DISSERTATION

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The fate of inorganic nitrogen fertilizers in agricultural soils

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Contents

Summary

Intensive agricultural crop production in central Europe largely depends on the input of nitrogen fertilizers, mainly provided in the form of NH_4^+ or NO_3^- or a combination thereof. Global estimates indicate that less than ~50% of the applied fertilizer N is used by the crop, while 2-5% are stored in the soil, ~25% are emitted to the atmosphere and ~20% are discharged to aquatic systems. In the last decade a wealth of studies have focused on ways to improve fertilizer use efficiency and on reducing adverse effects, namely the losses of fertilizer N to the environment. Other studies have focused on fates of N inputs during one or more growing seasons, or on predicting N availability to crops.

In the first step of the present work a new microcosm system was evaluated for its suitability to investigate nitrogen dynamics between soils, plants and microbes. Five different agricultural soils were homogenized and transferred in the test tubes, and kept under controlled conditions in a climate chamber for four weeks. Soils differed clearly in nitrogen pools and microbial population structures but less in their activities. Bacterial and fungal community compositions and soil properties, except gross N transformation rates, remained stable and reproducible during the test period in all soils. This proved that the microcosm system is suitable to study soil N cycling or other biogeochemical cycles as well as microbial populations and functions.

In the second step the present work examined how crop plants and soil microbes vary in their ability to take up and compete for fertilizer N on a short time scale (hours to days) using ^{15}N tracer techniques. Single plants of barley (*Hordeum vulgare* L. cv. *Morex*) were grown on two agricultural soils in microcosms which received either NH_4^+, NO_3^- or NH_4NO_3. Within each fertilizer treatment traces of $^{15}NH_4^+$ and $^{15}NO_3^-$ were added separately. During 8 days of fertilization the fate of fertilizer ^{15}N into plants, microbial biomass and inorganic soil N pools as well as changes in gross N transformation rates were investigated. One week after fertilization 60-80% of initially applied ^{15}N was recovered in crop plants compared to only 1-10% in soil microbes, proving that plants were the strongest competitors for fertilizer N. In terms of N uptake soil microbes out-competed plants only during the first 4 hours of N application independent of soil and fertilizer N form. Within one day microbial N uptake declined substantially, probably due to carbon limitation. In both soils, plants and soil microbes took up more NO_3^- than NH_4^+ independent of initially applied N form. Surprisingly, no inhibitory effect of one N form on the uptake of the other in both, plants and microbes, was observed. Compared to plant and microbial NH_4^+ uptake rates, gross nitrification rates were 3

to 75-fold higher, indicating that nitrifiers were the strongest competitors for NH_4^+ in both soils. The rapid conversion of NH_4^+ to NO_3^- and preferential use of NO_3^- by soil microbes suggest that in agricultural systems with high inorganic N fertilizer inputs the soil microbial community could adapt to high concentrations of NO_3^- and shift towards enhanced reliance on NO_3^- for their N supply.

In the third step the present work dealt with the impact of different forms of inorganic N fertilizer on emission of CO_2, CH_4 and N_2O. In both soils fertilization increased CO_2 and N_2O emissions, and consequently soil derived global warming potential (GWP). Thereby N_2O emissions were highest when N was supplied as NH_4NO_3, indicating that both, nitrification and denitrification significantly contributed to total N_2O emissions. The two soils were differently affected by fertilization, probably due to different soil microbial communities, suggesting that archaea potentially contribute to total soil N_2O emissions. Increased CO_2 emissions, on the other hand, were mainly the result of enhanced root respiration due to fertilization. Still, it was not possible to distinguish root and microbial respiration from the rhizosphere and needs to be addressed in future studies. CH_4 oxidation was only slightly decreasing soil-derived GWP and was not controlled by fertilizer N form but mainly by soil water content. Thereby, a 2 to 5 % change of water filled pore space resulted in marked, but short-lived increases of CH_4 consumption rates. Overall, for a detailed understanding of mechanisms responsible for greenhouse gas emissions future research should cover an interdisciplinary field, combining biochemical techniques with molecular based analyses, as well as [15]N (and ^{18}O) tracer studies to follow the fertilizer into the different compartments of soil-plant-atmosphere systems.

Zusammenfassung

Der Einsatz von Stickstoffdüngern in der Landwirtschaft Europas ist nicht mehr wegzudenken. Allerdings werden von den aufgetragenen Düngern, hauptsächlich in der Form von Ammonium, Nitrat oder Ammoniumnitrat, nur 50% von den Nutzpflanzen genutzt, während 2-5% im Boden gespeichert, ~25% als Stickstoffgase emittiert, und ~20% ausgewaschen werden. In den letzten Jahren wurde in verschiedensten Studien nach Wegen gesucht, die Stickstoffnutzungseffizient durch die Nutzpflanzen zu steigern und die Stickstoffverluste zu minimieren.

Der erste Teil dieser Arbeit zeigt die Zuverlässigkeit eines neuen Mikrokosmos Systems, im Bezug auf Untersuchungen von Stickstoffdynamiken zwischen Boden, Pflanze und Mikroorganismen. Fünf Ackerböden aus der Umgebung Wiens wurden homogenisiert und in die Mikrokosmen gepackt, und anschließend vier Wochen lang unter kontrollierten Bedingungen in einer Klimakammer gehalten. Die fünf Böden zeigten klare Unterschiede in den Stickstoffpools und in ihrer mikrobiellen Zusammensetzung, aber wenig Unterschied in ihren Aktivitäten. Bakterielle und pilzliche Gemeinschaftsstrukturen, sowie Bodeneigenschaften (mit Ausnahme von Brutto-Stickstofftransformationsraten) blieben während der Testperiode unverändert und reproduzierbar. Diese Ergebnisse zeigten, dass das neu entwickelte Mikrokosmossystem für Untersuchungen des Boden-Stickstoffkreislaufes sowie microbieller Strukturen und Aktivitäten genutzt werden kann.

Der zweite Teil dieser Arbeit untersucht mit Hilfe von ^{15}N Tracern Unterschiede zwischen Nutzpflanzen und Mikroorganismen im Bezug auf Stickstoffaufnahmeraten sowie Konkurrenzeffekte über eine kurze Zeitspanne. Jeweils ein Gerstenkeimling (*Hordeum vulgare* L. *cv. Morex*) wurde in je einem Mikrokosmos angebaut. Verwendet wurden zwei Böden, die mit unterschiedlichen Stickstoffformen gedüngt wurden (Ammoniun, Nitrat oder Ammuniumnitrat). Zu jedem Düngeansatz wurden zusätzlich Spuren von ^{15}N-Ammonium oder ^{15}N-Nitrat zugesetzt. Anschließend wurde dieser ^{15}N Tracer acht Tage lang in Pflanzen, Stickstoffpools im Boden und in der mikrobiellen Biomasse gemessen und Änderungen in Brutto-Stickstoffmineralisierung und Brutto-Nitrifizierungsraten untersucht. Eine Woche nach der Düngung konnten 60-80% des Düngers in den Pflanzen wieder gefunden werden, während nur 1-10% in der mikrobiellen Biomasse gebunden war. Das zeigt, dass die Pflanzen in diesem System die stärksten Konkurrenten für Stickstoffdünger waren.Außerdem waren Bodenmikroorganismen den Pflanzen im Bezug auf Stickstoff Aufnahmeraten ebenfalls unterlegen, mit der Ausnahme der ersten vier Stunden nach der Düngung. In diesem Fall

nahmen die Mikroorganismen mehr Stickstoff auf als die Pflanzen, unabhängig von Bodentyp oder Düngerform. Anschließend nahmen die Stickstoff Aufnahmeraten gravierend ab, höchstwahrscheinlich aufgrund von Kohlenstofflimitierung. In beiden Böden wurde ebenfalls unabhängig von der Düngerform mehr Nitrat als Ammonium von den Pflanzen und von Mikroorganismen aufgenommen. Überraschenderweise kam es zu keiner Inhibierung der Ammonium Aufnahme durch Nitratzugabe, und umgekehrt. Im Vergleich zu mikrobieller Ammonium Immobilisierung waren die Brutto-Nitrifizierungsraten 3-75 mal höher, was ein Zeichen dafür ist, dass die Nitrifizierer in beiden Böden das zugegebene Ammonium am besten nutzen konnten. Diese Ergebnisse zeigen, dass sich die Mikroorganismen in gedüngten Ackerböden an hohe Nitratkonzentrationen anpassen, und Nitrat immer mehr als Stickstoffquelle nutzen.

Im dritten Teil dieser Arbeit wurden die Auswirkungen von verschiedenen anorganischen Stickstoffdüngern auf die Ausgasung von den potentiellen Treibhausgasen Kohlendioxid (CO_2), Methan (CH_4) und Lachgas (N_2O) untersucht. In beiden Böden führte die Düngung zu gesteigerten Ausgasungen von CO_2, CH_4 und N_2O, und folglich auch zu einer Erhöhung des Potentials der globalen Erwärmung (global warming potential, GWP). Die Emissionen von N2O stiegen am stärksten nach der Düngung mit Ammoniumnitrat an, ein Zeichen dafür, dass sowohl Nitrifikation und Denitrifikation gleichzeitig zu den N_2O Emissionen beigetragen haben. Die beiden Böden reagierten unterschiedlich auf die verschiedenen Düngevarianten, sehr wahrscheinlich da sich die beiden Böden stark in ihrer mikrobiellen Gemeinschaft unterschieden. Das weist weiters darauf hin, dass Archaeen potentiell zu N_2O Emissionen beitragen könnten. Erhöhte CO_2 Emissionen konnten hauptsächlich auf eine erhöhte Wurzelatmung durch die Düngung zurückgeführt werden. Trotzdem war eine Unterscheidung zwischen Wurzelatmung und mikrobieller Atmung in der Rhizosphäre nicht möglich und bedarf noch zusätzlicher Studien. Die Oxidation von CH_4 verringerte das GWP nur zu einem sehr geringen Maß und wurde nicht von der Form des zugegebenen Düngers beeinflusst, sondern hauptsächlich vom Bodenwassergehalt. Eine Erhöhung dessen um 2 bis 5% führte dennoch zu einem starken, wenn auch kurzlebigen, Anstieg der CH_4 Aufnahme beider Böden. Um ein detailliertes Verständnis der für die Treibhausgasemissionen verantwortlichen Mechanismen zu bekommen, wird empfohlen integrative Studien durchzuführen, die sowohl biochemische und molekulare Methoden beinhalten, und zusätzlich mit dem Einsatz von stabilen Isotopen (^{15}N und ^{18}O) unterstützt werden.

1. Introduction

The element nitrogen (N) is an essential nutrient for all organisms, and as a critical component of proteins and nucleic acids, N is fundamental to the structures and biochemical processes that define life. Therefore it is not surprising that this nutrient and its cycle have been studied more extensively than any other nutrient element. In the last decades a vast amount of studies have addressed all kinds of aspects of the N cycle which resulted in numerous books and a nearly uncountable number of publications, including several excellent former and recent reviews. Although our knowledge is great already, recent improvements in methodology and technical equipment allowed further, so far unexpected, insights revealing new pathways and players in the N cycle. Micro-organisms are known to directly catalyze the processes of nitrification and denitrification, and these processes as well as the responsible microbial communities received special attention because of their crucial role in the N cycle (e.g., Bedard and Knowles, 1989; Conrad, 1996; De Boer and Kowalchuk, 2001; Barnard et al., 2005; Philippot et al., 2007; Philippot et al., 2008). However, in the past years our understanding of N cycling processes and the micro-organisms that mediate them has changed dramatically, as the processes of anaerobic ammonium oxidation (anammox) (Mulder et al., 1995), fungal (co-)denitrification (Shoun and Tanimoto, 1991; Shoun et al., 1992) and ammonia oxidation within the domain Archaea (Konneke et al., 2005; Leininger et al., 2006), have been recognized as new links in the global N cycle. Hayatsu et al. (2008) and Francis et al. (2007) give good reviews about these new processes and players in the N cycle.

Plants, on the other hand, were long considered to use only inorganic N and to be week competitors for N against soil microbes. However, recent research provided evidence that plants indeed compete successfully against soil microbes (e.g., Schimel and Bennett, 2004) and that they also take up organic N forms directly (e.g., Bardgett et al., 2003; Dunn et al., 2006; Harrison et al., 2007; Näsholm et al., 2009).

