the biosphere ((Smil 1996), p 194)

Terrestrial ecosystems strive towards steady state with respect to incoming and outgoing flows of matter and energy, constituting resiliency towards external perturbations. When natural or anthropogenic disturbances take place, biological and geochemical processes start to alleviate imbalances and resources or nutrients are either lost or scavenged and stored. Large disturbances can be detrimental and reaching a steady state requires time, sometimes occurring so slowly that an ecosystem can remain unproductive throughout a human lifetime. Cultivation constitutes continuous perturbation of soils, and replenishing lost nutrients through fertilization and liming does not necessarily alleviate all the imbalances imposed by cultivation. The long-term exhaustion of soils and the demands on all natural resources have become greater threats as the human population continues to grow. Losing natural resources is ultimately a threat to our survival. Therefore, we have to question what the carrying capacities of ecosystems are, and we need to find ways to avoid the overexploitation of our valuable resources. Sustainability (the ability of ecosystems to maintain ecological processes, functions, productivity, as well as biodiversity into the future) is the key objective to focus on when evaluating human activities.

The continuous increase in greenhouse gas concentrations in the atmosphere, due to human activities, is an indicator that our demands on ecosystems exceed the planet's capabilities to sustain its current equilibrium. One of the gasses increasing is nitrous oxide (N_2O), a gas which concentration in the atmosphere has risen from 270 ppb to 316 ppb within the time period between the industrial revolution (1800's) and the year 2000 (Gitay 2002). Nitrous oxide is relatively inert and harmless to life in the troposphere (it is also known under the name "laughing gas"), but once it dissipates to the stratosphere it contributes to a catalytic cycle of ozone destruction. In 1970, Paul Crutzen identified the process in which nitrous oxide reacts with excited oxygen to produce nitric oxide (NO) which then drives a catalytic cycle of ozone destruction (Smil 1996). Additionally, nitrous oxide is a potent greenhouse gas with a 300-fold higher global warming potential than carbon dioxide over the next 100 years (UEA 2008). In 1987, N_2O was estimated to be responsible for 6% of the anthropogenic greenhouse effect (Cicerone 1989). Modelling from the mid 1970s to the mid 1980s already suggested that if stratospheric nitrous oxide concentrations doubled, stratospheric ozone levels would be reduced by 10 to 16% (Smil 1996). Because N_2O has a direct effect on climate through absorbing radiative energy and destroying the ozone layer, anthropogenic effects on N_2O emissions are also effects on global warming and reducing greenhouse gas emissions is essential to global sustainability.

1.2 Anthropogenic activities altering the nitrogen cycle

Without anthropogenic disturbances, terrestrial ecosystems are relatively closed systems in which the amount of nutrients retained in the system greatly outweighs the amount of nutrients cycling in and out of the system. Most nutrients in soils including nitrogen are bound and relatively unavailable to plants and microbes for uptake. Most of the nitrogen in soil is in an organic form, bound to carbon complexes or incorporated into stabilized structures through humification. Nitrogen is not released and mineralized unless the soil organic matter is also broken down. In a similar fashion, mineral nitrogen can be bound to clay particles and even built into the clay structure when caught between sheets. Nitrogen species mobility in soils is distributed along a continuum of these kinds of associations, and relatively mobile nitrogen species are the ones whose movements among and within ecosystems are of importance for leaching and gaseous losses. These mobile nitrogen species (NH_4^+ , NO_3^- , NO_2^-), including dissolved organic nitrogen, support biomass, cause eutrophication when leached from land to water bodies, cause pollution of drinking water, or are volatilized in the form of NO , N_2O , N_2 or NH_3 . The reactive forms are valuable nutrients in N-limited ecosystems. As soon as N is released through mineralization, it is taken up by plants and microbes, and "... nitrogen accumulates in a system until nitrogen availability comes into approximate equilibrium with other resources..." (Chapin, Matson et al. 2002) such as phosphorous which often acts as the limiting nutrient. Similarly, the amount of biomass adapts to the amount of bio-available nutrients in an ecosystem until a steady state is reached, leading to the development of a closed nutrient cycle.

An increased flux of reactive nitrogen through the biosphere administered by man opens up the tight nitrogen cycle that has naturally evolved over time (Galloway, Aber et al. 2003). Formation and release of additional reactive nitrogen into the environment has global effects such as increasing atmospheric N_2O loading, as evidenced by the congruence of anthropogenic nitrogen fixation and atmospheric N_2O concentration (see below). Nitrous oxide is only one reactive species among the many forms of reactive nitrogen. Because they are readily converted to other reactive forms through the activities of microbes, the observed increase in atmospheric N_2O is perhaps a direct result of increased fertilizer use.

Disturbances including cultivation can cause sudden increases in labile nitrogen in an ecosystem. Slash and burn, planting of legumes, and the application of organic wastes are activities that have caused large local fluxes in pre-industrial times and remain in practice today. Overall fluxes can be temporarily altered by as much as an order of a magnitude by these activities and nitrogen saturation may occur (Bakken and Bleken 1998) if no precaution is taken to sequester the sudden release by the growing crop. As a result, N leaching and gaseous nitrogen losses occur (Chapin, Matson et al. 2002) which also affect the amount of labile nitrogen species in adjacent ecosystems.

Because reactive nitrogen is doubly mobile, meaning that it can be 1) transported by gravitational movement (through leaching of NO_3^- , NO_2^- and DON) or wind energy (through erosion when the nitrogen species is bound to soil particles or humus) and 2) transported through atmospheric pathways in the form of gases (NH_3 , NO , N_2O), it has the potential of being re-deposited close by or far away from its sources. Of all the pathways for movement of labile nitrogen, atmospheric pathways are the fastest, and reactive nitrogen species liberated from one ecosystem can affect ecosystems that are not adjacent

as well. Ultimately, increases in nitrous oxide emissions (or emissions of other reactive forms of nitrogen such as ammonia or nitrate) from non-agricultural lands are indirect anthropogenic sources of additional reactive nitrogen. This is because even pristine ecosystems receive elevated nitrogen amounts through deposition from other areas that are more directly affected by anthropogenic practices and from combustion of fossil fuels.

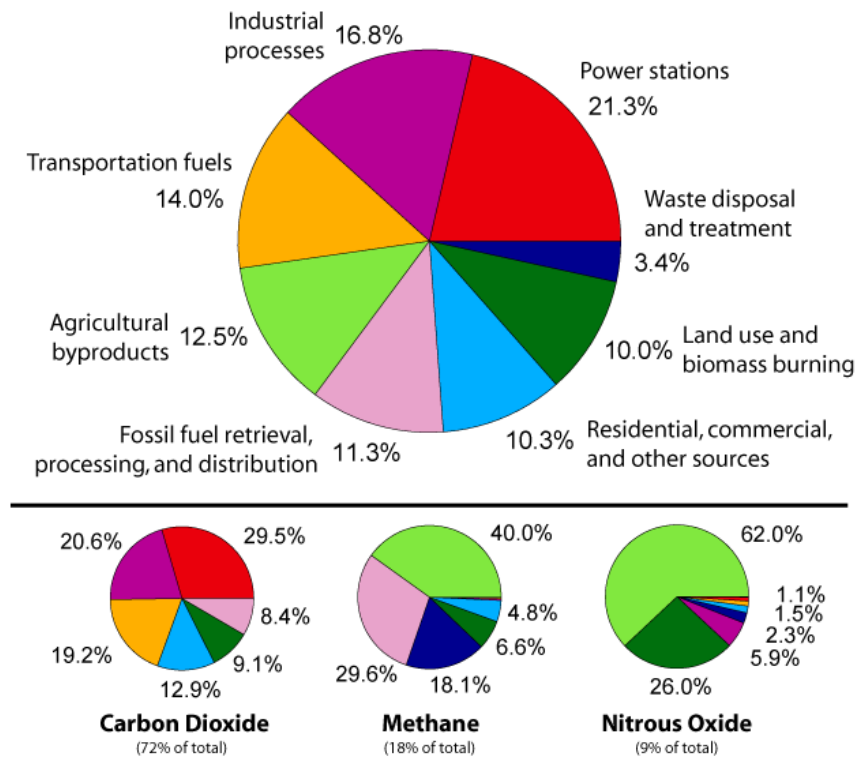
Since the industrial revolution, anthropogenic flows of nitrogen on a global scale have reached a magnitude comparable to natural biospheric fluxes, and humankind has become a driver of biogeochemical change. Humans have more than doubled the annual transfer of nitrogen into biologically available forms (Chapin, Matson et al. 2002) by fixing reactive nitrogen from the vast atmospheric pool via the Haber-Bosch process developed in 1913 and through mining deposited geological pools of fuel. This means that the amount of reactive nitrogen that we introduce in form of synthetic fertilizers and combustion gasses is comparable to the total amount of nitrogen fixed by all bacteria in natural terrestrial ecosystems (Smil 1996).

1.3 Anthropogenic sources of nitrous oxide

To reduce our effects on global warming, stabilization of the concentration of N_2O in the atmosphere needs to take place, but this can only be achieved through reducing its sources. This is because the average atmospheric lifetime of N_2O is 120 years and it is a greenhouse gas that is eliminated from the biosphere only through photochemical decomposition. No successful strategies have been found for minimizing emissions of N_2O from soil, and emissions have increased by nearly 17% from 1990 to 2005 (Smith 2007).

Anthropogenic additions of reactive nitrogen to the biosphere are occurring through three major pathways: the combustion of fossil fuels (just over 20 Mt N per year in the early 1990's), through the addition of fertilizers (80 Mt N per year), and through a widespread cultivation of nitrogen-fixing leguminous crops (30 Mt N per year) (Smil 1996). Therefore, synthetic fertilizers are responsible for the largest share of reactive nitrogen introduced into the biosphere. The figure below shows estimated sources of greenhouse gasses with N_2O shown separately on the bottom right.

Annual Greenhouse Gas Emissions by Sector



Please note that nitrous oxide represents 9% of the total greenhouse gases emitted of which 62.0% is characterized as "agricultural byproducts"

(Rhode 2006)

Figure 1: Sources of anthropogenic greenhouse gas emissions to the biosphere

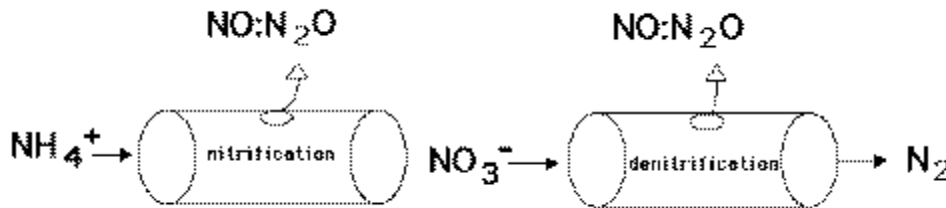
Agriculture is the most significant contributor of greenhouse gases to the biosphere, and the dominant source of nitrous oxide. Among the N_2O emissions from agriculture, direct soil emissions account for the greatest share (IPCC, 2001). Therefore attempts to reduce N_2O emissions should focus on refining agricultural soil management practices.

The extent of anthropogenic effects on the amount of N_2O emitted from soils is unclear because it happens naturally and microbial activity ultimately controls the emissions of this gas. Smaller sources of N_2O from combustion of fossil fuels and the production of adipic acid and nitric acid (HNO_3) are clearly anthropogenic in comparison. As mentioned previously, the amount of greenhouse gases emitted from soils is directly influenced by agricultural practices such as fertilization, cultivation intensity, cropping variety, liming, and tillage. According to the US environmental protection agency, agricultural soil management is responsible for 250 TgCO₂ equivalents of N_2O emissions in the United States alone (2006). Humans directly affect 40 to 50% of the Earth's land surface through agricultural production (lands consisting of cropland, managed grassland and permanent croplands such as used for agroforestry and bio-energy) (Smith 2007). Most difficult to estimate is the indirect effect of deposition from the overall increased amount of reactive nitrogen species in the atmosphere on N_2O emissions from

soils which are not obviously altered by agricultural management. Again, even though the emitted gasses themselves are produced by microbes, the increased emissions of N_2O from natural soils are anthropogenic when viewing the nitrogen cycle on a broader scale. This thesis focuses on the effects of selected direct management practices on N_2O emissions, but the even larger implications of increasing the reactive nitrogen pool is not to be forgotten.