Another important aspect of the N cycle is the loss of reactive N from the soil-plant system in the form of hydrological or gaseous losses. Especially the emission of N_2O, a highly potential greenhouse gas, was studied intensely in various ecosystems and at different scales (e.g., Hall and Matson, 1999; Flessa et al., 2002; Müller et al., 2002; Dobbie and Smith, 2003; Yanai et al., 2003; Ambus et al., 2006; Kitzler et al., 2006; Pilegaard et al., 2006; Rosenkranz et al., 2006; Jones et al., 2007; Soussana et al., 2007; Russow et al., 2008).

But nevertheless, despite decades of investigations of the different steps and pathways of the N cycle, many of these are still poorly understood and also insufficiently quantified. The

global N cycle has been altered markedly by humans in the last decades, mainly due to artificial N fertilizer inputs and fossil fuel combustion. As the human population is growing, resulting in increasing food and energy demands, it is likely that the global N cycle will be further changed in future. Consequently, and because of the significant role of N in climate change, it is absolutely crucial that research on the N cycle continues with tremendous effort.

1.1 The global nitrogen cycle

One striking aspect of the N cycle is the coexistence in nature of different oxidation states of the N atom ranging from reduced compounds, e.g., –III as in ammonia (NH_3) to fully oxidized state, e.g., +V as in nitrate (NO_3^-). The conversion between the different forms of N is mediated by processes performed by soil micoorganisms and plants and together these processes form the global N cycle.

With 79% of gaseous dinitrogen (N_2) the atmosphere is the largest reservoir of N. However, atmospheric N_2 is metabolically unavailable to the higher plants which do not have the ability to break the triple covalent bond of N_2. Due to that and due to continual losses of N by denitrification, soil erosion, leaching or chemical volatilization, N is also frequently found to be limiting for plant nutrition. Atmospheric N_2 can enter the terrestrial N cycle through the action of several unique types of microbes that can convert N_2 gas to NH_3. This conversion of molecular N into the plant available form is known as biological N fixation which is mainly performed by diazotrophs and is represented by the following equation (equation 1):

Equation 1: $N_2 + 8H+ + 8e^- + 16ATP \longrightarrow 2NH_3 + H_2 + 16\ ADP + 16Pi.$

NH_3 produced is generally used directly by free N-fixing bacteria or exported to plants by symbiotic N-fixing bacteria. Subsequently this N is used by plants to synthesize organic N compounds. After the death of an organism organic N is mineralized by microbes to NH_3/NH_4^+.

Once NH_3 is produced there are several possible fates: (1) it can be fixed by clays or by soil organic matter, (2) volatilized as NH_3, (3) assimilated by plants and mircoorganisms or finally (4) converted to NO_3^- during nitrification.

The NO_3^- formed by nitrification can than be assimilated by plants and soil microbes or used as a terminal electron acceptor by microbes when oxygen is limiting. Further, the conversion of the relatively immobile N form ammonium (NH_4^+) to the highly mobile ions nitrite (NO_2^-)

and NO_3^- are of high ecological significance because they increase the potential for N losses through increased leaching and N gas emissions.

The reduction of NO_3^- into gaseous N is performed by a four-step reaction process, namely denitrification, in which NO_3^- and NO_2^- are reduced into nitric oxide (NO), nitrous oxide (N_2O) and dinitrogen (N_2):

$$NO_3^- \longrightarrow NO_2^- \longrightarrow NO \longrightarrow N_2O \longrightarrow N_2.$$

Denitrification is the main biological process responsible for the return of fixed N to the atmosphere, thus completing the N cycle (Bowden, 1986).

1.2 Nitrogen transformations in the soil

It is necessary to understand the main mechanisms of microbially mediated N transformations in soils before studying the complex N cycle in a more complete form. Most processes are occuring simultaneously, but at different rates depending on a large variety of controlling factors, including soil physical and chemical properties and N availability. Without some basic knowledge about each specific transformation process, it would not be possible to understand the whole system. This chapter therefore gives a short introduction into the major processes relevant in agricultural soils, namely N mineralization, nitrification, denitrification and the recently discovered pathways of denitrifier nitrification, fungal and archaeal denitrification, chemical denitrification, anaerobic ammonium oxidation (anammox), and dissimilatory nitrate reduction to ammonia (DNRA). A simplified diagram of the N cycle with these key processes and involved functional genes recently appeared in Francis et al. (2007, Figure 1).
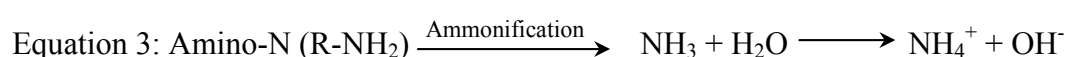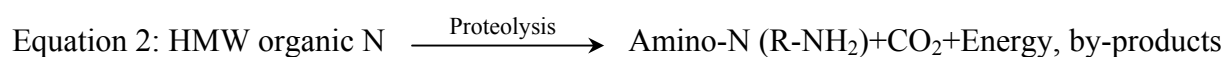
1.2.1 Nitrogen mineralization and immobilization

Nitrogen (N) mineralization refers to the conversion of complex organic N forms to simple, plant available N forms by a range of aerobic and anaerobic heterotrophic microbes.
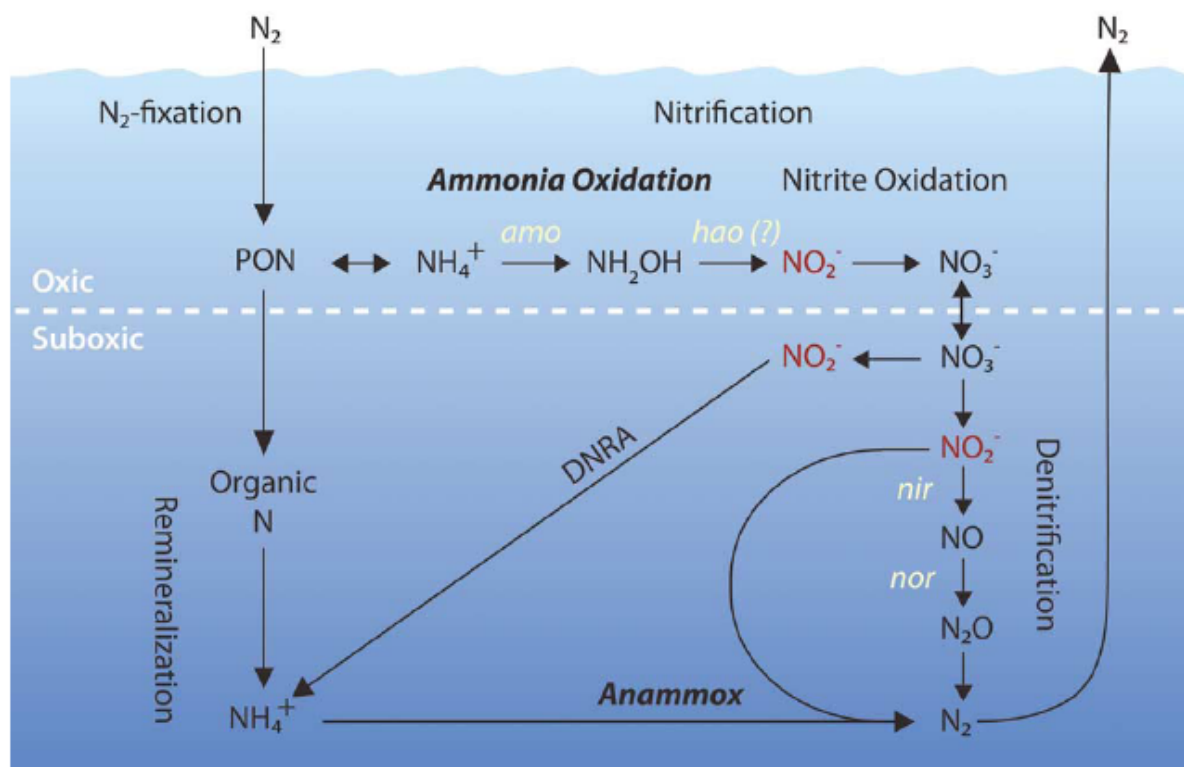
Figure 1 Microbial nitrogen transformations above, below and across an oxic/anoxic interface. *PON*, particulate organic N; *amo*, ammonia mono-oxygenase; *hao*, bacterial hydroxylamine oxidoreductase; *nir*, nitrite reductase; *nor*, nitric oxide reductase (Francis et al., 2007).

Traditionally, NH_4^+ has been viewed as the only immediate product of mineralization, and in older literature mineralization was often referred to as ammonification. Recent studies, however, concluded that plants are also able to take up available organic N, leading us to broaden this definition of mineralization by including anterior depolymerisation of high molecular weight (HMW) organic N to simple organic N forms that can be taken up by plants (Schimel et al., 2004; Näsholm et al., 2009). The reaction involved in the mineralization of HMW organic N to inorganic NH_4^+ consists of two steps and can be summarized as follows (equation 2 + 3):

Equation 2: HMW organic N $\xrightarrow{\text{Proteolysis}}$ Amino-N $(R\text{-}NH_2) + CO_2 + $ Energy, by-products

Equation 3: Amino-N $(R\text{-}NH_2)$ $\xrightarrow{\text{Ammonification}}$ $NH_3 + H_2O \longrightarrow NH_4^+ + OH^-$

Mineralization generally occurs under three conditions in soil: when (1) microorgganisms are carbon starved and utilize the keto-skeletons of amino acids for energy generation, (2) fluctuations in water and temperature cause cell death and lysis resulting in subsequent N-mineralization, and (3) microbes are consumed by predators that release excess NH_4^+.

Generally the mineralization process is accompanied by the assimilation of NH_4^+ and NO_3^- by the soil microbial biomass, known as immobilization. Since mineralization and immobilization take place simultaneously and at rather small scales, it is important to distinguish between gross and net mineralization and immobilization. Gross N mineralization is the total amount of soluble inorganic N produced by microbes, and gross N immobilization is the total amount of consumed soluble inorganic N. If gross mineralization exceeds gross immobilization the concentration of soluble inorganic N in the soil is increasing, resulting in net mineralization (Powlson and Barraclough, 1993). On the other hand, when gross N immobilization exceeds gross N mineralization, inorganic soluble N in the soil is decreasing, resulting in net immobilization.
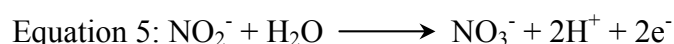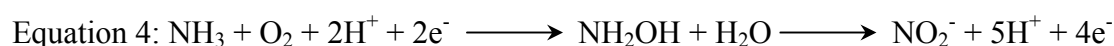
Both, mineralization and immobilization are carried out by a wide array of microbes. Additionally, soil fauna plays an important role in mineralization and immobilization processes, as they are responsible for much of the preliminary decomposition of detritus. They can also regulate populations of bacteria and fungi directly by feeding on them, and they can create and modify habitats for many organisms.

The reason for the wide distribution of N mineralization and immobilization is that these processes are so fundamental. All heterotrophic soil organisms consume organic materials to gain energy and carbon, and N is immobilized and mineralized as a by-product (Robertson and Groffman, 2007). Due to this wide distribution of both processes, their regulation is relatively straightforward. Factors generally influencing microbial activities in soils, like soil temperature, moisture, pH, O_2 concentration, and availability of other soil nutrients, also strongly affect N mineralization and immobilization rates (Canali and Benedetti, 2006). Activities increase with temperature and are optimal at intermediate water contents, although low activities are also found at extremes of both temperature and soil moisture (Robertson et al., 2007). Another main factor controlling the rates and patterns of N mineralization and immobilization is the availability of soil organic matter, as large inputs of organic matter increase rates of microbial activity. If the substrates have a C/N ratio of about 25:1 net mineralization is common, whereas substrates with greater ratios are associated with net immobilization of N (Killham, 1994; Paul and Clark, 1996; Myrold, 1998).

Besides that, edaphic and climatic influences on net N mineralization and immobilization are well documented (Haynes, 1986; Kumar and Goh, 2000) with the effects of specific soil characteristics and residue composition having been recently reviewed by (Cabrera et al., 2005).

1.2.2 Nitrification

Nitrification is a two-step process, consisting of the conversion of ammonia (NH_3) to nitrite (NO_2^-) and subsequently to nitrate (NO_3^-). The pioneering work of (Winogradsky, 1890) established that this process is performed by chemolithotrophic bacteria that respire with oxygen (O_2) and assimilate carbon dioxide (CO_2). These chemolithotrophic bacteria are classified into two groups, based on their ability to oxidize NH_3 to NO_2^- (ammonia-oxidizing bacteria, AOB) or NO_2^- to NO_3^- (nitrite-oxidizing bacteria, NOB) (Kowalchuk and Stephen, 2001). AOB convert NH_3 via hydroxylamine (NH_2OH) to NO_2^- (equation 4), with the responsible enzymes being ammonium monooxygenase (AMO) and hydroxylamine oxido-reductase, respectively. The conversion of NO_2^- to NO_3^- (equation 5), is catalyzed by the enzyme nitrite oxido-reductase.

Equation 4: $NH_3 + O_2 + 2H^+ + 2e^- \longrightarrow NH_2OH + H_2O \longrightarrow NO_2^- + 5H^+ + 4e^-$

Equation 5: $NO_2^- + H_2O \longrightarrow NO_3^- + 2H^+ + 2e^-$

Until recently, the slow growth, low yield and difficulty of isolating AOB and NOB have restricted physiological and ecological studies of these bacteria. In the past decade, however, the development of molecular biological techniques for identifying and quantifying soil bacteria has led to a better understanding of the distribution, diversity and population dynamics of AOB and NOB (Kowalchuk et al., 2001).

The majority of nitrifying bacteria are phylogenetically affiliated to the β- and γ-proteobacteria, represented by the genera *Nitrosomonas*, *Nitrospira* and *Nitrosococcus* (Aakra et al., 2001; Koops and Pommerening-Röser, 2001). The AOB in soils have been studied mainly by targeting 16 rRNA and *amoA* genes. The *amoA* gene encodes subunit A of AMO, which catalyzes the first step of ammonia oxidation. The determination of this gene proved to be a powerful molecular tool because of its fine-scale resolution of closely related populations and its functional trait rather than a phylogenetic trait (Purkhold et al., 2003). Because autotrophic nitrifying bacteria are dependent on NH_4^+ or NO_2^- as specific energy sources, the addition of NH_4^+ fertilizer to soils can increase the population size of nitrifying bacteria (e.g. Hastings et al., 1997; Chu et al., 2007b).

Besides by the size of available NH_4^+, nitrification is greatly influenced by edaphic factors such as pH, moisture, temperature and aeration (e.g., De Boer et al., 2001). The optimum pH for nitrifying bacteria in pure culture is in the range of 7-9, and the lower limit of growth was

shown to be approximately pH 6 (Allison and Prosser, 1993). Other studies, however, showed that nitrification occurred in strongly acidic soils with pH values ranging from 3-5 (Walker and Wickramasinghe, 1979; Hayatsu and Kosuge, 1993; Laverman et al., 2001; Nugroho et al., 2005). Soil texture, i.e. the spatial heterogeneity of the soil, is another important factor influencing nitrification, as nitrification is a result of a high degree of spatial compartmentalisation of NH_4^+ production and consumption sites. This, coupled with diffusional constraints between microsites, additionally controls the nitrification rate (Bramley and White, 1991).

For a long time it has been believed that ammonia oxidation was limited to bacteria, but recent discoveries demonstrated that crenarchaea, members of the domain Archaea, are also potential ammonia oxidizers in soil as they also carry the *amoA* gene (Rotthauwe et al., 1997; Schleper et al., 2005; Treusch et al., 2005). Potential *amoA* genes of Crenarcheota, as well as 16S rRNA genes, have been found in environments ranging from marine to estuarine, freshwater, sediment and soil (Francis et al., 2005; Beman and Francis, 2006; Park et al., 2006), suggesting that nitrification of Crenarcheota may contribute considerably to net nitrification in a wide range of environments. Another study has shown that *amoA* gene copies of Crenarchaeota were up to 3000-fold more abundant than those of AOB in pristine and agricultural soils from three climatic zones (Leininger et al., 2006). However, it still remains unclear whether archaeal or bacterial nitrification is the main contributor to nitrification activity in soil ecosystems (Francis et al., 2007). Up to date, there are no experimental methods, such as the use of specific inhibitors (acetylene, nitrapyrin), to distinguish archaeal and bacterial nitrification activity in soil. Therefore, further physiological, biochemical and genomic studies will be necessary to determine the relative contribution of nitrifying archaea to the nitrification activity in soils (Hayatsu et al., 2008).

NOB appear in a broader array of phylogenetic groupings than do the AOB and, based on cell morphology and the phylogenetic relationships of 16S rRNA gene sequences, NOB have been classified into four genera (Teske et al., 1994), but only the genera *Nitrobacter* and *Nitrospira* have been detected in soil (Freitag et al., 2005).

In addition to autotrophic nitrification, a wide phylogenetic range of bacteria and fungi possess the potential for heterotrophic nitrification, potentially oxidizing both inorganic and organic N compounds. However, heterotrophic nitrification is not linked to cellular growth, as it is for autotrophic nitrification, and apparently heterotrophic nitrifiers do not obtain any energy from this process (Paul et al., 1996). There is evidence for two pathways for heterotrophic ammonia oxidation. The first is similar to that of autotrophic oxidation, in that
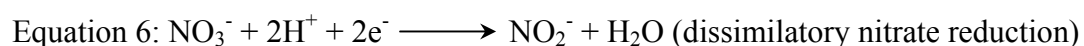
the nitrifying bacteria have similar ammonia- and hydroxylamine-oxidizing enzymes (Moir et al., 1996a; Moir et al., 1996b). The other pathway is also known as fungal nitrification, within which N compounds react with hydroxyl radicals (Wood, 1987). Although an increasing number of studies are focusing on heterotrophic nitrification the physiological role and the phylogenetic diversity still remain unclear.

In agricultural soils autotrophic nitrification is considered to predominate (e.g., Tortoso and Hutchinson, 1990), and evidence for heterotrophic nitrification in soils is limited mostly to acidic and organic matter-rich forest soils where autotrophic nitrification can be inhibited (e.g. Killham, 1986; Pedersen et al., 1999). Further, organic N addition promoted nitrification in acidic forest soils, but the addition of NH_4^+ had an inhibitory effect unless a readily available carbon source was supplied, indicating that the microbes responsible for nitrification in these soils were heterotrophs (Brierley and Wood, 2001). However, recent research has demonstrated the occurrence of heterotrophic nitrification in a fertilized agricultural soil as well (Bateman and Baggs, 2005).

1.2.3 Denitrification

Denitrification is the respiratory process by which NO_3^- is reduced to NO, N_2O, and N_2, via NO_2^- (equations 6 to 9). The sequential reduction steps are catalyzed by different enzymes, which are encoded by specific gene clusters (Philippot, 2002).

Nitrate reductase

Equation 6: $NO_3^- + 2H^+ + 2e^- \longrightarrow NO_2^- + H_2O$ (dissimilatory nitrate reduction)

Nitrite reductase

Equation 7: $NO_2^- + 2H^+ + e^- \longrightarrow NO + H_2O$

Nitric oxide reductase

Equation 8: $2NO + 2H^+ + 2e^- \longrightarrow N_2O + H_2O$

Nitrous oxide reductase

Equation 9: $N_2O + 2H_+ + 2e^- \longrightarrow N_2 + H_2O$
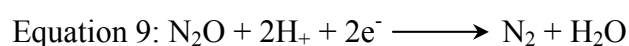
The reduction of NO_3^- to NO_2^- is catalyzed by different types of enzymes which are different in their location and biochemical attributes (Richardson et al., 2001; Stolz and Basu, 2002; Philippot, 2005). Three structurally distinct subclasses of bacterial NO_3^- reductase have been identified, which have an Fe-S center that binds Mo (in the form of the bis-molybdopterin guanine dinucleotide cofactor) and a hem-group (Richardson and Watmough, 1999). One of the NO_3^- reductases is membrane-bound and active under anoxic conditions, while the other is located in the periplasma and important under oxic conditions (Richardson et al., 1998). However, the reduction of NO_3^- to NO_2^- is not specific to denitrification, but also occurs in dissimilatory nitrate reduction to ammonia (Tiedje et al., 1983).

Researchers have identified three mutually exclusive bacterial NO_2^- reductases (Zumft, 1993; Richardson et al., 1999): a tetra-haem cytochrome *cd1* enzyme, a Cu-containing homo-trimer, and a multi-haem cytochrome *c* reductase, which are all located in the periplasm. The intermediate product of NO_2^- reduction is NO, which is actually toxic to denitrifiers. Therefore it is kept at low steady-state concentrations by a highly efficient NO reductase. The reduction of NO to N_2O is taking place on the outside of the cytoplasmatic membrane and two subclasses have been identified. Both are closely related to the haem-Cu oxidases but contain nonhaem-Fe instead of a haem-Cu in one metal center of the catalytic subunit (Zumft, 1997; Richardson et al., 1999).

The enzyme catalyzing the reduction of N_2O to N_2, nitrous oxide reductase, is a dimeric Cu-containing enzyme which is located in the periplasma, but still has to be fully characterized.

Denitrification occurs in a wide range of bacterial species: facultative phototrophs, chemoheterotrophs, and chemolithotrophs, mostly facultative anaerobes. Those bacteria use NO_3^- rather than O_2 as a terminal electron acceptor during respiration. Because NO_3^- is a less efficient electron acceptor than O_2, most denitrifiers undertake denitrification only when O_2 is otherwise unavailable. Further, denitrifying enzyme activities are inhibited by O_2 and the expression of the responsible genes are strictly surpressed. Therefore, denitrification typically starts to occur at water-filled pore space concentrations of 60% and higher. However, several bacteria, e.g. *Paracuccus denitrificans*, have been shown to reduce NO_3^- or NO_2^- to N_2O and N_2 even in the presence of O_2. This denitrifying bacterium has been characterized extensively already. In the presence of 92% O_2 in the atmosphere, *Paracoccus denitrificans* ATCC 35512 reduced 27% of added NO_3^- to gaseous N (Su et al., 2004). Other aerobic denitrifying bacteria could be isolated from soils and were identified as *Burkholeria cepacia* (Matsuzaka et al., 2003) and *Mesorhizobium* sp. (Okada et al., 2005). These results indicate that aerobic

denitrification can occur in a variety of environments indeed, and that the influence of O_2 concentration on denitrifying activity is dependent on the species of bacteria involved. Thus, the occurrence of aerobic denitrification is now considered to be more common than only a rare exception (Hayatsu et al., 2008). This seems to be valid also in arable soils, as was suggested previously in a soil incubation experiment (Bateman et al., 2005).

Besides the concentration of O_2 in the soil and the soil water content, other critical factors regulating the rate and duration of denitrification in soils are soil pH and temperature and the availability of the substrate NO_3^- and available carbon, the source of energy and electrons (Beauchamp et al., 1989; Firestone and Davidson, 1989; Aulakh et al., 1992; Smith and Doran, 1996).

Conversely, denitrification can be inhibited by any process that restricts NO_3^- production (e.g., slows nitrification) or enhances NO_3^- removal (leaching, plant uptake), or promotes aerobic conditions (artificial drainage, plant depletion of soil moisture). Rapid O_2 consumption by soil microbes during the decomposition of organic matter can produce anaerobic zones adjacent to aerobic areas where NO_3^- is being produced. When NO_3^- moves into the anaerobic zone by diffusion or by mass flow, it can then be denitrified. Although denitrification can occur in wide range of temperatures, the optimum temperature is ~30°C. The optimum range in pH for denitrifying organisms is 6 to 8, but similar to nitrification, denitrification has also been measured in highly acidic soils.

Denitrification can provide a valuable ecosystem service, e.g., by mediating N removal from NO_3^--polluted waters. In agricultural systems, however, denitrification can have negative effects as it causes fertilizer N losses, thus contributing to decreasing the efficiency of fertilization (Reddy and Patrick, 1986; Cassman et al., 1993). Denitrification also results in emissions of NO and N_2O, two trace gases that contribute to global warming (Wang et al., 1976) and ozone reduction (Crutzen and Ehalt, 1977; Conrad, 1990). In managed ecosystems it is therefore usually desirable to minimize denitrification in order to conserve N further for plant uptake. The ecological role of denitrifiers in arable soils and especially in the rhizosphere of crop plants therefore received increased attention of researchers in the last years and has only recently been reviewed in detail (Philippot et al., 2007; Philippot et al., 2008).