1.4 Microbial production of nitrous oxide

Nitrous oxide emissions from soils are governed by processes related to microbial nitrogen transformations, such as microbial N-fixation, nitrification and denitrification. Denitrification provides the major output of nitrogen to the atmosphere (Chapin, Matson et al. 2002). The two microbial processes responsible for the biogenic production of N_2O in soils are nitrification and denitrification. Nitrification converts ammonium to nitrate, a mobile and bio-available form for plant uptake, and denitrification uses nitrate as an electron acceptor in the absence of oxygen for respiration and produces NO , N_2O , and N_2 . Nitrous oxide and nitric oxide are co-produced during nitrification and are true intermediates in denitrification. A model named “hole in the pipe” was developed by Firestone and Davidson for conceptualizing both processes (Fig. 2).



Adapted from Firestone and Davidson 1989 as cited by (Keller 1995)

Figure 2: “Hole in the pipe” conceptual model

Together, these two processes are responsible for the elimination of excess reactive nitrogen from soils as inert nitrogen. The model suggests that the more nitrogen flows through the pipe(s), the more material is squeezed through the hole. “The production of N_2O in soil is reported to be mainly controlled by the availability of NH_4^+ (substrate for nitrification) and NO_3^- (substrate for denitrification)” (Zaman, Nguyen et al. 2007); (Firestone, Firestone et al. 1980). In other words, the more labile nitrogen is present in a soil (added directly as fertilizer or deposited indirectly through cycling), the more nitrous oxide is emitted from the soil through microbial activity (through nitrification and denitrification, with bacteria being the most significant contributors to microbial activity in agricultural soils).

The amount of N_2O “leaking from the pipe” is however not always in constant proportion to the amount of NH_4^+ oxidized during nitrification or the amount of NO_3^- reduced during denitrification (i.e. the size of the “hole” can vary). More specifically, in addition to the concentration of substrates, other factors such

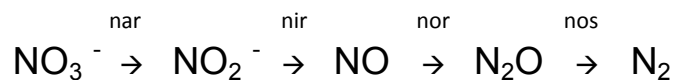
as pH, available organic carbon, availability of oxygen, and concentrations of intermediates affect the relative amount of N_2O produced. A meta-study on N_2O emissions from cultivated soils suggested that “...factors that significantly influence agricultural N_2O emissions were N application rate, crop type, fertilizer type, soil organic C content, soil pH and texture” (Stehfest and Bouwman 2006). Of the two mentioned processes, denitrification will be investigated in this thesis. The term “denitrification product ratio” is used to describe the ratio $N_2O/(N_2+N_2O)$, and the quantity represents the efficiency with which soil denitrifying communities are able to perform complete denitrification (i.e. reducing NO_3^- all the way to N_2). The following section is a brief review about effects of pH, fertilization, and tillage on the activity and product ratio of denitrifying bacteria, and its effects on nitrous oxide emissions from soils.

2. Background

2.1 Denitrification

Denitrification is an important biogeochemical process dissipating reactive nitrogen in terrestrial and aquatic ecosystems. The process was named by the French microbiologist Ulysse Gayon, who suggested closure of the nitrogen cycle by denitrification in 1886 (Smil 1996). Denitrification is carried out by facultative heterotrophic or chemolithotrophic bacteria, and some fungi and archaea under anaerobic conditions, during which oxidized forms of nitrogen are reduced (though oxygen is a more energetically favorable electron acceptor). Electron donors such as organic matter or reduced inorganic compounds are oxidized in return. The most common denitrifying heterotrophic genera are *Pseudomonas*, *Bacillus*, and *Alcaligenes* (Smil 1996). Some of the nitrogen-fixing rhizobia and ammonia-oxidizing bacteria are also capable of denitrification *i.s.s.*, though their roles are much less significant (Philippot, Hallin et al. 2007). Only under high pH has dissimilatory nitrate reduction to ammonia (DNRA) been reported to be significant (Stevens, Laughlin et al. 1998). Chemodenitrification and other types of denitrification are likely to play a minor role for nitrous oxide production in agricultural soils (Beauchamp 1997).

Denitrification seems to have evolutionary advantages for microorganisms in habitats being frequently exposed to shifting pO_2 conditions. Bacteria responsible for denitrification are the most abundant functional group within the nitrogen cycle representing up to 5% of the total microbial biomass (Henry, Bru et al. 2006; Philippot, Hallin et al. 2007). Even though denitrification is performed by a diverse group of bacteria, archaea, and even some fungi, the transformations of the nitrogen species always occur in the following sequence.



The three-letter abbreviations above the arrows represent the enzymes responsible for each of the reactions: nar: Nitrate reductase, nir: Nitrite reductase, nor: Nitric reductase, and nos: Nitrous oxide reductase (also N_2OR)

Figure 3: Series of steps in denitrification

Interestingly, both the activity and synthesis of denitrification enzymes (shown above the arrows in the reaction scheme) are repressed by oxygen (Payne 1973 as cited by (Smith and Tiedje 1979)), but the sensitivity to oxygen varies widely among different denitrifiers (Cavigelli and Robertson 2001). This shows that not only factors such as fertilization, pH, and tillage influence how much N_2O is emitted from a plot, but also the types of denitrifying bacteria present. Denitrifiers can also function through truncated pathways, only reducing N_2O to N_2 for example (Bazylinski et al 1986 as cited by (Chapuis-Lardy, Wrage et al. 2007)). In general however, "... the enzymes involved in the denitrification chain are differentially affected by various stress factors, with N_2O reductase being the most sensitive" (Philippot, Hallin et al. 2007). Therefore, denitrifying bacteria produce N_2O in varying amounts, even when they have the enzymatic potential to carry out the entire sequence shown above, resulting in different ratios

of denitrification products from the different denitrifying communities in response to the different environmental influences.

In turn, this opens for a theoretic possibility to selectively manipulate denitrification product ratios through targeted agricultural practice. It is therefore important to study how various agricultural management practices affect the functioning of denitrifiers, and especially to note any apparent differences in product stoichiometry in response to contrasting management regimes. Inherent differences in denitrifier product stoichiometries may be connected to differences in taxonomic composition (Cavigelli and Robertson 2001) which in turn may be affected by long-term soil conditions. However, the present study will not evaluate community composition directly; instead the relative production of N_2O as affected by the various treatments will be studied as a 'phenotypic trait' which will be linked to long-term site conditions at the experimental plots. Apparent contrasts can then be interpreted as indicators for possible differences in community composition, the molecular evidence of which has to be scrutinized in future studies.

The following is a brief overview of the important factors related to the three agricultural practices addressed in this thesis, fertilization, liming or pH, and tillage methods, and their effects on the denitrification process.

2.2 Known Effects of liming and pH on denitrification

Soil pH is most directly altered through liming, and pH has the biggest influence on denitrification along with soil aeration (Focht 1974). A review written about the influence of soil pH on denitrification leads to the conclusion that both the rate of denitrification and the ratio of gaseous products produced are affected (Simek and Cooper 2002). Most noticed is that under acidic conditions (such as pH 4.5), denitrification activity is usually reduced, thereby lowering the amount of nitrous oxide, nitric oxide, and nitrogen gas emissions (Simek and Cooper 2002). At the same time however, the ratio between nitrous oxide and nitrogen gas ($N_2O:N_2$) increases with increasing acidity (shown through liming of soil, (Zaman, Nguyen et al. 2007)), meaning that it is not certain whether overall N_2O emissions are higher or lower from an acidified soil compared to a neutral soil. In their review about the influence of soil pH on denitrification, Simek and Cooper (2002) show reports of contradictory results, indicating that the effects are not entirely clear. This may be because pH affects denitrification directly as well as indirectly. Soil pH may directly affect the amount of N_2O emitted through the regulation of N_2O reductase (N_2OR) activity. Alternatively, high proton concentration could suppress the expression of this enzyme. That the synthesis of this reductase enzyme is inhibited under acidic conditions is visible through a longer lag-phase of enzyme expression (Ellis et al. (1998) as cited by Simek and Cooper (2002)).

Soil pH is a master variable and also has indirect influences since it is linked to many other soil properties such as nutrient availability (in the form of trace metals, mineral nitrogen, organic carbon, etc.) and reduction potential. Soil pH also affects microbial processes and organic matter formation. It has been suggested that "the rate of carbon mineralization, rather than pH, controls the rate of denitrification in systems limited by storage of carbon, i.e. in most soils" (Koskinen and Keeny 1982 as

cited by Simek and Cooper (2002)). Soil pH influences the availability of carbon sources (Curtin, Campbell et al. 1998) and supports higher microbial biomass and diversity (Fierer and Jackson 2006). Since carbon acts as the electron-donor for denitrification processes, the availability of carbon may be a regulating factor for denitrification functioning in terms of the relative expression or activity of the denitrification enzymes, which in turn affects denitrification product ratios.

The mentioned review by (2002) also commented on the composition of denitrifying populations in response to long-term regular application of fertilizer and the resulting decrease in pH: "Evidently the denitrifier communities are resilient to changes in pH and there is no marked change of the optimal pH for potential denitrification". More recently however, it has been found through functional gene analysis that the soil pH has effects on the structure of the nitrate-reducing community while other factors such as CO₂ enrichment, plant species or fertilizer treatment have no apparent effect (Deiglmayr, Philippot et al. 2004). When looking at soil bacterial communities in general, it is evident that ecosystem type determines the diversity and richness of soil bacterial communities, and that pH can largely explain these differences (Fierer and Jackson 2006). Enwall et al. observed differences in denitrifier community composition in response to fertilization and hypothesized that the difference is at least partially attributed "...to an indirect effect of soil acidification, which has resulted in a selection of bacteria adapted to low pH" (2005). Soil acidity is a fundamental variable influencing all other soil qualities including soil formation.

2.3 Known Effects of mineral fertilization on denitrification

Applying fertilizer (inorganic or organic) increases the amount of reactive nitrogen in the soil, providing substrate for soil microbial processes in general. Nitrogen fertilization has also been shown to stimulate denitrification activity (Mulvaney, Khan et al. 1997), thereby increasing nitrous oxide emissions (Skiba, Smith et al. 1993). This trend is explained by the 'hole in the pipe model' from Firestone and Davidson, as mentioned previously.

Inputs of nitrogen may also change the pH of a soil. Ammonia fertilizers are known to cause acidification by stimulating nitrification, but different fertilizers affect pH differently, and they can be classified as alkaline forming or acid forming fertilizers. For example, Enwall and co-workers used long-term fertilization field trials (started in 1956) to study denitrification and pointed out that ammonium-sulfate treatment resulted in soil pH of 3.97 while fertilizing with calcium nitrate produced pH of 6.26 (2005). Based on a holistic view of nitrogen cycling in soil, K.R. Helyar suggested that "since the cycling of nitrogen in a closed ecosystem is neutral, the effects of nitrogen on soil acidity are associated with gains or losses of nitrogen from the system"(1976). More specifically, if the nitrogen species entering the system are the same as the nitrogen species exiting the system (in both type and quantity), acidification does not occur. Nitrogen in arable land is lost by plant uptake, nitrate leaching, and denitrification and added amounts of nitrogen through fertilizer (especially acid-forming fertilizers such as ammonium) increase the likelihood of acidification. Soil acidification is associated with slow dissolution of basic compounds which buffer the soil. Therefore, different soils having better or worse buffering capacities are affected by acidifying fertilizers in different ways.