1.2.4 New players and pathways in the nitrogen cycle

1.2.4.1 Nitrifier denitrification

It is interesting to note that the denitrification capacity can also be present in N-fixing bacteria and in nitrifiers. Thus, denitrification is very common in rhizobia species such as *Bradyrhizobium japonicum* and *Rhizobium fredii* (O'Hara and Daniel, 1985; Tiedje, 1988). In nitrifier denitrification, the oxidation of NH_3 to NO_2^- is followed by the reduction of NO_2^- to N_2O and/or N_2. However, the physiological role of nitrifier denitrification still remains unclear. Three hypothesis have been proposed (summarized in Hayatsu et al., 2008): (1) nitrifier denitrification may be a strategy to reduce competition for O_2 from NOB by removing their substrate NO_2^- (Poth and Focht, 1985), (2) AOB use NO_2^- as an electron acceptor to obtain energy for their growth in low-oxygen environments (Schmidt and Bock, 1997; Schmidt et al., 2004), (3) nitrifier denitrification is used to protect AOBs' own cells from toxic NO_2^- produced during NH3 oxidation (Beaumont et al., 2002; Cho et al., 2006). These hypotheses still need to be tested to estimate the contribution of nitrifier denitrification in different environments.

1.2.4.2 Fungal and archaeal denitrification

Denitrification is not only restricted to bacteria but can also be carried out by a range of fungi and archaea. The soil fungi *Fusarium oxysporum* and *Fusarium solani* reduced NO_2^- in growing cultures and simultaneously released N_2O under conditions of low O_2 concentrations (Bollag and Tung, 1972). Within several other phyla, including ascomycota such as *Cylindrocarpon tonkinese* and *Gibberella fujikuroii* and the basidiomycota *Trichosporon cutaneum*, denitrifying activity was found as well (Shoun et al., 1992). Continuing research showed that fungi also contribute significantly to the denitrification in soils, therefore also being potential contributors to N_2O emissions. An overview of the fungal denitrification system is given in Hayatsu et al. (2008).

Similarly, several archaea have been shown to reduce NO_3^- to N_2 through the dissimilatory nitrate reduction pathway (Cabello et al., 2004). Nevertheless, despite their potential capability to denitrify, the ecology and importance of denitrifying archaea still is poorly understood.

1.2.4.3 Chemodenitrification

Chemodenitrification occurs when NO_2^- in soil reacts to form N_2 or NO_x, which can occur by several aerobic pathways. In the Van Slyke reaction, amino groups in the α-position to carboxyls yield N2 (equation 10). In a similar reaction, NO_2^- reacts with NH_4^+, urea, methylamine, purines, and pyrimidines to yield N_2 (equation 11). Additionally, chemical decomposition of HNO_2 may also occur spontaneously (equation 12).

Equation 10: $RNH_2 + HNO_2 \longrightarrow ROH + H_2O + N_2$

Equation 11: $HNO_2 + NH_4^+ \longrightarrow N_2 + 2H_2O$

Equation 12: $3HNO_2 \longrightarrow HNO_3 + H_2O + 2NO$
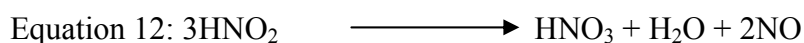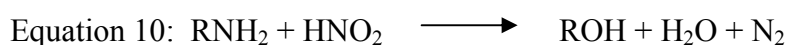
Chemodenitrification is generally thought to be of minor importance in most ecosystems, including agricultural lands (Bremner, 1997). However, it might be potentially significant in soils at pH<5 and in any soil under situations conducive to NO_2^- accumulation, e.g., in alkaline soils treated with acid hydrolysing fertilizers, in urine patches, and in unfrozen water of freezing soils (Chalk and Smith, 1983). Until now chemodenitrification still remained hard to study, and therefore its actual role and significance is still poorly understood.

1.2.4.4 Anammox

The conversion of NH_4^+ and NO_2^- to N_2, known as anaerobic ammonium oxidation (anammox), has been only recently discovered (Mulder et al., 1995). Therefore it is not surprising that its environmental significance is not fully known except in oceanic systems (Kuypers et al., 2003).

The reaction combines NH_4^+ and NO_2^- directly into N_2 gas under anoxic conditions (equation 13).

Equation 13: $NH_4^+ + NO_2^- \longrightarrow N_2 + 2H_2O$

Van de Graaf et al. (1995) used $^{15}NH_4^+$ and $^{14}NO_2^-$ and subsequently measured evolved $^{29}N_2$ to quantify the rate of anammox. Anammox bacteria grow very slowly in enrichment culture

and only under strict anaerobic conditions. It would therefore be likely that anammox is of significance only in periodically or permanently submerged soils. However, recent studies indicate that anammox bacteria are more widely distributed than previously assumed, and that they may play an important role in both artificial and natural environments (Toh et al., 2002; Meyer et al., 2005; Penton et al., 2006; Schubert et al., 2006; Schmid et al., 2007).

1.2.4.4 DNRA

Dissimilatory nitrate reduction to ammonia (DNRA) is an anaerobic process and can be considered as an alternative mechanism to denitrification. Several studies have studied the relative contribution of DNRA compared to denitrification during NO_3^- reduction in soils in relation to environmental conditions, particularly oxygen and carbon availability. Yin et al. (2002) demonstrated that the key influencing factor is available soil carbon. They found that in cultivated and normally aerated soils kept under anaerobic conditions, DNRA corresponds to only a few percent of denitrified N, while it can reach 15% of reduced NO_3^- in soils naturally rich in biodegradable organic carbon. Similarly, in another study rates of DNRA were found to increase after addition of labile carbon sources (Fazzolari et al., 1998).

Moreover, DNRA can be an important source of N_2O emissions (Tiedje, 1988; Fazzolari et al., 1990).

Dissimilatory nitrate reduction to ammonia (DNRA) is typical for the Enterobacteriaceae, but is also present in *Bacillus*, *Pseudomonas*, and *Neisseria* species (Tiedje, 1988). Enzymes which catalyze NO_3^- dissimilation are of two types: hexa-haem *c* type cytochromes located in the periplasm and cytoplasmic flavoproteins with sirohaem as the prosthetic group (Cole, 1990).

However, data on the ecology of DNRA microbes in soils are scarce, and it is often assumed that conditions in soil are more favourable for denitrification than DNRA (Tiedje, 1988).

1.3 Plant N acquisition

Plant N uptake is a key process in the global N cycle and also a topic that challenges the researcher with a number of problems not encountered in other areas of plant mineral nutrition research. The diversity of N forms present in the soil, their interconversions, their different chemical and physical characteristics and not the least the multitude of adaptations

and acclimatizations that plants display to optimize acquisition of various N forms all contribute to the complexity of plant N nutrition (reviewed by Näsholm et al., 2009). Recent research emphasized that plants can use a wide array of chemical N forms, ranging from simple inorganic N compounds to organic N forms such as aminoacids or even proteins (Paungfoo-Lonhienne et al., 2008). While in fertilized agricultural soils plant available N is present mainly in soluble inorganic forms (NO_3^-, NH_4^+, NO_2^-), the pool of soluble organic N in unfertilized agricultural soils can be as large as the mineral N pool and might be strongly involved in mineralization and immobilization processes (reviewed by Murphy et al., 2000). Nevertheless, unlike in several other ecosystems, the importance of organic N for plant nutrition seems to be negligible (<1%) in agricultural systems that receive high quantities of inorganic N fertilizer (Xu et al., 2008). Especially this strong abundance of inorganic N fertilizers used in agriculture as well as the importance of crop plant production resulted in a strong focus of research on the role of inorganic N forms for plant uptake, but nevertheless, there are still many open questions in this field.

1.3.1 Nutrient movement to the root

A fundamental requirement for studying plant N acquisition is the knowledge of some basic, mechanistic processes. Before plant roots can absorb any nutrients (including N) from the soil solution, it is necessary that the required nutrients first get in direct contact with living root cells. There are three major mechanisms responsible for the movement of nutrients through the soil to the root surface: diffusion, mass flow and root interception (Chapin III et al., 2002). Briefly, **diffusion** is the movement of molecules or ions along a concentration gradient. On the one hand, plants take up nutrients at the root surface and consequently deplete nutrient concentrations in the rhizosphere. On the other hand, in the bulk soil mineralization processes and other inputs lead to an increase of nutrients. Together, these processes provide the major driving forces for diffusion of nutrients to the root surface. Depending on the buffering capacity of the soil, around each absorbing root a cylinder of soil, a so-called diffusion shell, is created from which nutrients are absorbed. The size of this shell depends on the nutrient in question, again depending on the soil cation and anion exchange capacity. Generally, the anion exchange capacity in soils is much lower than the cation exchange capacity, so most anions, like NO_3^-, diffuse more rapidly in soils than do cations, like NH_4^+. Therefore, NO_3^- is typically depleted in a diffusion shell 6 to 10mm in radius around the absorbing root, whereas NH_4^+ is depleted over a radius of only 1 to 2mm (Chapin III et al., 2002).

As diffusion is the process that delivers most nutrients to plant roots, the major way in which a plant can enhance acquisition of slow-diffusing nutrients is to increase root density.

Mass flow simply is the transport of dissolved nutrients to the root surface in the soil water, with plant transpiration being the major driving force. Although mass flow can be of importance for acquiring micronutrients, it contributes only little to the necessary supply of macronutrients, such as N. Even in agricultural soils, with high macronutrient concentrations due to fertilizer inputs, mass flow supplies less than 10% of this growth-limiting nutrient for plants.

Root interception takes place when growing roots are exploring new soil areas, thereby gaining nutrients that were bound in this soil. However, like mass flow, root interception is not an important mechanism for plant nutrient acquisition, as generally the construction of new roots demands higher amounts of macronutrients than would become available from the new soil. Even so, root growth is important as it allows the plant to increase the root surface and consequently also the diffusion shell (see above).

1.3.2 Plant N uptake

As soon as soil nutrients come into contact with living root cells plants can take up these nutrients. In fertilized agricultural fields NO_3^- and NH_4^+ are the major sources of inorganic N taken up by the roots of crop plants. The assimilation of NH_4^+ or NO_3^- begins with its uptake from the soil solution by epidermal and cortical cells of the root. NO3- has to be reduced to NH4+ before it can be incorporated into amino acids via the pathway known as glutamine synthetase (GS)/ glutamate synthase (GOGAT) pathway. A brief overview of NH4+ and NO3- assimilation is given in figures 2 and 3, respectively.

Uptake of NH_4^+ and NO_3^- is mediated by a range of transporters and, for both ions, high- and low- affinity transport systems (also called HATS and LATS) have been identified (Siddiqi et al., 1990; Aslam et al., 1992; Williams and Miller, 2001; Miller and Cramer, 2004).

In the case of NO_3^-, HATS displays Michaelis-Menten kinetics, saturating at 0.2 to 0.5 mM NO_3^- with a K_m typically between 10 and 100 µM. HATS is further divided into a constitutive component, which is expressed in the absence of NO_3^-, and an inducible component, which is induced by high concentrations of NO_3^-. The activity of the low-affinity transport system becomes evident at NO_3^- concentrations above 0.5 mM, a concentration exceeded at least temporarily in virtually all cropping systems, and usually displays non-saturating uptake kinetics.
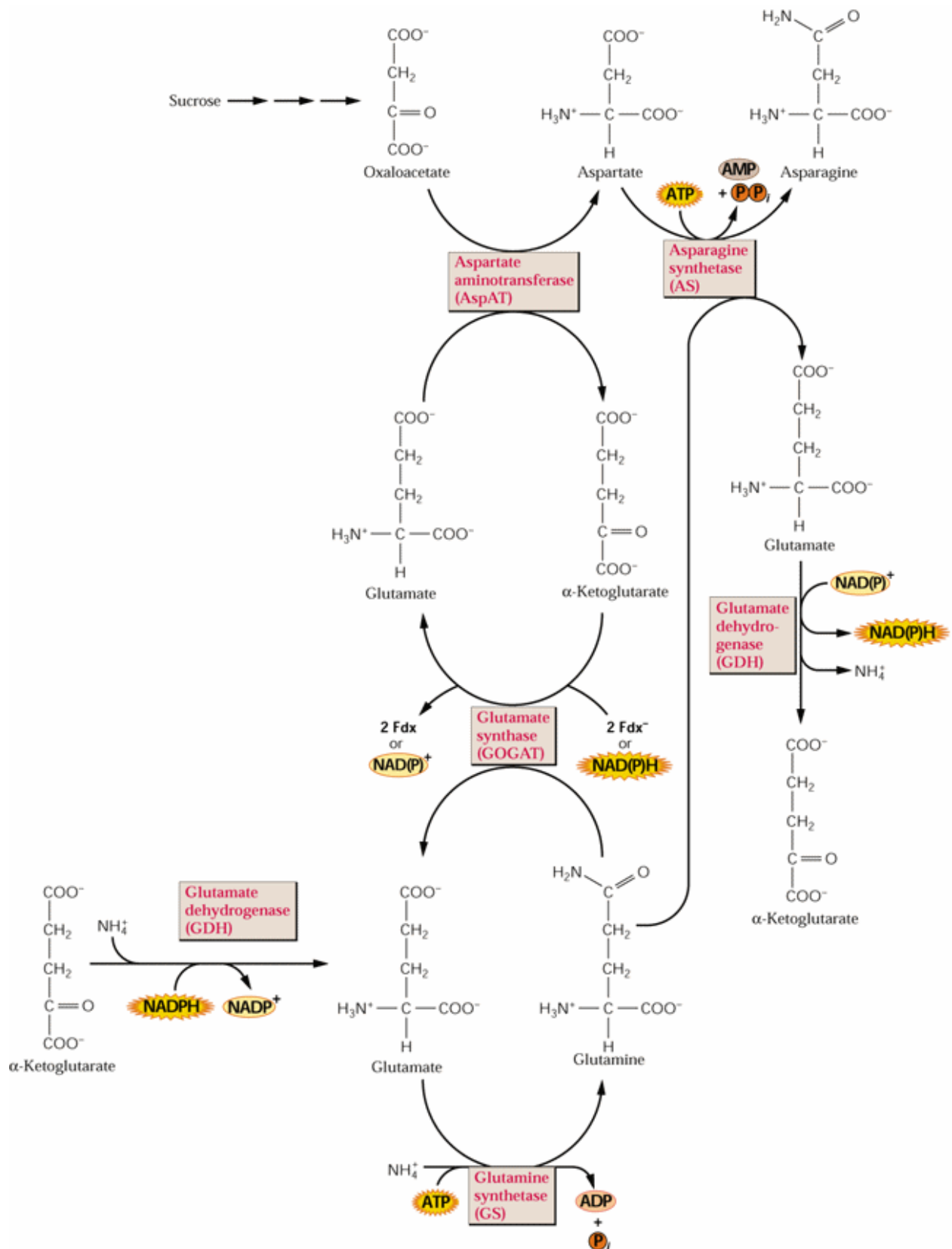
Figure 2 An overview of the enzymes that participate in ammonium assimilation into glutamate, glutamine, aspartate, and asparagines, the N-transport amino acids in plants. *Fdx*, ferredoxin (Coruzzi and Last, 2000)

In combination, these two transport systems allow plants to accommodate a wide range of external NO_3^- concentrations (5 µM to 50 mM) without experiencing severe deficiency or toxicity. So far, two NO_3^- transporter gene families, *NRT1* and *NRT2*, have been discovered. Briefly, the *NRT1* family is quite complex encoding NO_3^- transporters of both, LATS and HATS. The *NRT2* genes encode NO_3^- transporters of HATS and are inducible by NO_3^- and are down-regulated by several forms of reduced N, including NH_4^+ and glutamine (Siddiqi et al., 1989). This regulation allows plants to adjust the concentrations of this important class of transporters in response to the form of N in the soil solution and the N needs of the plant.
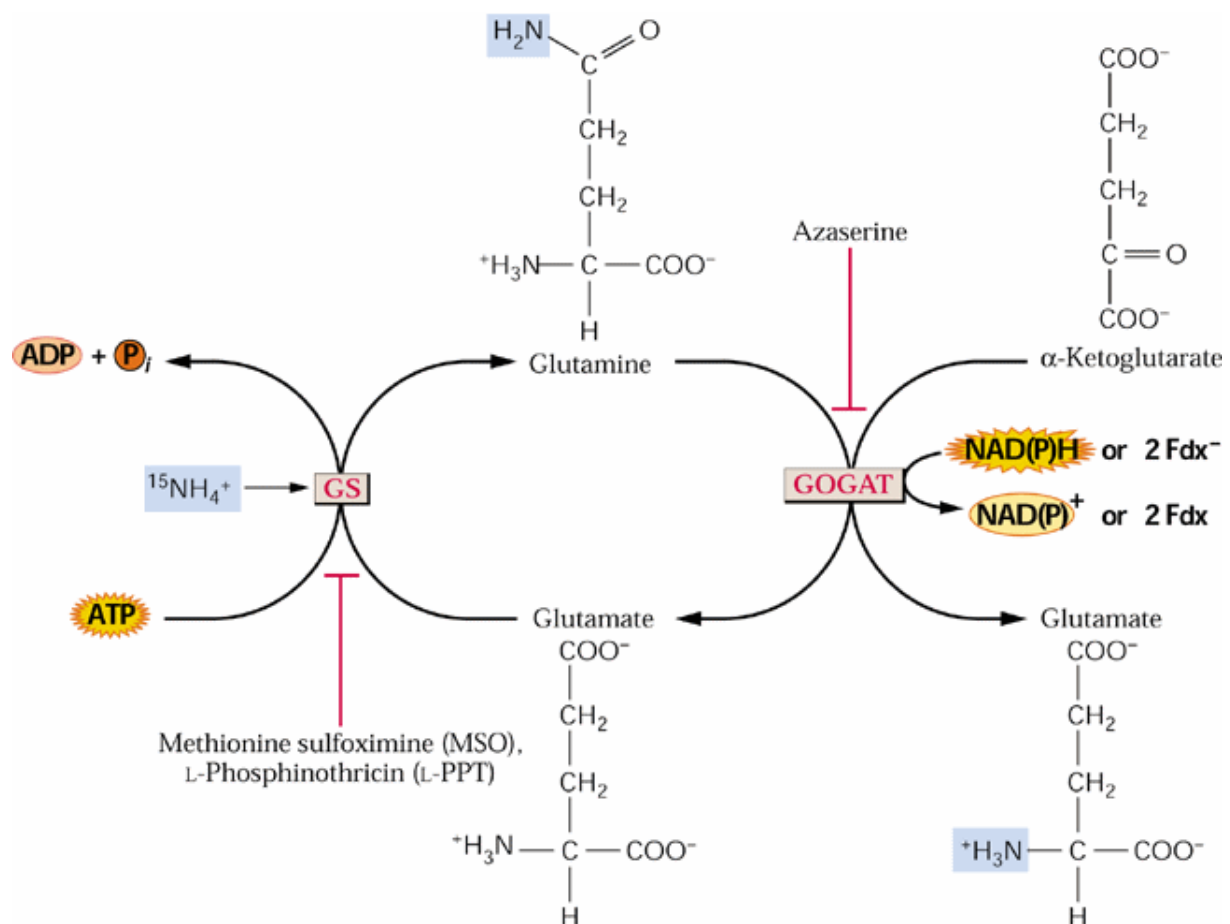
Figure 3 The glutamine synthetase-glutamate synthase (GS/GOGAT) pathway is thought to be the principal mechanism of primary and secondary ammonium assimilation. Sites of action are shown for several enzyme inhibitors. *Fdx*, ferredoxin (Coruzzi and Last, 2000)

Once within the symplasm, NO_3^- can be transported into the vacuole where temporal storage of high concentrations (more than 20 mM) is possible, or it can be loaded into the xylem for long-distance transport to the shoot and subsequent reduction. Diverse physiological, genetic,

and environmental factors determine how NO_3^- is allocated and stored throughout the plant. In general, as the external NO_3^- concentration increases, the proportion that is transported to the shoot for reduction increases. NO_3^- may also be immediately reduced and further converted into amino acids in the root, although this requires more energy and higher rates of root respiration (Bloom et al., 1992).

NO_3^- itself can not be incorporated into organic compounds directly, but first has to be reduced to NH_4^+ in a two-step process. Plants reduce NO_3^- and NO_2^- in both root and shoot tissues, with the oxidation state of N dropping from +5 to -3 during this eight-electron transfer. Significant quantities of N are assimilated through this pathway, although it consumes large amounts of energy, carbon and protons.

The first step, the reduction of NO_3^- to NO_2^- (equation 14), is catalyzed by the enzyme nitrate reductase (NR, Figure 4). The intracellular location of NR has been the subject of intense study and considerable discussion. The majority of investigations have demonstrated that the enzyme is present in the cytoplasm, while studies that indicated that the enzyme is loosely bound to the plasma or chloroplast membrane, have in general, been discounted (Askerlund et al., 1991).
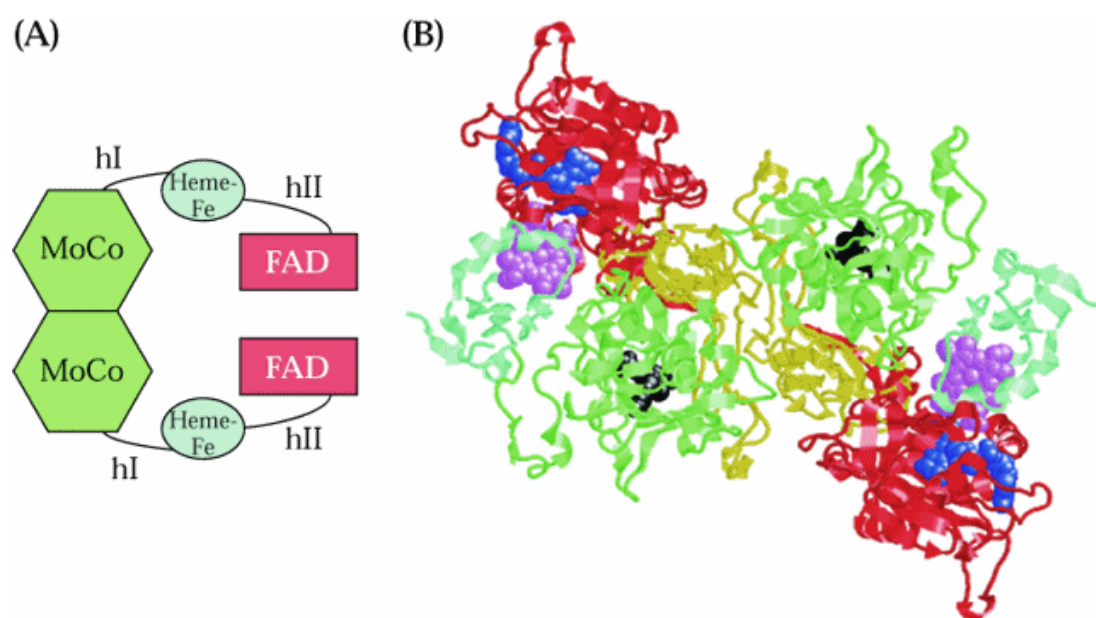
Figure 4 Domain structure (A) and ribbon diagram (B) of nitrite reductase. *MoCo*, molybdenum cofactor; *FAD*, flavin adenine dinucleotide; *hI* and *hII* refer to hinge I and hinge II, which separate the functional domains. In (B) the haem prosthetic group is shown in purple, FAD in blue, and MoCo in black. The interface between the two monomers is shown in yellow (Crawford et al., 2000).

The most common form of NR uses only NADH as an electron donor, while another form of the enzyme that is found predominantly in non-green tissues, such as roots, can use either NADH of NADPH (Warner and Kleinhofs, 1992).

Equation 14: $NO_3^- + NAD(P)H + H^+ + 2e^- \longrightarrow NO_2^- + NAD(P)^+ + H_2O$
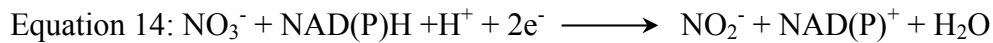
Briefly, the NR of higher plants is composed of two identical subunits, each containing three prosthetic groups: flavin adenine dinucleotide (FAD), haem, and a molybdenum cofactor (Campbell, 1999). These three cofactors provide the redox centers that facilitate the chain of electron transfers in the reaction.

The regulation of NR plays a key role in NO_3^- assimilation. It has been known for nearly 50 years that NR activity is induced by the presence of NO_3^- and light (Hageman and Flesher, 1960). Additionally, the concentration and activity of NR is regulated in response to N metabolites (especially glutamine), CO_2, carbon metabolites (especially sucrose), and cytokinins (e.g., Sivasankar and Oaks, 1996). With molecular biological techniques, it is possible to follow the induction of NR activity at the gene level. Thereby it was found that NO_3^- leads to an increase in the steady-state level of mRNA encoding NR in a variety of plant species and tissues (Crawford et al., 1986; Cheng et al., 1991). This in turn, results in NR protein synthesis and increased activity, shortly after NO_3^- supply to the roots and after a lag in the leaves. In barley seedlings, for example, NR mRNA was detected approximately 40 minutes after addition of NO_3^-, and maximum levels were attained within 3 hours (Kleinhofs et al., 1989). In contrast to the rapid mRNA accumulation, there was a gradual linear increase in NR activity, reflecting the slower synthesis of the protein. In addition, the protein is subject to posttranslational modification (involving a reversible phosphorylation). Light, carbohydrate levels, and other environmental factors stimulate a protein phosphatase that dephosphorylates a key serine residue in the hinge 1 region of NR (between the molybdenum complex and haem-binding domains) and thereby activates the enzyme.