On a similar line of thought, the application of alkaline forming fertilizers has been observed to affect the dissolution of organic matter (Norman et al 1987, Sen and Chak 1994 as cited by Philippot et al.(2007)) which also influences denitrification. The alkaline forming fertilizer seems to cause a temporary boost in microbial respiration which leads to the solubilization of more carbon and nitrogen. The resulting anoxic conditions in the soil may increase denitrification activity with more overall N₂O emissions compared to acid-forming fertilizers (Mulvaney et al 1997 as cited by Philippot et al. (2007)). The influence of labile organic carbon in the soil on denitrification is discussed in a following section about tillage effects.

The amount of available nitrogen species alone through fertilization has not been found to have an effect on the community composition of denitrifying bacteria. The type of mineral fertilizer on the other hand has been shown to cause detectable genetic differences among denitrifying bacteria (Wolsing and Prieme 2004).

2.4 Known Effects of tillage on denitrification

“More than nine-tenths of the nitrogen in soil is bound in organic matter, dead or living” ((Smil 1996) pg.65)

No-till (NT) is also known as zero tillage, and along with reduced tillage (RT), it is a form of conservation tillage. Compared to conventional tillage (CT), conservation tillage methods aim to reduce the amount of disturbance to the soil. Conservation tillage has recently become popular because of its ability to enhance carbon sequestration in soil, reduce erosion, and reduce soil compaction.

Effects of tillage method on denitrification could be explained by changes in carbon and nitrogen availability. Ploughing is known to change the chemical and physical composition of soil organic matter (SOM) through increasing the rate at which organic matter is decomposed (Szajdak et al. (2003) as cited by Doelman (2004)). Soil aggregates are thought to be disrupted through tillage, leading to the exposure of physically protected soil organic matter. “Nitrogen is directly bonded to the carbon skeleton of organic matter (C-N) and is generally released by breakdown of the carbon skeleton into amino acids and other forms of dissolved organic nitrogen” ((Chapin, Matson et al. 2002)). This may explain the observation that microbial respiration as well as denitrification increase after tillage, at least temporarily, based on a soil-core study at 0 to 30 cm depth (Calderon, Jackson et al. 2001). Calderon et al. hypothesized that “...low C:N ratio compounds may have been mineralized following tillage” (2001), indicating the preferential breakdown of fulvic acids over humic acids (humic acids are less water soluble and have a lower mobility in soil (Doelman 2004)). This would also alter the chemical composition of soils in the long term, as seen through the analysis of humic compounds from long-term tillage field trials (Tatzber, Stemmer et al. 2008). In another study where soil cores were taken up to 45 cm depths, less nitrate was detected in the soil profile under NT compared to RT and CT (Elmi, Madramootoo et al. 2003), but RT was unique in that more nitrate had been shown to leach to lower layers compared to the other tillage methods.

The redistribution of substrates through the disruption induced by tillage is also thought to lead to the formation of denitrification “hot-spots” (Calderon, Jackson et al. 2001). Even though tillage initially aerates the soil and reduces its density, anaerobicity has been shown to dominate following tillage (Calderon, Jackson et al. 2001), providing optimal conditions for denitrification to take place. On a deeper level, it has been shown that tillage rearranges pore space and the associated water film, which in turn affects “diffusion rates to and from active microbial populations” (Young and Ritz 2000). Liu et al. (2007) in another study on soil cores up to 10 cm depth showed that N₂O ratios were higher for CT compared to NT, indicating less efficient denitrification under the more intense tillage. This may lead back to the Calderon et al study where the mineralization of low C:N ratio compounds temporarily creates more substrate for denitrification following tillage while less carbon is available to act as the electron donor in the denitrification process. Therefore, tillage practices have been shown to have the potential to temporarily affect denitrification on two levels: by increasing the overall denitrification rate and by changing the product ratio. Contradictory results from another study where only the top 5 cm of soil were sampled showed that more N₂O was emitted from RT than CT (D'Haene, Sleutel et al. 2009).

Tillage is also known to have long-term effects on the physical properties of soil with possible indirect effects on denitrification. For example, NT is known to result in lower soil temperatures and higher soil water content compared to CT (Liu, Mosier et al. 2007). Bacteria are also better fitted for tilled soils compared to fungi, and because fungi form compounds that help form and stabilize aggregates, aggregate stability is influenced (Baere et al 1992 as cited by Young and Ritz(2000)). As a result of higher soil water retention as well as better soil structure supporting anoxic microsites in undisturbed soils, anoxic conditions are more prominent under NT management, enhancing denitrification activity as well as efficiency.

Purpose

The purpose of this study is to evaluate the effects of three common types of agricultural practices (tillage, fertilization, and liming) on potential nitrous oxide emissions from denitrifying bacteria. First, it is my hypothesis that acidity of soil results in a higher denitrification product ratio (N₂O/(N₂+N₂O)), and I will try to separate this pH effect from a fertilizer effect if there is one. Then using soil from another long-term field experiment, I study effects of tillage on the denitrification rate and product ratio. Once an effect has been identified, the significance of the results is evaluated within an agro-ecological context and agro-ecological suggestions for better management practices are provided.

3. Materials and Methods

3.1 Soils and sites

Existing long-term agricultural studies were selected for soil sampling, one in Hungary (Nyírlugos) and the other in Austria (Fuchsenbigl). For the purpose of studying management effects on denitrifying populations, it was important that the field experiments were long-term. One of the field trials had been devised for studying fertilization and liming while the other focused on tillage effects. Selected site and soil parameters are given in table 1. The soils differed in texture, pH, and carbon and nutrient content, while climatic conditions were relatively similar.

Table 1: Site and soil properties (^a from Tatzber et al. 2008, ^b from Kadar et al. 2007)

Climate			Soil chemical properties				Soil physical properties					
Elevation	MAT	MAP	pH	CEC	C	N	sand	silt	clay	BD	soil type	
m ab.sea	°C	m m	in KCl	me/100g	%	%	%	%	%	g cm ⁻³		
Nyirlugos fertilization and liming field trial in Hungary												
control	100 ^b	9,8 ^b	550-600 ^b	4,3	5-10 ^b	0,43	poor ^b	70-85 ^b	8-20 ^b	3-6 ^b	n.d.	brown forest soil, acid sand ^b
Fuchsenbigl tillage field trial in Austria												
MT	150 ^a	9,4 ^a	529 ^a	7,6	n.d.	2,18	0,23 ^a	37 ^a	41 ^a	22 ^a	1,38 ^a	fine sandy loamy Haplic Chernozem ^a
CT				7,7		1,57	0,19 ^a				1,44 ^a	
RT				7,7		1,49	0,20 ^a				1,35 ^a	

Nyirlugos fertilization and liming field trial

The field trial in Hungary was started in 1962 (46 years ago) and is named “Fertilization and liming effects on sandy acid soil”(Kadar 2007). The field consists of 128 parcels, a random block design of 32 treatments in 4 replicates each, and the size of each parcel is 50 m² (5m wide and 10m long).

Of the 32 treatments, 7 were sampled on all four replicates resulting in 28 samples. The 7 treatments were selected for the purpose of providing clear contrasts in fertilizer application rate (a control without fertilizer and three levels of NPK) and lime application to soil fertilized at a medium N rate (fertilizer without liming acts as the control). Treatment levels are given in table 2:

Table 2: Fertilizer and lime application rates

levels	Applied nutrients (kg*ha ⁻¹ *yr ⁻¹)			
	N	P ₂ O ₅	K ₂ O	CaCO ₃
0	0	0	0	0
1	50	60	60	250
2	100	120	120	500
3	150	180	180	1000

Table 3: Treatment names based on fertilizer and lime application rates

	Fertilization	Liming
Control	control	N₂P₂K₂*
Level 1	N₁P₁K₁	N₂P₂K₂Ca₁
Level 2	N₂P₂K₂*	N₂P₂K₂Ca₂
Level 3	N₃P₃K₃	N₂P₂K₂Ca₄

The levels 1, 2, and 3 appear as subscripts after the compound N, P, K, or Ca to indicate these respective application concentrations. *Please notice that N₂P₂K₂ is repeated because it acts as an intermediate level in the fertilization series and as the control in the liming series, yielding a total of seven treatments for both fertilization and liming increments.

The nitrogen mineral fertilizer used is ammonium nitrate, phosphate fertilizer is in the form of superphosphate, potassium fertilizer is in the form of potassium oxide, and lime is in the form of powdered limestone.

Fertilizer and lime application are performed by hand in autumn before tillage (with the exception that half of the nitrogen fertilizer is applied in the spring). Herbicides and other plant protection methods are applied only when needed. Crop rotation history since 1963 included potato, rye, wheat, lupin, sunflower, grass, barley, and tobacco, and since 1991 the rotation has been triticale monoculture.

The soil type is a *sandy acid brown forest soil* (tab. 1) with thin interstratified layers of colloid and sesquioxide accumulation holding 10 to 18% of the total clay content. This sandy soil is poor in humus (0,5 – 0,8%) and major nutrients, and the pH without fertilization and liming is 4.3 (measured in KCl). The climate indicates extreme drought sensitivity with yearly 1900 to 2000. sunny hours.

Fuchsenbigl tillage field trial

The field trial in Austria was started in 1988 and is part of a project named “Impacts of different tillage experiments on molecular characteristics of humic acids”(Tatzber, Stemmer et al. 2008). Three tillage methods, minimum tillage (MT, with a cultivation depth of 5-8 cm), conventional tillage (CT with a ploughing depth of 25 to 30 cm), and reduced tillage (RT with a cultivation depth of ~ 15 cm), are used in three replicates each giving a total of nine parcels of 720 m² (12 m wide and 60 m long). For more details including a scheme of the tillage field experiment and crop rotation history until 2005, see Spiegel (2007). Sugar beet was the cultivated crop in the season before sampling. All of the nine parcels were sampled yielding three treatments with three true field replicates.

Fertilizer is applied early in the year to all parcels, 80 kg/ha nitrogen in the form of NAC, 55kg/ha phosphate in the form of triple phosphate, and 80 kg/ha potassium in the form of 60Kali (potassium oxide).

The three tillage treatments have been described in detail by Kandeler et al. (1999) in another study: Conventional ploughing is performed in autumn where crop residues are incorporated while the soil is turned over. During cultivation under reduced tillage, the soil is loosened and mixed but not turned over. In both of these treatments, the seed bed is prepared with a zig-zag draw harrow and a crumbling roller immediately before the seed is sown. Minimum tillage proceeds with a rotary driller without any preliminary treatment before seeding. During the minimum tillage treatment, the soil is loosened to a depth of 5-8 cm and whirled up into the air, falling upside down to cover the seed and incorporated crop residues.

3.2 Sampling method and storage

The top 10 cm of soil were sampled at each location using a soil core sampler with a diameter of about 3 cm. Each plot was probed 28 times, the contents of which were mixed in a bucket to yield a composite sample. The samples, consisting of 200 to 300 grams of soil were placed in PE plastic bags, shipped to Norway and stored at 4°C. The bags were not closed and stored together with moist paper towels to prevent drying. Austrian soil at the Fuchsenbigle site was sampled on November 27, 2008 and Hungarian soil at the Nyirlugos site was sampled on December 5, 2008. Laboratory incubation measurements were carried out in the period March 4th to May 1st of 2009.

3.3 Measurements

Soil pH

Soil pH was determined following a protocol for dried homogenized samples (Peeche 1965) with slight modifications. Approximately 5 g of soil (between 4 and 5 g dry weight) were weighed into screw-top conical vials and relative amounts (twice the g dry weight in ml), between 8 and 10 ml of 0.01 M CaCl₂ were added. The samples were shaken and left overnight. The samples were shaken again one hour before and again directly before measurement by an ORION model SA720 electrode pH meter.