Operating in the reverse direction, darkness and Mg^{2+} stimulate a protein kinase that phosphorylates the same serine residues, which then interact with a 14-3-3 inhibitor protein, and thereby inactivate NR (Kaiser et al., 1999).

The advantage of this system is that the regulation of NR activity through phosphorylation and dephosphorylation provides more rapid control than can be achieved through synthesis or degradation of the enzyme. For example, this might be the case when plants are exposed to darkness or low concentrations of CO_2 leading to NR inhibition within minutes.

Therefore, by regulating NR transcription (within hours to days) and activity (minutes to hours), plants are able to fine-tune the amount of NO_3^- reduction depending on temporal requirements.

After NO_3^- reduction, the next step in the NO_3^- assimilation pathway is the reduction of NO_2^- to NH_4^+, which is catalyzed by nitrite reductase (NiR, Figure 5). NO_2^- is a highly reactive, potentially toxic ion and therefore plant cells immediately have to transport the NO_2^- generated by NO_3^- reduction from the cytosol into chloroplasts in leaves and plastids in roots. In these organelles, NiR reduces NO_2^- to NH_4^+ transferring six electrons reduced ferredoxin (Fd_{red}) produced in chloroplasts by photosynthetic non-cyclic electron transfer acting as electron donor (equation 15).

Equation 15: $NO_2^- + 6Fd_{red} + 8H^+ + 6e^- \longrightarrow NH_4^+ + 6Fd_{ox} + 2H_2O$
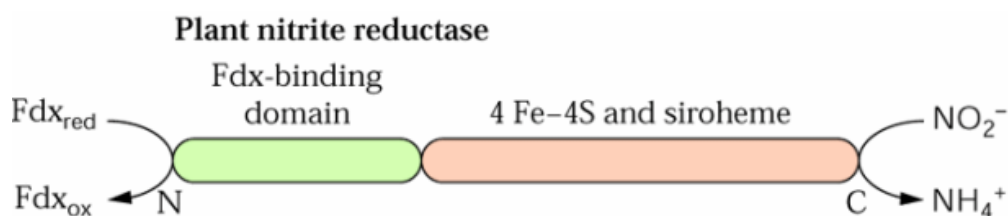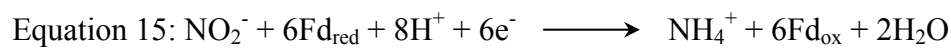
Figure 5 Structure of nitrite reductase from plants. The N-terminal region oxidizes ferredoxin. The C-terminal region, which binds a 4Fe-4S centre and a sirohaem group, reduces nitrite to ammonium (Crawford et al., 2000).

Chloroplasts and root plastids contain different forms of the enzyme, but both forms consist of a single poly-peptide containing two prosthetic groups: an iron-sulfur cluster (Fe_4S_4) and a specialized haem (Siegel and Wilkerson, 1989). These groups act together to bind NO_2^- and reduce it to NH_4^+.

NiR is regulated transcriptionally, usually in coordination with NR, because cells must contain enough NiR to reduce all the NO_2^- produced by NR. Thus, plants maintain an excess of NiR activity whenever NR is present by inducing NiR gene expression in response to elevated concentrations of NO_3^- and exposure to light. Accumulation of the end products in the process – asparagine and glutamine – repress this induction. The toxic nature of NO_2^-, which has a deleterious effect on metabolism, is stressed by the fact that barley mutants lacking NiR activity in the leaves and roots can only be maintained by growth on NH_4^+ and glutamine but not on NO_3^-.

In the case of NH4+ assimilation, physiological studies have revealed multiphasic NH_4^+ uptake in diverse plant species, implying multiple transport systems, with K_m values for the transporters ranging from 10 to 70 µM (e.g., Glass et al., 2002; Orsel et al., 2006). NH_4^+, either coming from NO_3^-/NO_2^- reduction or supplied directly as fertilizer, is toxic to plants if it accumulates to high levels in plant tissues as it dissipates transmembrane proton gradients that are required for both photosynthetic and respiratory electron transport and for sequestrating metabolites in the vacuole. Plants avoid NH_4^+ toxicity by rapidly converting the NH_4^+ generated from NO_3^- assimilation or photorespiration into amino acids near the site of absorption or generation. The primary pathway of this conversion involves the sequential actions of glutamine synthetase (GS) and glutamate synthase, also called glutamine-2-oxoglutarate aminotransferase (GOGAT) (Lea et al. 1992).

GS catalyzes the ATP-dependent assimilation of NH_4^+ into glutamine, using glutamate as a substrate (equation 16).

Equation 16: glutamate + NH_4^+ + ATP $\longrightarrow$ glutamine + ADP + Pi

This reaction requires the hydrolysis of one ATP and involves a divalent cation as co-factor (e.g., Mg^{2+}, Mn^{2+}, or Co^{2+}). Two classes of GS can be found in plants either in the cytosol or in root plastids or shoot chloroplasts. Cytosolic GS produce glutamine for intracellular N transport while root plastid GS generates amide N for local consumption. The GS in shoot chloroplasts, on the other hand, is responsible for re-assimilating NH_3 produced during photorespiration and is therefore essential for plant survival (Lam et al., 1996). GS has a very high affinity for NH_4^+ and therefore can operate at the low NH_4^+ concentrations present in living cells (K_m 3 to 5 µM). Labelling studies that trace the fate of $^{15}NH_4^+$ confirm that the label is incorporated primarily into the amide group of glutamine, subsequently appearing in the amino groups of glutamate and other amino compounds, including glutamine.

GS functions in a cycle with GOGAT, which catalyzes the reductive transfer of the amide group from glutamine to α-ketoglutarate, yielding two molecules of glutamate.

Plants contain two types of GOGAT, with one accepts electrons from NADH and the other from reduced ferredoxin (equations 17 and 18).

Equation 17: glutamine + 2-oxoglutarate + NADH + H^+ $\longrightarrow$ 2 glutamate + NAD^+

Equation 18: glutamine + 2-oxoglutarate + Fd_{red} + H^+ $\longrightarrow$ 2 glutamate + Fd_{ox}
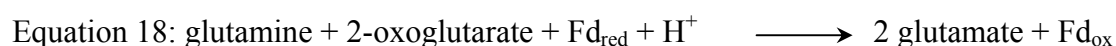
NADH-GOGAT is located in the plastids of non-photosynthetic tissues such as roots or vascular bundles of developing leaves. In roots, NADH-GOGAT is involved in the assimilation of NH_4^+ absorbed from the rhizosphere, while in vascular bundles of developing leaves, NADH-GOGAT assimilates glutamine translocated from roots or senescing leaves. Fd-GOGAT is found in chloroplasts and, together with chloroplast GS, serves in photorespiratory N metabolism. Roots also have Fd-GOGAT in plastids, particularly those under NO_3^- nutrition, where it presumably functions to incorporate the glutamine generated during NO_3^- assimilation.

However, NH_4^+ can be assimilated via an alternative pathway: Glutamate dehydrogenase (GDH) catalyzes a reversible reaction that synthesizes or deaminates glutamate (equation 19).

Equation 19: 2-oxoglutarate + NH_4^+ + NAD(P)H $\longleftrightarrow$ glutamate + H_2O + NAD(P)$^+$

The NADH-dependent form of GDH is found in mitochondria, while the NADPH dependent form is localized in the chloroplasts of photosynthetic organs. Although both forms are relatively abundant, they cannot substitute for the GS-GOGAT pathway of assimilation of NH_4^+. Evidence suggests that GDH plays primarily a catabolic role but may assimilate NH_4^+ when its supply is plentiful (Figure 6).
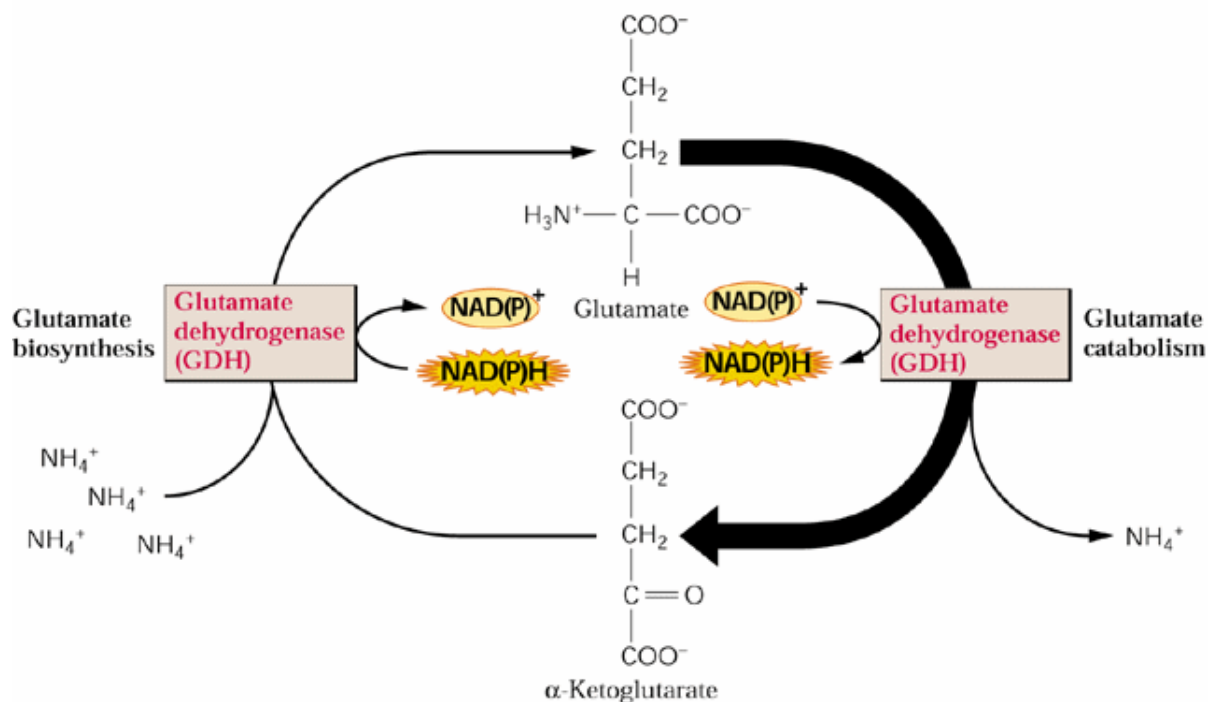
Figure 6 Glutamate dehydrogenase is thought to function primarily in glutamate catabolism (deamination) but can also assimilate inorganic nitrogen into glutamate when ammonium concentrations are high. (Coruzzi and Last, 2000)

Whether NH_4^+ or NO_3^- as sole source of N supply is better for plant growth and yield formation depends on many factors (Kirkby, 1981). Compared with NH_4^+, NO_3^- has the advantage of also being a storage form in plants with no necessity to be assimilated in the roots. In addition, NO_3^- nutrition induces an increase rather than a decrease in rhizosphere pH. This is the result of approximately equimolar production of OH^- or consumption of protons correlated with NO_3^- assimilation (Raven, 1986). Assimilation of NH_4^+, on the other hand, produces protons in about equimolar ratio. As NH_4^+ assimilation takes place in the roots the protons are excreted into the external solution (Marschner et al., 1991). As a consequence the pH in the external solution is strongly affected whether N is supplied as NH_4^+ or NO_3^-. Further, when both NH_4^+ and NO_3^- are supplied, soil pH may not change strongly due to similar rates of proton production during NH_4^+ assimilation and proton consumption during NO_3^- assimilation (Raven, 1985; Allen et al., 1988).

On the other hand, reduction and assimilation of NO_3^- have a high energy requirement and are costly processes when carried out in roots. When expressed in ATP equivalents, this requirement represents 15 mol ATP for the reduction of one mole of NO_3^- and an additional 5 mol ATP for NH_4^+ assimilation (Salsac et al., 1987). In barley, where a high proportion of NO_3^- reduction occurs in the roots, up to 23% of the energy from root transpiration is required for absorption (5%), reduction (15%) and assimilation of the reduced N (3%), as compared with only 14% for assimilation when NH_4^+ is supplied (Bloom et al., 1992). Therefore, from an energetic point of view, it is more beneficial for the plant to take up N in the form of NH_4^+ rather than NO_3^-.