C-org content measurement

Percent soil organic carbon content was determined by an element analyser, burning soil samples after acidification at 1300 to 1400°C, and measuring CO₂ with the help of infra-red light (an IR-cell). The instrument Leco Carbon analyzer EC12 and method described by (1982) were used for this purpose.

Soil samples were dried at 65°C until constant weight was achieved (about one week), sieved with a 2 mm sieve and ground using a *Retsch* grinder to a homogeneous powder. Each sample was weighed onto a Whatman GF/F filter on a glass funnel (approximately 0.13 g for Hungarian soils, and 0.02 g for Austrian soils). The filters and soil samples were washed six times with 2M HCl by dripping the acid on the sample using a pasteur pipette, and again six times with water to remove traces of Cl from the soil and filters. Blank filters without soil were also washed. The samples were then dried for two days at 60°C and measured using the Leco Carbon analyzer (EC12, St. Joseph, Michigan, USA). The instrument was calibrated using a 1 g metal ring from LECO corporation containing 0,803% organic carbon, and the amount of sample weighed onto the filters was accounted for in the measured values of organic carbon content using the equation:

$$\% C_{org} = (1/\text{weight}) * (\text{reading of sample} - \text{reading of blank})$$

Soil gravimetric water content

Water content at field condition was calculated gravimetrically from the weight of the soil before and after drying at 105°C. Mass of the soil water divided by the bulk material mass is expressed by the equation

$$u = \frac{m_{wet} - m_{dry}}{m_{dry}}$$

The same method was applied to calculate water content after washing soil samples with a nitrate solution (see below). The moisture content was used for calculations of flask headspace and for correcting N₂O and CO₂ production rates for dissolution (Wilhelm, Battino et al. 1977).

3.4 Incubation measurements

Keeping basic parameters such as temperature, soil moisture, pO_2 and NO_3^- and carbon source equal, absolute and relative N_2O production rates can be considered to reflect the intrinsic performance of the inducible denitrifying populations in a soil sample. This concept is commonly applied when determining denitrification enzyme activity, using anoxic incubation of agitated soil slurries with and without acetylene (C_2H_2) which blocks the N_2O reducing enzyme (Smith, Firestone et al. 1978). Using soil slurry eliminates soil matrix effects and diffusion constraints, thus targeting the microbial populations independently of their habitats.

The soil incubation method used in the present study uses coarsely homogenized soil samples and will be referred to as 'solid phase incubation'. Even though the overall aggregate structure is not maintained, this approach preserves basic matrix effects (pH, microbial – mineral associations, etc.), thus allowing to study denitrifier community performance in a site-specific context. To exclude substrate limitation with respect to electron-acceptors, soil samples were saturated with a NO_3^- solution and drained to equal suction. This allows denitrifying populations to function at their maximum capacity, bio-available carbon being their only limitation. Altering the nitrate content does not affect the interpretation of the results regarding long-term effects on denitrifier populations since denitrifier community structure and abundance is primarily controlled by factors other than NO_3^- supply (Wallenstein, Myrold et al. 2006).

Short-term versus long-term measured denitrification rates (phase 1 and phase 2, see Smith and Tiedje (1979)) each provide different information about denitrifying bacteria. The first can be referred to as denitrification enzyme activity (DEA) while the second is called denitrification potential (DP). The length of incubation in the present study was 50 to 60 hours which is beyond DEA measurements, thus estimating DP rather than DEA in solid phase soil. DP was estimated from the linear portion of the N_2+N_2O accumulation curve (about 20 hours after the onset of oxygen depletion) in the presence of acetylene.

Two types of incubation experiments were performed. First, the soil samples were incubated in a He atmosphere with approximately 5 percent oxygen to determine microbial respiration (consumption of oxygen and production of carbon dioxide) under oxic conditions. Thereafter, the incubation bottles were He-washed and incubated in the absence of oxygen for measuring denitrification.

Pre-treatment of soil: Nitrate washing

In order to eliminate differences in N-oxide availability, all soil samples were washed with a 2 mM KNO_3 solution. 30 g of soil were placed on GF/C Whatman filters in a Buckner funnel, and NO_3^- solution was added until soil samples were immersed. Thereafter, the samples were drained equally by applying vacuum. This process was repeated three times with the vacuum left on for a final drying of the soil to near field-capacity.

Pre-treatment of soil: Helium washing

After NO_3^- washing and drying, approximately 15 g of the samples were transferred to 120 ml serum bottles and crimp-sealed with butyl septa. Prior to incubation, the bottle atmosphere was He-washed to remove all gases. This was achieved by repeated cycles of evacuation and He-filling by means of an automated manifold (Molstad, Dorsch et al. 2007). The overpressure from the last He-filling was released once the flasks had equilibrated to 15°C in the water bath using a syringe without plunger filled with some ml of water.

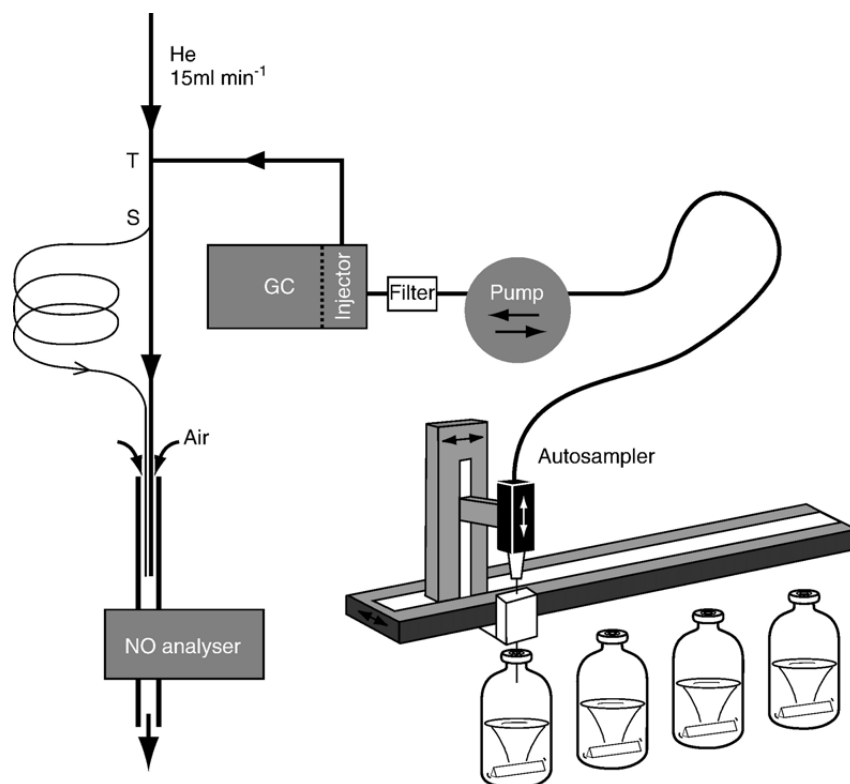
Pre-treatment of soil: Addition of O₂ and acetylene

Despite the reported ability of the incubation system to quantify N_2 production directly, acetylene blockage was used to measure total denitrification (N_2O and N_2). A preliminary study performed on soil slurries and solid phase soil from some of the samples revealed that measured N_2 production did not correlate with N_2 production calculated from the acetylene blockage technique, suggesting that atmospheric N_2 could not completely be removed from the soils by He-washing. Soil samples were therefore incubated in duplicate flasks to which acetylene (10 ml of C_2H_2 gas at 1 atm) was added to one of the duplicates. Acetylene was generated freshly by adding DI water to grains of 80% calciumcarbide in evacuated flasks.

To assess respiration activity, O_2 equivalent to 5 vol% of bottle headspace was added during a first round of incubation. 5 vol% O_2 rather than atmospheric O_2 concentration (21 vol%) was chosen to facilitate the detection of O_2 uptake (particularly in the acid Hungarian soils with little microbial activity) while simultaneously ensuring oxic conditions during a short term incubation (around 20 hrs). Estimation of respiration by O_2 uptake was necessary because bicarbonate-equilibria in liming-treatments and neutral soils are difficult to calculate in solid phase, thus preventing respiration measurements by CO_2 accumulation.

Instrumentation: GC and Autosampler

To assess denitrifier performance in the soil samples, the automated incubation system described by Molstad et al. (2007) was used: Briefly, accumulation kinetics of O_2 , CO_2 , N_2 , and N_2O were observed semi-continuously in 21 parallel bottles using the robotic arm of an autosampler (Gilson Model 222; Gilson, leBel, France) coupled to a gas chromatograph.



(Molstad, Dorsch et al. 2007)

Note: the NO analyzer was not used during these experiments

Figure 4: Partial diagram of autosampler and gas chromatograph

Automatic sampling by rotary peristaltic pump (Gilson Minipuls 3) was programmed for up to 60 hours at 3 hour intervals by a Gilson Model 222 (Gilson, leBel, France). For headspace analysis, the auto-sampler pierces the bottle septum with a small diameter steel needle (0.4×40 mm (276×11/2), Braun, Braun-Melsungen Germany) (Fig. 4) coupled to a gas line. Piercing is randomized within a pre-defined radius (0.7 mm in this case) to avoid that the needle pierced the septa at the same spot which could cause leakage. After pumping up approximately 0.6% of headspace gas, the pump is returned automatically and returns an equal volume of helium to the flasks in order to maintain atmospheric pressure. The resulting dilution and dissolution of gases in soil water are accounted for when calculating production rates from the accumulation kinetics (Molstad, Dorsch et al. 2007). Sample dilution and leakage occurring throughout the incubation is evaluated from blank and standard bottles included in the experiment.

Denitrification product ratio

The product ratio of denitrification can be expressed as the ratio of N₂O net production to total denitrification (N₂O/(N₂+N₂O)) and is expressed as a percentage in this study. Through utilizing the acetylene inhibition method, this ratio is found by dividing the amount of N₂O accumulated *without* acetylene with the amount of N₂O accumulated *with* acetylene at various time steps. This means that the denitrification ratio can be calculated for different phases of the incubation, thus taking account for

its dynamic nature caused by delays (lags) in denitrification enzyme induction after the onset of anaerobiosis. In the present study, ratios obtained 10 hours and 40 hours after onset of anoxic conditions were found to be most significant with respect to treatment effects.

Estimation of maximized emission potential

Potential emission was calculated by multiplying the denitrification product ratio with the measured potential denitrification rate. These values are not comparable to field values and are rather estimations obtained under optimized laboratory conditions with fully anoxic conditions.

3.5 Data analysis

Data were analyzed using a spreadsheet developed by Lars Bakken and colleagues (ROBOTSORT). Using this spreadsheet, respiration measurements based on both CO₂ production and O₂ consumption were adjusted for pH and soil water content using a constant for solubility of gas in liquid (Wilhelm, Battino et al. 1977). Additionally, all measurements were adjusted using calculated response factors, leakage factors, dilution factors, and biological consumption of gasses during the incubations.

The response factors for the gas chromatograph were found empirically by sampling standard gasses with known concentrations (in ppm) during a test run. These constants were then used for converting peak areas to gas concentrations for the rest of the measurements. A correction for dilution was also calculated during the test run using standards, resulting in a constant dilution factor which could then be adjusted to changes in the headspace in the flasks when varying amounts of soil were added. Correction factors for leakage were calculated separately for each experiment by including a helium standard in every run. Differences between leakage rates of Oxygen and Nitrogen could be used to assess the nature of the leak because diffusion rates through the rubber septa used in the experiments differ for the two gasses. Based upon this, oxygen consumption rates from one of the experiments were excluded from the final results due to irregular leakage rates.