Further, the presence of NH_4^+ inhibits NO_3^- uptake (Gessler et al., 1998; Gazzarrini et al., 1999; Siddiqi et al., 2002), although the mechanism underlying the immediate inhibition of NO_3^- uptake still remains unclear. As mentioned above, the products of NH_4^+ assimilation within the GS/GOGAT cycle (mainly glutamine) may have a role as feedback inhibitors of NO_3^- uptake.

Accordingly, in many cultivated soils it has been observed that in plants and microbes NH_4^+ assimilation exceeds NO_3^- assimilation (e.g. Azam et al., 1993). However, other studies showed that NH_4^+ utilization by plants was lower or similar to NO_3^- utilization (Burger and Jackson, 2003; Song et al., 2007), presumably due to higher mobility of NO_3^- in soils as compared to NH_4^+ (Hodge et al., 2000). These opposing results as well as the importance of controls of N uptake mechanisms in agriculture, indicate the there is still need of elucidating the remaining uncertainties in this field.

1.4 Competition for nitrogen between soil microbes and plants

Many ecologists have hypothesized that plants and heterotrophic microbes do not compete for soil resources because heterotrophic microbe are C limited, while temperate plants are known to be N limited (Vitousek and Howarth, 1991). Others have assumed that heterotrophic microbes are superior competitors for soil inorganic N than plants (Nadelhoffer et al., 1985; Hart and Firestone, 1991; Johnson, 1992). Based on this view, plants assimilate N that is "left over" after heterotrophic microbes are no longer N limited. Rather than competing with plants for N, heterotrophic microbes mediate plant N uptake by controlling available N. This theory is bolstered by several short-term ^{15}N experiments, which showed that inorganic N uptake by heterotrophic mircoorganisms was unaffected by plants (Zak et al., 1990; Norton and Firestone, 1996).

However, an increasing number of experiments suggest that plants and soil microbes are both limited by inorganic N, even on relatively fertile sites and also in agriculture (Schimel et al., 2004). Therefore, soil microorgansisms are influencing the rate of plant N uptake not only indirectly by continuously transforming plant available soil N pools, but also directly by competing for fertilizer N with plants. Evidence for plant-microbe competition for inorganic N again comes from long- and short-term ^{15}N experiments. In short-term ^{15}N experiments, the soil inorganic N pool is enriched with [15]NH_4^+ or [15]NO_3^-, and the label is measured in plant, microbial and inorganic N pools after a few days. Long-term experiments use the same tracer techniques, but the fate of the ^{15}N is determined after weeks or months (Kaye and Hart, 1997). Therefore it depends on the aim of a study which approach is better suitable. Long-term ^{15}N tracer studies are able to determine the fate of fertilizer N during one or more growing seasons, or with regard to agriculture, to determine how much of the added fertilizer N is actually recovered in harvested grains. With this approach it is possible to see the net outcome of overall competition effects, but to directly assess plant-microbe competition, which takes place at much shorter time scales (minutes to days), short-term ^{15}N tracer studies proved to be the best way.

Competition and other interactions between plants and soil microbes are rather complex. E.g., plants may compete for both, NH_4^+ and NO_3^- with heterotrophic microorgansisms, and at the same time for NH_4^+ with autotrophic nitrifiers. On the other hand, autotrophic nitrifiers also produce highly mobile NO_3^- which may be more accessible to plants (Schimel et al., 1989). As nitrification rates in agricultural soils were often found to be high, it is difficult to distinguish between the sources of N (NH_4^+ or NO_3^-) for plant N uptake. Therefore, there are

still inconsistencies regarding the preferred N source for uptake by soil microbes and plants. While several studies found that soil microbes preferentially use NH_4^+ over NO_3^- in many cultivated soils (e.g. Recous et al., 1990; Azam et al., 1993; Paul et al., 1996), other studies found no difference between NH_4^+ and NO_3^- assimilation (Harrison et al., 2007; Song et al., 2007; Harrison et al., 2008) or even preferential uptake of NO_3^- (Burger and Jackson, 2003). Comparisons of rates of root uptake of NH_4^+ and NO_3^- have been made for several species in different ecosystems and the general conclusion from these comparisons is that NH_4^+ is absorbed at higher rates compared to aminoacids and NO_3^- (e.g., Falkengren-Grerup et al., 2000; Öhlund and Näsholm, 2001; Thornton and Robinson, 2005). Further, the presence of NH_4^+ often inhibits uptake of NO_3^- (Gessler et al., 1998; Gazzarrini et al., 1999; Siddiqi et al., 2002), indicating the importance of abundance and availability of inorganic soil N pools. In agriculture soils NH_4^+ pools are often very low, whereas NO_3^- is the predominant N form (Schimel et al., 2004). Therefore, crop plants might rely on NO_3^- rather than NH_4^+ to meet growth demands.

Despite these different findings in uptake preferences, results from plant-microbe competition studies are often inconsistent as well. With the help of ^{15}N tracer techniques it is possible to follow the fate of inorganic N fertilizer into plants, soil microbial biomass, soil N pools, and N lost by leaching or gas emission. Two short-term experiments (Jackson et al., 1989; Schimel et al., 1989) in the laboratory and in the field found that recovery of added $^{15}NH_4^+$ and $^{15}NO_3^-$ 24h after N application was greater in the microbial biomass (46-61% and 37-50%, respectively) than in plants (9-11% and 17-26%, respectively). The reason for higher ^{15}N uptake by microbes compared to plants may at least partly be explained by higher substrate affinities, larger surface area to volume ratios, and faster growth rates of microorgansims compared to plant roots (Hodge, 2004; Schimel et al., 2004). Several other short-term ^{15}N tracer studies (up to several days) investigating grassland ecosystems found similar results, with microbes assimilating more ^{15}N-labelled inorganic N than plants. However, after an initially rapid N capture, microbial biomass appeared to reach a steady state, probably because of insufficient available C to maintain the fast initial growth rates (Hodge et al., 2000; Harrison et al., 2008). The same studies showed that during the following period, microbial ^{15}N was gradually released by microbial decay and re-mineralization into the soil, eventually becoming available for plant root uptake. After longer time periods (weeks to months), plants contained an increasing proportion of the added ^{15}N, showing that plants indeed utilized this N resource (Harrison et al., 2007). In other words, the turnover rate of

plant biomass was much slower than that of microbes, which allowed plants to compete for the same N for extended periods, therefore enhancing plant competitiveness for N.

1.5 The role of nitrogen in agriculture

Nitrogen (N) is a fundamental component of living organisms, but it is often in short supply in forms that can be assimilated by plants. As a result, N has a critical role in controlling primary production in the biosphere. N is therefore also a limiting factor for crop plants grown by humans in agricultural systems (Gruber and Galloway, 2008). To sustain the increase of the global population, food production increased enormously over the past century. This would not have been possible without the production and application of nitrogenous fertilizers. Over the past four decades, the doubling of agricultural food production worldwide has been associated with a 7- fold increase in the use of N fertilizers (Hirel et al., 2007), indicating the magnitude and importance of synthetic fertilizers. Without the production of synthetic fertilizers, world food production could not have increased at the rate it did and more natural ecosystems would have been converted to agricultural systems already (Tilman et al., 2002). Intensive agricultural plant production still largely depends on the input of mineral fertilizers, mainly provided in the form of urea, NH_4^+ or NO_3^-.

Fritz Haber and Carl Bosch developed the first high pressure industrial process, which uses energy from fossil fuels to convert N_2 to ammonia gas (NH_3) using H_2 gas produced from CH_4 (equation 20 and 21) on a large scale in the Haber-Bosch process (Erisman et al., 2007; Erisman et al., 2008).

Equation 20: $CH_4 + H_2O \longrightarrow CO + 3H_2$

Equation 21: $N_2 + 3H_2 \longleftrightarrow 2NH_3$

Shortly after the invention and implementation of the Haber-Bosch process the production of NH_3 increased rapidly (Smil, 2001). This process now fixes more N than any other anthropogenic process. Industrial fixation of N by this process increased substantially in the 1940s, reaching 30 teragrams (Tg) per year by 1970, 80 Tg per year by 2000 and it is projected to increase to 120 Tg per year by 2025 (Galloway et al., 1995). If the amount of N, which is emitted to the atmosphere during fossil-fuel combustion, is added to the N industrially fixed, these two sources of anthropogenic N to the environment amounted to more than 160 Tg N per year in the 1990s. On a global basis, this is more than that supplied by

natural biological N fixation on land (110 Tg N per year) or in the ocean (140 Tg N per year) (Gruber et al., 2008). Overall, 80% of the chemical production of NH_3 is used to produce N fertilizer in various forms, e.g., ammonium nitrate, urea, calcium nitrate, ammonium bicarbonate and several varieties of NPK (mixtures of nitrogen, potassium, and phosphorus). Fertilizer N production is still accelerating, a trend unlikely to change in the near future.

 A major drawback of fertilization in agriculture is that only 30-50% of the applied N fertilizer is taken up by the crop plants (Smil, 1999; Cassman et al., 2002) while a significant amount of the applied N is lost from the agricultural fields. The negative consequences of these N losses are substantial and manifold, affecting climate, the chemistry of the atmosphere, and the composition and function of terrestrial and aquatic ecosystems (Vitousek et al., 1997). Moreover, because a single molecule of reactive N can "cascade" through the environment, it can contribute to more than one of these environmental responses (Galloway et al., 2003). For example, an emitted molecule of nitrous oxide can first cause photochemical smog and then, after it has been oxidized in the atmosphere to nitric acid and deposited on the ground, can lead to ecosystem acidification and eutrophication (Gruber et al., 2008). Additionally, we know that environmental changes caused by excess fertilizer N can feed back to affect human health and welfare, both directly, for example through increased production of atmospheric particulate matter, and indirectly through impacts on food production (Townsend et al., 2003). Thus, a major challenge is how to maximize the benefits of fertilizer N application by increasing food production while minimizing its unwanted consequences. In the last decade a wealth of studies has therefore addressed the consequences of intensive N fertilization, ranging from field studies (e.g., Chu et al., 2007a) to laboratory experiments (e.g., Well et al., 2006). The overall aim of these studies was to find ways to improve fertilizer use efficiency, largely in cereal grain production, and/or on reducing adverse effects, namely losses of fertilizer N to the environment (Tilman et al., 2002; Mosier et al., 2004). Other studies have focused on fates of N inputs during one or more growing seasons, or on predicting N availability to crops (Mosier et al., 2004; Jackson et al., 2008). Plant N availability proved to be hard to predict, as the inorganic N pools in agricultural soils are highly dynamic (e.g., Jackson et al., 1989). Responsible for the high turnover rates of plant available N pools in these soils are high N transformation rates, mediated by soil microbes. Some of these processes can further lead to increased N losses to the environment, e.g., nitrification can rapidly transform the relatively immobile soil NH_4^+ pool to highly mobile NO_3^-, which might result in increased NO_3^- leaching. High rates of nitrification and denitrification can also significantly increase gaseous losses of fertilizer N to the atmosphere.

However, the magnitude of these soil processes is dependent on a huge variety of influencing factors (see previous chapters).

The diversity and variability of N transformations as well as plant-microbe competition effects indicate the complexity of the N cycle in soils, and make it difficult to investigate the underlying mechanisms and to elucidate the fate of fertilizer N.

1.6 Greenhouse gas emissions from agricultural soils

Dealing with global climate change and therefore reduction of global greenhouse gas emissions is undoubtly one of the most challenging but also one of the most pressing aims in current ecology research. Global atmospheric concentrations of carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) have increased markedly as a result of human activities since 1750 and now far exceed pre-industrial values determined from ice cores spanning many thousands of years. The global increases in CO_2 concentration are primarily due to fossil fuel use and land-use change, while those of CH_4 and N_2O are primarily due to agriculture (IPCC, 2007).

N_2O has been shown to contribute to global warming accounting for approximately 6% of the total greenhouse effect (IPCC, 2001) and is also involved in the destruction of stratospheric ozone (Cicerone, 1987).The global atmospheric N_2O concentration increased from a pre-industrial value of about 270 pb to 319 ppb in 2005 and is currently increasing at a rate of 2% per year (IPCC, 2007). Soils have been identified to be the dominant source of N_2O, contributing about 57% (9 Tg per year) of the total annual global emissions, of which about 27% (2.4 Tg per year) originates from agricultural soils (IPCC, 2001).