Statistical analysis

Multivariate regression analysis using the Statistical Analysis System (SAS/STAT TEST) was performed for the data sets.

4. Results

4.1 Site Effects and Activities

Soils from the two sites differ greatly in their physical and chemical properties. The soil from the Hungarian site (see fertilization and liming results in table 4) is extremely poor and acidic (pH_{KCl} 4 to 4.4 for plots without added lime) while the soil from the Austrian site (see tillage results) is neutral to slightly basic (pH_{KCl} 7.7) with more nutrients and better soil structure (while handling the soils it was noticed that while the Hungarian soils were light in color and easily fell apart, the dark Austrian soils had an aggregated structure with differences between tillage methods used, the most obvious being that minimum tillage soils had larger clumps than the reduced or conventional tillage soils). The soils differed markedly in organic carbon content, with 0.38 to 0.72 mg C_{org} *g⁻¹ soil for the sandy acidic Hungarian soils and between 1.3 and 2.5 mg C_{org} *g⁻¹ soil for the Austrian soils. Despite these differences however, all levels of oxygen consumption, carbon dioxide production and measured potential denitrification are comparable for both soils with the exception of values obtained for minimum tillage where values are higher.

Apart from the organic carbon content, large differences were seen in the denitrification product ratio of the two different soils. Sandy soils from Hungary produced N_2O at higher ratios (40% to 100%), whereas the loamy Austrian soils produced nitrous oxide at ratios between 0 and 40%. As a result, the estimated emission potentials also greatly differed between the two soils, with initial emission potential (after 10 hrs of anoxic incubation) being up to four times higher from the Hungarian soils than from the Austrian. This difference becomes even greater when considering product ratios observed after 40 hours of incubation.

Table 4: Soil Properties and activities. n=3, except n=2 where *. Fertilization and liming data was obtained from the Hungarian long-term field trial, whereas tillage data was obtained from the Austrian long-term field trial.

	Soil Properties				5 to 20 hrs		5 to 35 hrs		First 20 hrs incub.		At 10 hrs of incub.		At 40 hours of incubation		Max. Emission potential nmol N ₂ O*g ⁻¹ soil*h ⁻¹	
	pH in KCl		SOC mg C*g ⁻¹ soil		O ₂ consumption * nmol O ₂ *g ⁻¹ soil*h ⁻¹		CO ₂ production nmol CO ₂ *g ⁻¹ soil*h ⁻¹		potential Denit. nmol N ₂ O*g ⁻¹ soil*h ⁻¹		product ratio N ₂ O/(N ₂ +N ₂ O)*100		product ratio N ₂ O/(N ₂ +N ₂ O)*100			
		std		std		std		std		std		std		std		std
fertilization																
Control	4,4	0,5	0,43	0,08	115	42	105	22	8,40	1,97	73	16	77	17	6,32	1,39
N ₁ P ₁ K ₁	4,1	0,2	0,45	0,06	139	32	108	13	8,53	1,52	79	11	80	7	6,75	0,62
N ₂ P ₂ K ₂	4,0	0,3	0,53	0,10	95	19	96	15	7,72	2,01	110	18	106	12	8,02	1,50
N ₃ P ₃ K ₃	4,1	0,4	0,63	0,10	111	48	121	6	9,74	1,15	88	9	89	9	8,61	0,64
liming																
N ₂ P ₂ K ₂	4,0	0,3	0,53	0,10	95	19	96	15	7,72	2,01	110	18	106	12	8,02	1,50
N ₂ P ₂ K ₂ Ca ₁	4,8	0,6	0,56	0,08	127	23	133	27	9,40	0,94	83	6	82	5	7,65	0,36
N ₂ P ₂ K ₂ Ca ₂	5,3	0,9	0,57	0,15	134	32	150	56	7,65	1,73	83	24	64	22	4,66	0,93
N ₂ P ₂ K ₂ Ca ₄	6,2	1,0	0,54	0,08	175	26	169	32	7,63	1,69	71	2	50	10	3,88	1,40
tillage																
Min. Till.	7,64	0,05	2,18	0,28	323	59	166	49	16,96	1,08	16	15	2	2	0,27	0,34
Conv. Till.	7,73	0,05	1,57	0,19	150	16	71	32	7,98	3,35	25	9	17	14	1,09	0,81
Red. Till.	7,71	0,04	1,49	0,24	73	28	54	20	6,81	4,56	41	15	17	15	0,95	0,68

4.2 Liming Effects

Liming of mineral fertilized soil results in a clear increase in pH from 4 (no lime) to 6 (maximum liming rate at $1000 \text{ kg Ca}_2\text{CO}_3 \cdot \text{ha}^{-1} \cdot \text{yr}^{-1}$). Respiration was significantly increased through liming, but potential denitrification rates and soil organic carbon content were not affected. Liming significantly decreased the denitrification product ratio (Fig. 5). In comparison, plotting denitrification product ratios observed after 10 hours of incubation resulted in lower values for slope and coefficient of variation (data not shown: slope = -12.8 , $R^2 = 0.55$).

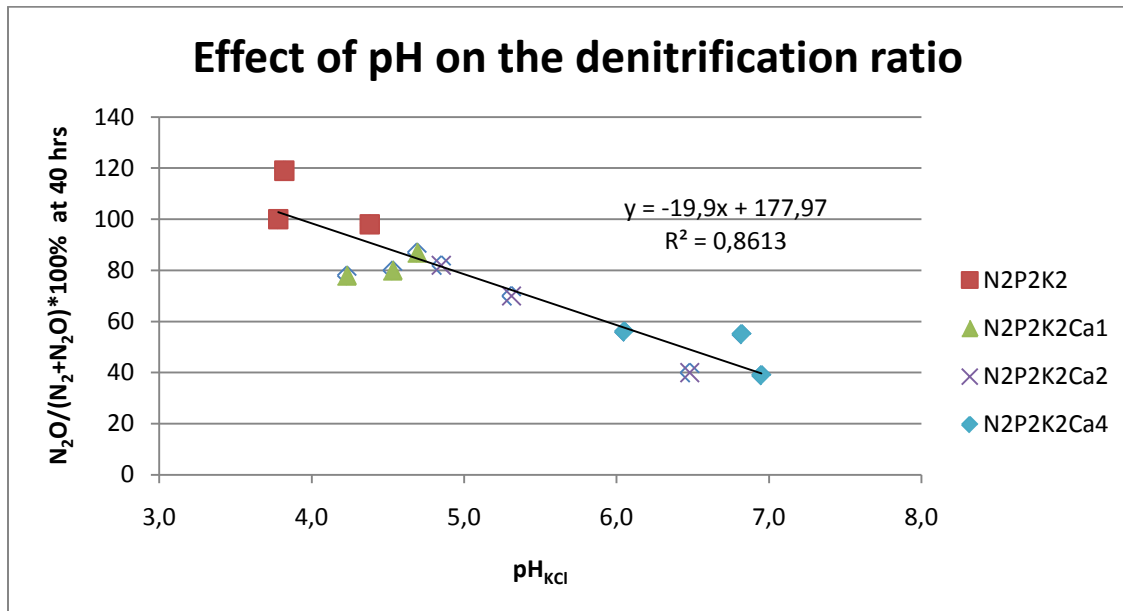


Figure 5: Liming effect on denitrification product ratio. Shown are denitrification product ratios obtained after 40 hours of anoxic incubation as a function of soil pH in individual samples from 3 liming levels and control. Note values for percent N₂O above 100 are an artifact of the calculation method, resulting from using the acetylene inhibition method.

An inverse correlation between respiration (CO₂ production) and percent nitrous oxide with an R² value of 0.51 was also observed (data not shown).

4.3 Fertilization Effects

Long-term fertilization did not result in consistent changes in pH in the acid Hungarian soil (tab. 4). pH variation was much smaller as compared with the liming experiment. Nevertheless, a weak trend between denitrification product ratio and soil pH could also be observed in the fertilization experiment (Fig. 6). The slope of this relation was not statistically different from the slope of the same relationship obtained for the liming experiment. There was no significant effect of fertilization level on denitrification potential (tab. 4). However, if multiplied with the product ratio, the estimated emission potentials

clearly increased with fertilization level (tab. 4). The soil organic carbon content increased with long-term mineral fertilizer addition while there was no effect on respiration (tab. 4).

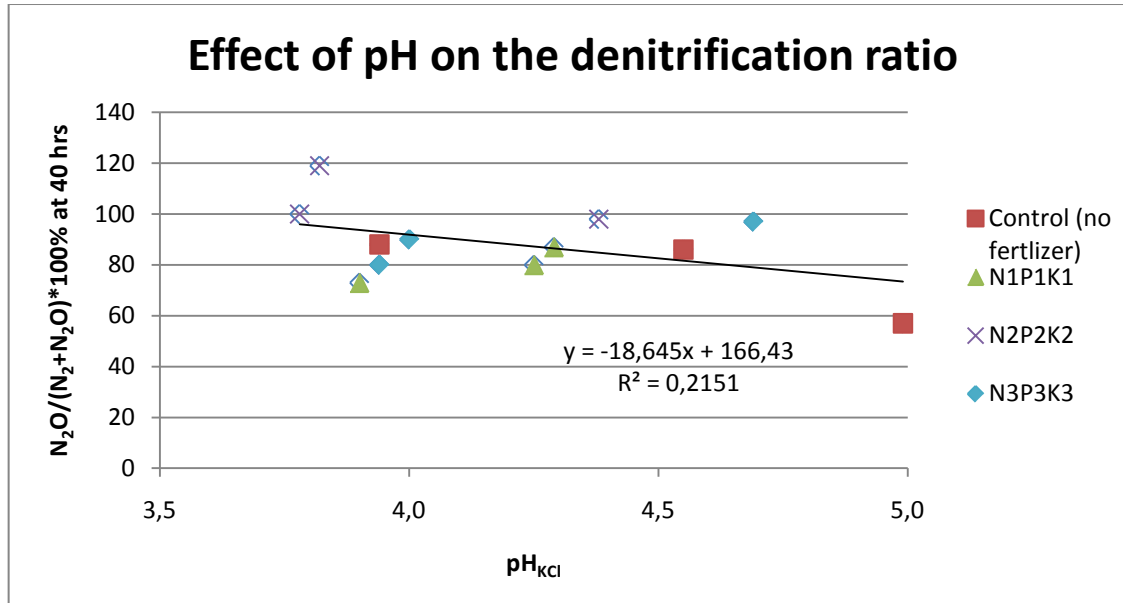


Figure 6: Fertilization effect on denitrification product ratios. Shown are denitrification product ratios obtained after 40 hours of anoxic incubation as a function of soil pH in individual samples from 3 fertilization levels and control.

4.4 Tillage Effects

The organic carbon content in the top 10 centimeters of soil sampled was higher in the minimum tillage as compared with conventional and reduced tillage which had comparable organic carbon contents (tab. 4). Respiration and potential denitrification were highest for minimum tillage (at least twice as high as compared to conventional and three times higher than reduced tillage), and the denitrification product ratio was lowest for minimum tillage. A positive correlation between percent organic carbon in the soil and measured potential denitrification was found (Fig. 7).

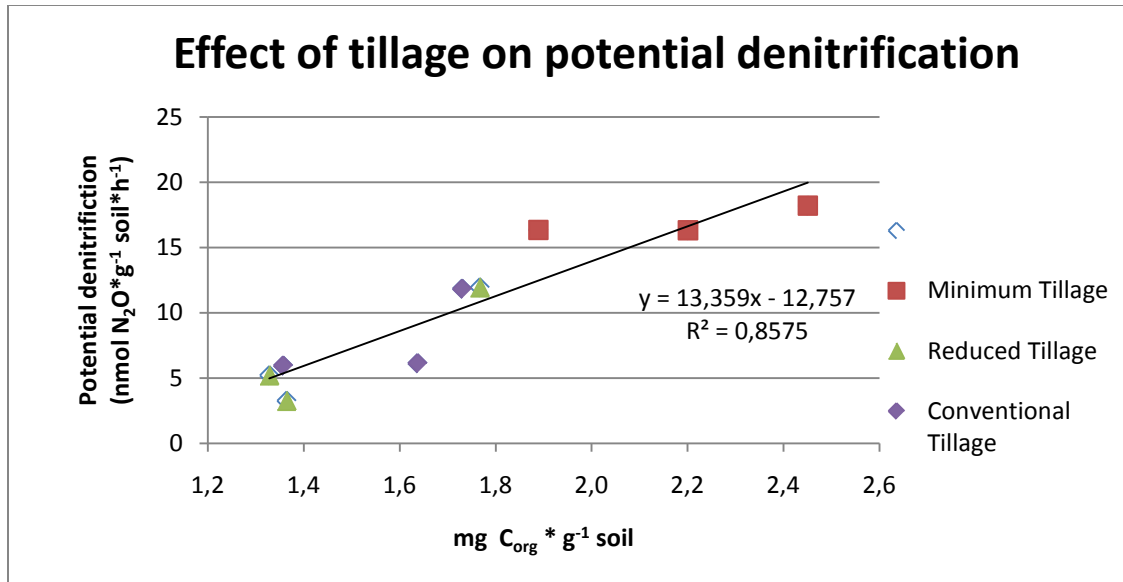


Figure 7: Tillage effect on potential denitrification. Shown are potential denitrification rates as a function of soil organic carbon content in individual samples from 3 tillage treatments.

Conversely, the denitrification product ratio was found to be inversely correlated to soil organic carbon content (Fig. 8). Plotting product ratio values obtained at 40 hours of incubation against soil organic carbon yielded a weaker correlation ($R^2=0.37$) and lower slope of -20 (not shown).

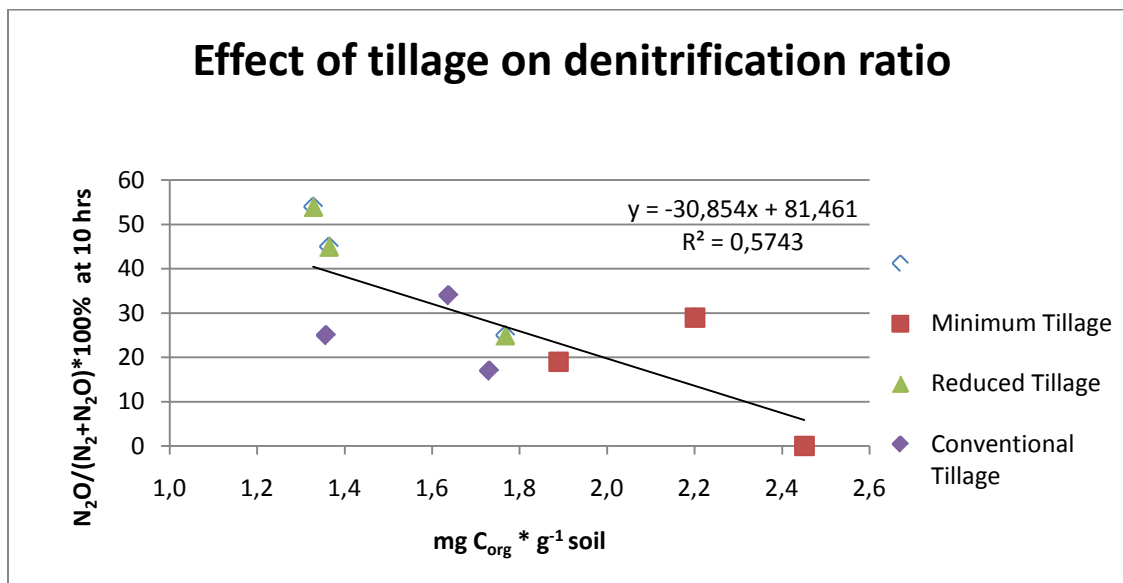


Figure 8: Tillage effect on denitrification product ratio. Shown are denitrification product ratios observed after 10 hours of incubation as a function of soil organic carbon content in individual samples from 3 tillage treatments.

As a result of the dependency of denitrification potential and product ratio, potential and product ratio become mutually dependent, showing a strong negative correlation: the higher the denitrification potential the lower its product ratio (Fig. 9). Again, a weaker relationship (slope =-1.7, R²=0.58) was found when plotting product ratios obtained at 40 hours against potential denitrification (not shown).

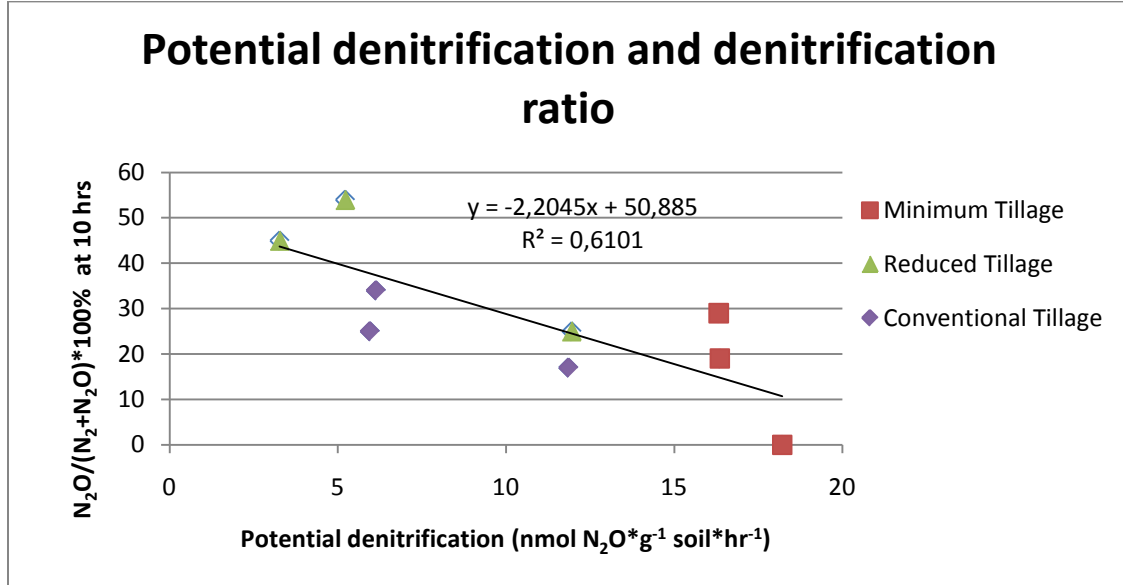


Figure 9: Dependency of denitrification product ratios on potential denitrification. Shown are denitrification product ratios observed after 10 hours of incubation as a function of denitrification potential in individual samples from 3 tillage treatments.

5. Discussion

5.1 Denitrification and pH

High variability of bulk soil pH was found within treatment groups in the Hungarian experiment (Tab. 4). The experimental setup of the Hungarian field trial may be responsible for this, the reason being that the plots are small with a high likelihood of carryover between the plots during tillage or through natural events such as erosion, leading to a range of pH values rather than well-confined treatment classes. Since the resulting variation results in a low statistical power and masks treatment effects, regression analyses based on individual soil samples was chosen rather than analysis of variance using mean values. This approach is valid to elucidate the principal effects of soil pH as affected by liming and fertilization rates in a sandy poor soil on denitrification product ratios.

Denitrification efficiency and the denitrification product ratio seem to be largely regulated by pH, a result that is in accordance with previous studies (for review see Simek and Cooper (2002)). When viewing the percent N_2O produced over a large pH range as represented by the Hungarian soils (Fig. 5), its inverse relation to pH is eminent. This may be interpreted as a direct pH effect on the denitrification process itself, because liming of these poor soils did not result in a buildup of SOC (tab. 4) that could explain the relationship by creating conditions supportive to a greater or more efficient denitrifier community. Long-term fertilization on the other hand seemed to not only affect soil pH but soil organic carbon content as well. Fertilization of the sandy soils resulted in higher crop yields and C returns to the soil while gradual acidification inhibited decomposition (Kadar 2007), likely explaining the higher soil organic carbon content at high fertilization rates. Here, confounding effects on the relationship between pH and denitrification product ratio can be expected (Fig. 6) which are difficult to disentangle. When performing a regression analysis of the linear relationship shown in Fig. 6, no significance (compared to the null hypothesis) could be stated, while the trend in Fig. 5 was significant. However, fertilization of the sandy-acidic Hungarian soil resulted in a much smaller variation of soil pH as compared to liming, also explaining why the relation between pH and denitrification product ratio was not significant for this experiment. It was tested whether the smaller range of pH values with their relatively high deviation could be pointing at the same trend as the trend between pH and product ratio in the liming plots. When testing the slopes of the two regressions, no significant difference between the trends from the liming and fertilization data was found. Therefore the question at hand whether a fertilization effect can be isolated from the liming effect remains unanswered and changes in the denitrification product ratio due to long-term fertilization are thought to be either solely due to pH or due to combined effects of pH and other factors such as soil organic matter turnover.

The better correlation between product ratios and pH determined at 40 hours compared to 10 hours of incubation in the liming experiments indicates that the pH effect on the product ratio depends on the duration of anoxic conditions. Obviously, soils limed to pH values above $pH=5.0$ ($N_2P_2K_2Ca_2$ and $N_2P_2K_2Ca_3$) were able to increase expression or functioning of N_2O reductase (N_2OR , the enzyme responsible for reduction of N_2O to N_2) between 10 and 40 hours of incubation as can be seen from comparing the 10 and 40 hour ratios in tab. 4. This suggests that liming affects the ability to express

N₂O foremost during longer anoxic spells. Interestingly, there was no difference between the 10 and 40 hour denitrification product ratios in the fertilization plots (tab. 4), confirming that the pH has to be raised above a certain threshold to allow for enhanced N₂O expression upon prolonged anaerobiosis. However, it has to be kept in mind that this finding is only valid for the poor sandy soil studied here, in which liming has not changed soil organic matter content. This raises the interesting question whether the time dependency (10 vs. 40 hr incubation) of the pH effect on the denitrification product ratio differs with other soil properties such as soil texture and soil organic carbon content (C_{org}).

Liming of poor sandy acidic soils as represented by this long-term field trial in Hungary seems to be a successful measure for reducing nitrous oxide emissions; calculated maximum emission potentials showed a significant decrease with increasing liming rate (tab. 4), suggesting a reduction potential of up to 50%. Considering liming resulted in an increase in grain yields compared to non-limed plots (up to 25% more grain obtained from limed fields between 1999 and 2006, see Kadar (2007)), a reduction potential per unit grain produced is even greater. Once liming is discontinued however, soil pH can quickly (in a matter of years) drop back to initial levels (Chan, Conyers et al. 2007) because liming itself does not build the buffering capacity of the soil. As seen from the results, liming of this soil also did not aid in increasing soil organic matter content. It is often observed that liming increases net primary production and organic matter inputs to the soil, but that soil respiration is also enhanced as observed in this study. The increased soil respiration would lead to the degradation of organic matter, preventing any buildup of soil organic matter from increased plant biomass and associated root exudates. Whether a buildup of organic matter and soil buffering capacity occurs is determined by the rates of both processes, and the rates are unique for different soils and cannot be generalized. Publications regarding the effects of liming on soil organic matter confirm this with large discrepancies in results (Chantigny 2003). While liming is a quick solution for treating soil acidity, the process of acidification can be avoided or reversed in the long term using other methods based on more inherent alkalizing effects as discussed later.

5.2 Tillage and soil organic carbon

While soils from the Hungarian long-term field trial varied greatly in pH, revealing a strong direct effect of pH on denitrification and its product ratio, soils from the Austrian long-term tillage field trial did not vary in pH. Overall, the Austrian soils had much lower N₂O product ratios than the Hungarian ones, suggesting that the denitrifier communities from the two sites differed fundamentally in their performance. Near neutral soils seem to support higher microbial activity although adaptation of microbial growth to pH has been demonstrated (Baath 1996). In the present study, microbial respiration rates did not differ grossly between the poor acidic Hungarian soil and the C-rich Austrian soil with the exception of the minimum tillage soil which exhibited roughly 3 times higher O₂ consumption rates as compared to the other soil treatments. Apart from microbial activity, near neutral conditions in the Austrian soils are likely to support a higher functional microbial diversity than what can be expected for the Hungarian soils (cf. (Fierer and Jackson 2006)). A link between functional genetic diversity of the denitrifier community and its functional performance has been suggested ((Cavigelli and Robertson

2001),(Wallenstein, Myrold et al. 2006)) and may be an explanation for the higher denitrification efficiency in the Austrian soils as compared with the Hungarian soils. In addition, higher C and N turnover rates at the more favorable pH in the Austrian soil may lead to a higher electron pressure which would favor a more complete reduction of electron acceptors such as N_2O (Granli 1994). Quantification of C and N turnover was beyond the scope of the present study, and the only parameter measured besides pH was SOC. Soil organic carbon content of the chernozemic Austrian soil was inherently three to four times higher than in the poor sandy acid Hungarian soil and was clearly affected by tillage practice (tab. 4). The distribution of SOM affects a range of other soil characteristics such as soil structure, aeration, CEC and others, all of which directly or indirectly affect denitrification. Measuring N_2O production rates as a function of tillage therefore compares overall effects, but the inherent difference in soil stratification between the tillage treatments adds a dimension which makes it difficult to compare the treatments directly. Assuming that most denitrification occurs in the uppermost soil layers would justify the simplified approach taken in this study in which the top ten centimeters of soil were sampled and used in incubation studies.

Denitrification performance appeared to be linked to SOM content as affected by tillage in the topsoil. Both potential denitrification and the denitrification product ratio correlated well with soil organic carbon content (Figures 7 and 8). Because increased soil organic carbon content reduced the ratio of N_2O produced and simultaneously increased denitrification activity, the overall effect of soil organic carbon on total N_2O emissions is not straight forward (pH in comparison had no effect on potential denitrification and the ratio was the only determinant of total emissions). To get an idea of overall effects, maximum emission potentials were calculated. The resulting values were not significantly different between treatments, however, due to the high standard error associated with the measurements. Whether increased denitrification with a lower product ratio is likely to result in reduced overall N_2O levels will be discussed from another angle in the section on denitrification efficiency.

As with the Hungarian soils, the denitrification product ratio became smaller at 40 hours of anaerobic incubation compared to 10 hours. This was most pronounced for soil under minimum tillage for which the denitrification product ratio dropped from 16% (at 10 hours) to close to zero (after 40 hours of incubation), illustrating the high potential of this topsoil to express N_2O . The correlation between soil organic carbon and the product ratio at 40 hours however is *much* weaker than at 10 hours, the opposite of what was observed with the Hungarian soils (the correlation between pH and the product ratio was *slightly* better at 40 hours than at 10 hours for the limed soils). Since the incubation measurements were run in parallel and anaerobicity was induced simultaneously, the experimental setup cannot be held accountable for this difference. It is hypothesized that the Austrian soil inherently supports denitrifier population being more conducive to efficient expression of the N_2O enzyme. As result, differences in denitrification performance are seen immediately after onset of denitrification.

5.3 Generalizability of results

Two vastly different soils were analyzed in this study, with pH values and organic carbon contents being at opposite extremes. When plotting the measured pH values of *all* soil samples (both sandy acidic soils

and loamy neutral soils) against the denitrification product ratio, regression analysis shows that the correlation between pH and the product ratio remains the same (constant slope, not shown). Performing the same with the organic carbon content shows that values for the carbon-poor Hungarian soils basically fall on the regression line determined by the Austrian soils alone (when this line is extrapolated to reach the lower carbon content range). This finding indicates that the two correlations (between soil pH and product ratio or soil organic carbon content, respectively) may represent a more general trend. If true (pending empirical confirmation), pH and C_{org} could be used to better parameterize the N_2O product ratio in N_2O emission models. As shown in the present study, this ratio appears to be a prominent variable determining the overall N_2O emission potential of cultivated soils. If the patterns found here with pH and C_{org} can be confirmed, the denitrification product ratio also opens for refining management strategies as to optimizing denitrification product ratios to mitigate N_2O emission in food production. However, the patterns found here cannot unequivocally be applied to field conditions since laboratory incubations were performed under fully anaerobic conditions with unlimited nitrate availability. It is hypothesized that because product ratios were investigated in a closed system (soils were placed in a small sealed flask), initial product ratios (10 hrs) are more relevant for predicting *in situ* product ratios since gaseous denitrification intermediates such as N_2O would escape the soil without complete reduction in a field situation. It also has to be considered that the onset of anaerobic conditions in the field would occur more gradually (in the laboratory, the soils were exposed to sudden anoxic conditions by He-washing). Oxygen is known to specifically repress N_2OR , likely leading to a mosaic of N_2OR activities depending on soil texture and moisture (Schurgers, Dorsch et al. 2006).

5.4 Denitrification Efficiency

“Denitrification is both a source and a sink for N_2O ” (Philippot, Hallin et al. 2007)

Both the Hungarian soils with their varying pH and the Austrian soils with their varying organic carbon contents show that as conditions for denitrification are improved (higher pH and organic carbon content), the product ratio decreases (see e.g. Fig. 9). This suggests that denitrifying populations optimize their denitrification product ratio on the long run towards complete N-oxide reduction to N_2 when noninhibited by pH or C-availability. Without this essential information, it may be thought that nitrous oxide emissions can be mitigated by repressing denitrification. For example, it has been suggested that the pH of acidic mineral soil should not be raised to values above 5.5-6 in order to avoid major N_2O evolution (Feng, Yan et al. 2003). Similarly, higher soil water content is viewed negatively because of the resulting anoxic conditions (prerequisite for denitrification).

Denitrification is viewed negatively because nitrogen fertilizers applied to soil or nitrogen from legumes is removed in the process. Denitrification is however essential for removing excess nitrates (which contaminate waters), and to close the N cycle by returning reactive N to the vast pool of atmospheric N_2 . Increasing demand for food production will lead to more reactive N loading globally and cultivation strategies therefore need to be optimized as to minimizing N_2O emissions. Cultivated soils would be the

primary target because they receive most of anthropogenically fixed N and because they are managed systems.

Denitrification should be viewed as a resource also because it is an alkalizing process, counterbalancing soil acidification (Helyar 1976). Soil acidification is a common process resulting from long-term mineral fertilization and nitrate leaching, and it could also be a problem with the planting of nitrogen fixing leguminous crops because nitrogen fixation is an acid-forming process. Soil acidification has other consequences besides increasing the denitrification product ratio (liming is widely practiced to control the availability of elements in soils as well as to repress certain disease causing agents). Denitrification ensures a positive feedback loop in terms of regulating soil pH, ensuring its own ability to function in the future by alleviating soil acidification.

Denitrification has importance as a key regulator of water and air quality at regional and global scales (Galloway, Dentener et al. 2004)

5.5 Sustainable management of pH and soil organic matter

Management practices that develop the soil's buffering capacity and lead to the buildup of soil organic matter lead to improved soil quality on the long term. Liming as a solution for reducing N₂O emissions may be limited to sandy acidic soils only. Liming soils rich in organic matter may be more problematic because increased microbial activity after pH up-shift may lead to overall loss of soil organic carbon which was shown to support denitrification efficiency in the Austrian Chernozem. Soil management strategies in general need to be considered in light of the soil type at hand. Charcoal amendments in another instance have been shown to improve the moisture availability of sandy soils but reduce moisture availability of clayey soils (Glaser, Lehmann et al. 2002). Tillage and liming are also likely to affect soils differently.

Alternative strategies to increase pH of acid soils while maintaining or building up SOM levels ought to be considered. Valuable studies are as follows:

- Application of chicken manure and charcoal to *highly weathered soils* has been shown to increase soil pH (Steiner, Teixeira et al. 2007).
- Application of composted and thermally dried sewage sludge increases both pH and total organic carbon content with a more evident effect with increasing number of applications (Fernandez, Senesi et al. 2009).
- Municipal solid waste compost amendments have been shown to increase both pH as well as soil organic carbon. Soil buffering capacity was also increased (Garcia-Gil, Ceppi et al. 2004).

These are long-term effects of amendments on soil pH and SOC and the long-term effects of adding such amendments on N₂O emissions may be different from the short-term effects. The addition of organic amendments together with mineral fertilizer has been shown to lead to high sudden N₂O emissions (Meng, Ding et al. 2005), and accumulative emissions on long-term scales should also be considered in

future studies on management options. The use of organic amendments is especially useful in efficiently recycling organic wastes and byproducts of industrial processes. Therefore if organic soil amendments do improve soil quality without increasing overall N₂O emissions in the long-term, this valuable function of recycling could be further utilized, especially if complete denitrification leads to the return of reactive nitrogen species (in the case of organic wastes high in nitrogen) to the biospheric pool of inert nitrogen. With more types of usable soil amendments, farmers have increased options for utilizing products in the vicinity of their farm.

Minimum tillage practices also offer advantages to farmers once initial investments (in new machinery such as seed drill and in farming skill for dealing with increased weeds) associated with conversion are surpassed. Thereafter, the field needs less frequent work with a potentially lighter tractor resulting in fuel savings as well as benefits for soil structure maintenance (Elmi, Madramootoo et al. 2003). With the right management skills such as the use of cover crops and crop rotation, annual yields can be maintained as well (Uri, Atwood et al. 1999). Conservation tillage systems are also more water efficient (Lindwall and Anderson 1981), alleviating crop loss under increased climatic fluctuations.

Poor soil management leads to soil degradation. Soils depleted of organic matter can give devastatingly lower yields compared to soils that are able to sustain their plant biomass (Steiner, Teixeira et al. 2007). Soil microbes are similarly susceptible to soil quality with poor acid soils supporting lower taxonomic diversity, which also has been shown to be the case for denitrifiers (Dörsch In prep.). This means that improving soil quality may contribute to the mitigation of N₂O emissions, putting this issue at the core of agroecology.

Bibliography

- Bakken, L. R. and M. A. Bleken (1998). "Temporal aspects of N-enrichment and emission of N₂O to the atmosphere." Nutrient Cycling in Agroecosystems **52**(2-3): 107-121.
- Beauchamp, E. G. (1997). Nitrous oxide emission from agricultural soils, Agr Inst Canada.
- Baath, E. (1996). "Adaptation of soil bacterial communities to prevailing pH in different soils." Fems Microbiology Ecology **19**(4): 227-237.
- Calderon, F. J., L. E. Jackson, et al. (2001). "Short-term dynamics of nitrogen, microbial activity, and phospholipid fatty acids after tillage." Soil Science Society of America Journal **65**(1): 118-126.
- Cavigelli, M. A. and G. P. Robertson (2001). "Role of denitrifier diversity in rates of nitrous oxide consumption in a terrestrial ecosystem." Soil Biology & Biochemistry **33**(3): 297-310.
- Chan, K. Y., M. K. Conyers, et al. (2007). "Improved structural stability of an acidic hardsetting soil attributable to lime application." Communications in Soil Science and Plant Analysis **38**(15-16): 2163-2175.
- Chantigny, M. H. (2003). Dissolved and water-extractable organic matter in soils: a review on the influence of land use and management practices, Elsevier Science Bv.
- Chapin, F. S., P. A. Matson, et al. (2002). Principles of terrestrial ecosystem ecology. New York, Springer.
- Chapuis-Lardy, L., N. Wrage, et al. (2007). "Soils, a sink for N₂O? A review." Global Change Biology **13**(1): 1-17.
- Cicerone, R. J. (1989). "ANALYSIS OF SOURCES AND SINKS OF ATMOSPHERIC NITROUS-OXIDE (N₂O)." Journal of Geophysical Research-Atmospheres **94**(D15): 18265-18271.
- Curtin, D., C. A. Campbell, et al. (1998). "Effects of acidity on mineralization: pH-dependence of organic matter mineralization in weakly acidic soils." Soil Biology & Biochemistry **30**(1): 57-64.
- D'Haene, K., S. Sleutel, et al. (2009). "The effect of reduced tillage agriculture on carbon dynamics in silt loam soils." Nutrient Cycling in Agroecosystems **84**(3): 249-265.
- Deiglmayr, K., L. Philippot, et al. (2004). "Structure and activity of the nitrate-reducing community in the rhizosphere of *Lolium perenne* and *Trifolium repens* under long-term elevated atmospheric pCO₂." Fems Microbiology Ecology **49**(3): 445-454.
- Doelman, P., Eijsackers, H.J.P. (2004). Vital Soil: Function, Value and Properties, Elsevier Science.
- Dörsch, P., Brake, G., Bakken, L. (In prep.). Functional differences in soil denitrifying communities: the role of pH.
- Ellis, S., M. T. Howe, et al. (1998). "Carbon and nitrogen dynamics in a grassland soil with varying pH: Effect of pH on the denitrification potential and dynamics of the reduction enzymes." Soil Biology & Biochemistry **30**(3): 359-367.
- Elmi, A. A., C. Madramootoo, et al. (2003). "Denitrification and nitrous oxide to nitrous oxide plus dinitrogen ratios in the soil profile under three tillage systems." Biology and Fertility of Soils **38**(6): 340-348.
- Enwall, K., L. Philippot, et al. (2005). "Activity and composition of the denitrifying bacterial community respond differently to long-term fertilization." Applied and Environmental Microbiology **71**(12): 8335-8343.
- EPA, U. S. (2006, October 19, 2009). "Nitrous Oxide: Sources and Emissions." Retrieved April 10, 2009, 2009, from <http://www.epa.gov/nitrousoxide/sources.html>.
- Feng, K., F. Yan, et al. (2003). "Nitrous oxide emission as affected by liming an acidic mineral soil used for arable agriculture." Nutrient Cycling in Agroecosystems **67**(3): 283-292.
- Fernandez, J. M., N. Senesi, et al. (2009). "Effects of Composted and Thermally Dried Sewage Sludges on Soil and Soil Humic Acid Properties." Pedosphere **19**(3): 281-291.

- Fierer, N. and R. B. Jackson (2006). "The diversity and biogeography of soil bacterial communities." Proceedings of the National Academy of Sciences of the United States of America **103**(3): 626-631.
- Firestone, M. K., R. B. Firestone, et al. (1980). "NITROUS-OXIDE FROM SOIL DENITRIFICATION - FACTORS CONTROLLING ITS BIOLOGICAL PRODUCTION." Science **208**(4445): 749-751.
- Focht, D. D. (1974). "EFFECT OF TEMPERATURE, PH, AND AERATION ON PRODUCTION OF NITROUS-OXIDE AND GASEOUS NITROGEN - ZERO-ORDER KINETIC-MODEL." Soil Science **118**(3): 173-179.
- Galloway, J. N., J. D. Aber, et al. (2003). "The nitrogen cascade." Bioscience **53**(4): 341-356.
- Galloway, J. N., F. J. Dentener, et al. (2004). "Nitrogen cycles: past, present, and future." Biogeochemistry **70**(2): 153-226.
- Garcia-Gil, J. C., S. B. Ceppi, et al. (2004). "Long-term effects of amendment with municipal solid waste compost on the elemental and acidic functional group composition and pH-buffer capacity of soil humic acids." Geoderma **121**(1-2): 135-142.
- Gitay, H., Suarez, A., Watson, RT., Dokken, DJ. (2002). IPCC Technical Paper V - Climate Change and Biodiversity. Geneva, Switzerland, IPCC: 85.
- Glaser, B., J. Lehmann, et al. (2002). "Ameliorating physical and chemical properties of highly weathered soils in the tropics with charcoal - a review." Biology and Fertility of Soils **35**(4): 219-230.
- Granli, T., Bøckmann, O. (1994). "Nitrous oxide from agriculture." Norwegian Journal of Agricultural Sciences **12**: 1-128.
- Helyar, K. R. (1976). "NITROGEN CYCLING AND SOIL ACIDIFICATION." Journal of the Australian Institute of Agricultural Science **42**(4): 217-221.
- Henry, S., D. Bru, et al. (2006). "Quantitative detection of the nosZ gene, encoding nitrous oxide reductase, and comparison of the abundances of 16S rRNA, narG, nirK, and nosZ genes in soils." Applied and Environmental Microbiology **72**(8): 5181-5189.
- I Page, A. L., Miller, R.H. & Keeney, D.R. (1982). Total Carbon, Organic Carbon and Organic Matter. Methods of Soil Analysis Part 2 Agronomy **9**. D. W. S. Nelson, L.E. Madison, Wisconsin, USA, American Society of Agronomy, Inc.: 539-579.
- Kadar, I., Marton, L., Nømeth, T., Szemes, I. (2007). "Effect of liming and mineral fertilization on the soil and plants in a 44-year long-term field experiment in Nyírlugos in Hungarian with English summary." Agrokémia és Talajtan **56**: 255-270.
- Kandeler, E., D. Tschirko, et al. (1999). "Long-term monitoring of microbial biomass, N mineralisation and enzyme activities of a Chernozem under different tillage management." Biology and Fertility of Soils **28**(4): 343-351.
- Keller, M. (1995). "Hole in the Pipe" conceptual model.
- Lindwall, C. W. and D. T. Anderson (1981). "AGRONOMIC EVALUATION OF MINIMUM TILLAGE SYSTEMS FOR SUMMER FALLOW IN SOUTHERN ALBERTA." Canadian Journal of Plant Science **61**(2): 247-253.
- Liu, X. J. J., A. R. Mosier, et al. (2007). "Dinitrogen and N₂O emissions in arable soils: Effect of tillage, N source and soil moisture." Soil Biology & Biochemistry **39**(9): 2362-2370.
- Meng, L., W. X. Ding, et al. (2005). "Long-term application of organic manure and nitrogen fertilizer on N₂O emissions, soil quality and crop production in a sandy loam soil." Soil Biology & Biochemistry **37**(11): 2037-2045.
- Molstad, L., P. Dorsch, et al. (2007). "Robotized incubation system for monitoring gases (O₂, NO, N₂O) in denitrifying cultures." Journal of Microbiological Methods **71**(3): 202-211.
- Mulvaney, R. L., S. A. Khan, et al. (1997). "Nitrogen fertilizers promote denitrification." Biology and Fertility of Soils **24**(2): 211-220.
- Peeche, M. (1965). "Hydrogen activity. Methods of soil analysis." Ser. Agron. **9**: 914-932.

- Philippot, L., S. Hallin, et al. (2007). Ecology of denitrifying prokaryotes in agricultural soil. Advances in Agronomy, Vol 96. San Diego, Elsevier Academic Press Inc. **96**: 249-305.
- Rhode, R. A. (2006). Greenhouse Gas by Sector, Global Warming Art
- Schurgers, G., P. Dorsch, et al. (2006). "Modelling soil anaerobiosis from water retention characteristics and soil respiration." Soil Biology & Biochemistry **38**(9): 2637-2644.
- Simek, M. and J. E. Cooper (2002). "The influence of soil pH on denitrification: progress towards the understanding of this interaction over the last 50 years." European Journal of Soil Science **53**(3): 345-354.
- Skiba, U., K. A. Smith, et al. (1993). "NITRIFICATION AND DENITRIFICATION AS SOURCES OF NITRIC-OXIDE AND NITROUS-OXIDE IN A SANDY LOAM SOIL." Soil Biology & Biochemistry **25**(11): 1527-1536.
- Smil, V. (1996). Cycles of life: civilization and the biosphere. New York, Scientific American Library.
- Smith, M. S., M. K. Firestone, et al. (1978). "ACETYLENE INHIBITION METHOD FOR SHORT-TERM MEASUREMENT OF SOIL DENITRIFICATION AND ITS EVALUATION USING N-13." Soil Science Society of America Journal **42**(4): 611-615.
- Smith, M. S. and J. M. Tiedje (1979). "PHASES OF DENITRIFICATION FOLLOWING OXYGEN DEPLETION IN SOIL." Soil Biology & Biochemistry **11**(3): 261-267.
- Smith, P., D. Martiono, Z. Cai, D. Gwary, H. Janzen, P. Kumar, B. McCarl, S. Ogle, F. O'Mara, C. Rice, B. Scholes, O. Sitotenko (2007). Agriculture, Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.
- Spiegel, H., Dersch, G., Hosch, J., Baumgarten, A. (2007). "Tillage effects on soil organic carbon and nutrient availability in a long-term field experiment in Austria." Die Bodenkultur **58**: 1-4.
- Stehfest, E. and L. Bouwman (2006). "N₂O and NO emission from agricultural fields and soils under natural vegetation: summarizing available measurement data and modeling of global annual emissions." Nutrient Cycling in Agroecosystems **74**(3): 207-228.
- Steiner, C., W. G. Teixeira, et al. (2007). "Long term effects of manure, charcoal and mineral fertilization on crop production and fertility on a highly weathered Central Amazonian upland soil." Plant and Soil **291**(1-2): 275-290.
- Stevens, R. J., R. J. Laughlin, et al. (1998). "Soil pH affects the processes reducing nitrate to nitrous oxide and di-nitrogen." Soil Biology & Biochemistry **30**(8-9): 1119-1126.
- Szajdak, L., A. Jezierski, et al. (2003). Impact of conventional and no-tillage management on soil amino acids, stable and transient radicals and properties of humic and fulvic acids, Pergamon-Elsevier Science Ltd.
- Tatzber, M., M. Stemmer, et al. (2008). "Impact of different tillage practices on molecular characteristics of humic acids in a long-term field experiment - An application of three different spectroscopic methods." Science of the Total Environment **406**(1-2): 256-268.
- UEA. (2008). "Nitrous Oxide focus group, Overview." Retrieved April 10, 2009, from <http://www.nitrousoxide.org/overview.html>.
- Uri, N. D., J. D. Atwood, et al. (1999). "The Environmental benefits and costs of conservation tillage." Environmental Geology **38**(2): 111-125.
- Wallenstein, M. D., D. D. Myrold, et al. (2006). "Environmental controls on denitrifying communities and denitrification rates: Insights from molecular methods." Ecological Applications **16**(6): 2143-2152.
- Wilhelm, E., R. Battino, et al. (1977). "LOW-PRESSURE SOLUBILITY OF GASES IN LIQUID WATER." Chemical Reviews **77**(2): 219-262.
- Wolsing, M. and A. Prieme (2004). "Observation of high seasonal variation in community structure of denitrifying bacteria in arable soil receiving artificial fertilizer and cattle manure by determining T-RFLP of nir gene fragments." Fems Microbiology Ecology **48**(2): 261-271.

- Young, I. M. and K. Ritz (2000). "Tillage, habitat space and function of soil microbes." Soil & Tillage Research **53**(3-4): 201-213.
- Zaman, M., M. L. Nguyen, et al. (2007). "Can soil amendments (zeolite or lime) shift the balance between nitrous oxide and dinitrogen emissions from pasture and wetland soils receiving urine or urea-N?" Australian Journal of Soil Research **45**(7): 543-553.