Still, its atmospheric abundance is increasing unabated due to intensification of agricultural activities in recent years, but the estimates of both source and sink strengths of N_2O are still highly uncertain (Verma et al., 2006).

Agriculture and related activities account for about two-thirds of all anthropogenic CH_4 emissions ((Duxbury et al., 1993; IPCC, 2007). In addition, agricultural practices may influence atmospheric concentration of CH_4 by strongly affecting CH_4 consumption rates in aerated soils. This biological CH_4 oxidation in aerobic soils is estimated to comprise 3–9% of the global atmospheric CH_4 sink (Prather et al., 1995).

 In recent years the contribution of agricultural soils to the global warming potential has received increasing attention. Advances in methodology and improvement of technical equipment allowed estimating greenhouse gas emissions and budgets from a magnitude of

ecosystems around the world. The impact of a variety of influencing parameters on greenhouse gas emissions have been studied, e.g., soil temperature, soil moisture and soil pH (Conrad et al., 1983; Smith et al., 1998; Papen and Butterbach-Bahl, 1999; Skiba and Smith, 2000; Wolf and Russow, 2000; Pilegaard et al., 2001; Dobbie et al., 2003; Pilegaard et al., 2006; Dannenmann et al., 2008), spatial variability in relation to microsites (Parkin, 1987; Gupta and Germida, 1988; Seech and Beauchamp, 1988; Beauchamp and Seech, 1990; Drury et al., 2004; Key et al., 2008) available carbon (Seto and Yanagiya, 1983; Myrold and Tiedje, 1985), or effects of the rhizosphere (Raich and Tufekcioglu, 2000; Chu et al., 2007c; Philippot et al., 2008).

Undoubtly, a major research focus was related to fertilization: Studies reached from investigating the impact of the form of fertilizer (e.g., solid vs. fluid) as well as the form and quantity of N (e.g., Steudler et al., 1989; Bouwman, 1990; Granli and Bockman, 1994; Matson et al., 1998; Mosier, 1998; Hall et al., 1999; Hu et al., 2002; Chu et al., 2004; Verma et al., 2006; Jones et al., 2007) to application and managing techniques (e.g., Mosier et al., 1997; Hosen et al., 2000; Hütsch, 2001; Korsaeth et al., 2002; Chu et al., 2007c; Abdalla et al., 2009). More and more studies are also trying to assess the net global warming potential of diverse ecosystems using an integrated approach, taking into account as many sources and sinks of major greenhouse gases as possible (e.g., Robertson et al., 2000; Smith and Dobbie, 2001; Flessa et al., 2002; Maljanen et al., 2004; Levy et al., 2007; Soussana et al., 2007; Key et al., 2008).

As expected, with every month our knowledge about responsible mechanisms and controlling parameters is increasing, but unfortunately, with many answers a lot of new questions arise. When following the increasing number of available literature, it is difficult to keep a sensible overview about this topic. However, additionally to several reviews (e.g., Conrad, 1996; Le Mer and Rogert, 2001), the IPCC (2007) report extensively summarizes current knowledge and comprehensively covers options for mitigation of N_2O and CH_4 in addition to CO_2 from agricultural systems.

1.7 Economical considerations

N fertilization is not only of importance in an ecological sense, but clearly also from an economical point of view. Because of the high-energy requirements of the Haber-Bosch process, the synthetic fertilizer price is strongly coupled to the natural gas and thus the oil price. Costs for these fossil fuels required for production of N fertilizers are rising, and

consequently farmers are facing increasing economic pressures (Hirel et al., 2007). It is therefore not surprising that often economics, more than any other factor, including the preservation of the environment, controls the form of N fertilizer selected. In general, fertilizers with higher N contents have lower costs of storage, transportation, handling, and application, and hence are more economic (Pierzynski et al., 2004). However, the properties of some high-N fertilizers can increase these costs, by time consuming application procedures or higher N losses (e.g., via NH_3 volatilization). Other factors that influence the selection of an N fertilizer include crop management practices, soil type and properties, the need to supply other nutrients simultaneously, the suitability of the material for existing application equipment and the proximity of fertilizer manufacturing and transportation infrastructure (Pierzynski et al., 2004). It is obvious that farmers will rather choose the cheapest and most convenient fertilizer option, even if this choice leads to higher environmental damages. Consequently, economical and ecological criteria for optimal N fertilizer are inherently in conflict. Often a significant proportion of applied fertilizer N is lost due to a lack of precise predictions of N availability for plants as a function of agronomic practice and weather conditions. If the synchronisation of fertilizer application with plant N demand is not achieved, this would therefore result in additional economical losses (Korsaeth et al., 2002).

The important question we are facing now is how such costs in agriculture can be minimized at the same time that food production is increased. In theory, the answer would be that crop and livestock production must increase without an increase in the negative environmental impacts associated with agriculture. However, together with optimum choices of crop plants and management techniques, this would only be possible by largely increasing nitrogen and water use efficiencies of crop plants as well as an integrated pest management that minimizes the need for toxic pesticides. In reality, achieving such a scenario represents one of the greatest scientific challenges facing humankind because of the trade-offs among competing economic and environmental goals, and inadequate knowledge of the key biological, biogeochemical and ecological processes (Tilman et al., 2002).

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2. Objectives

Intensive agricultural crop production in central Europe largely depends on the input of nitrogen (N) fertilizers, mainly provided in the form of ammonium (NH_4^+) or nitrate (NO_3^-) or a combination thereof. However, only ~50% of the applied fertilizer N is used by the crop, while the rest is lost to the environment leading to a variety of adverse effects. Present research is therefore aiming at improving fertilizer use efficiency of crop plants and reducing losses of fertilizer N to the environment. To accomplish these goals it is crucial to understand and investigate the key N transformation processes in fertilized agricultural soils. A simplified overview of the major N pools and N transformations as well as the influence of inorganic N fertilizer input in agricultural soils is given in Figure 7.
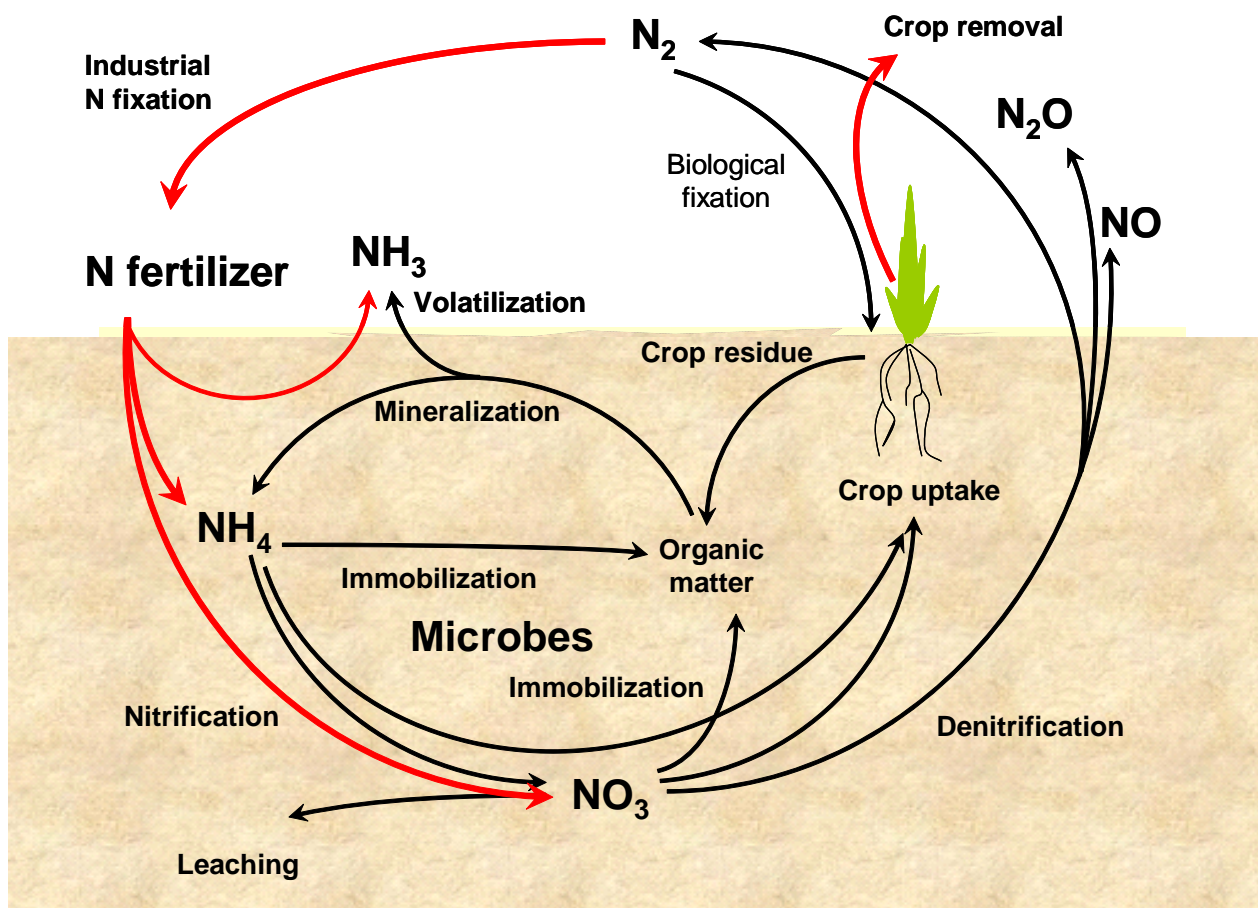
Figure 7 Major N pools, N transformations and pathways of N losses in agricultural soils. Both, N pools and transformation processes are strongly influenced by N fertilizers provided as NH_4^+ and/or NO_3^-.

As these processes are mediated by microbes, it is obviously necessary to characterize the microbial population structure in these soils as well. The rapid N turn-over in agricultural soils leads to an extremely dynamic N cycle. Further, all N transformation processes are running simultaneously and their rates are changing rapidly in response to changing soil conditions. Therefore, to study soil N dynamics, as well as direct competition effects between crop plants and soil microbes, short-term (hours to days) experiments proved to be the best suitable approach. Due to the heterogeneity of soils, and due to unpredictability of climatic conditions in the field (e.g., heavy rainfall events, significant temperature changes), field studies are hardly able to achieve the aforementioned goals, or to reliably predict the influence of one specific parameter (e.g., fertilizer N form) on soil N dynamics. Rather, it is recommendable to conduct microcosm incubation studies in the greenhouse, which allow controlling a multitude of otherwise undesirable parameters, in order to preliminary determine the N cycle in homogenized soils before going to the field. When keeping in mind that microcosms generally represent artificial systems, the results gathered from such experiments can potentially contribute to elucidating the complexity of soil-plant-microbe interactions.

In the present work a new, simple and cost-effective laboratory format microcosm system is presented, which allows standardized high-throughput analyses of N dynamics in soil-plant-microbe systems.

In the first part of this work the system was evaluated for its suitability, reproducibility and reliability to simultaneously study soil N pools, N transformation processes, N losses, bacterial and fungal populations, as well as plant growth and N uptake using the model plant barley (*Hordeum vulgare* L. cv. *Morex*). Five different agricultural soils from the vicinity of Vienna, Austria, were homogenized and transferred in the test tubes, and kept untreated (except daily adjusting the soil water content gravimetrically) under controlled conditions in a climate chamber for 4 weeks. During this period samples were taken and analyzed continuously, proving that after an initial equilibration period the variability of soil parameters between individual microcosms remained constant.

The second part of the present work investigates the impact of different inorganic N fertilizers (NH_4^+, NO_3^- or NH_4NO_3) on soil N dynamics. Applying ^{15}N tracer techniques this part of the work examines how crop plants and soil microbes vary in their ability to take up and compete for fertilizer N on a short time scale (hours to days). Within each fertilizer treatment traces of $^{15}NH_4^+$ and $^{15}NO_3^-$ were added separately. During 8 days of fertilization the fate of fertilizer ^{15}N into plants, microbial biomass and inorganic soil N pools as well as changes in gross N

transformation rates were investigated. Overall, the main objectives were to assess (1) the effect of applied inorganic N form on the degree of competition for fertilizer N between crop plants and soil microbes, (2) the population response of bacteria and fungi to the different N treatments, as determined by genome equivalent measurements (quantitative PCR), (3) preferences in uptake of NH_4^+ or NO_3^- by plant roots and soil microbes as well as possible inhibitory effects of one N form on uptake of the other form and (4) fertilizer induced changes of microbially mediated N transformation rates that control N distribution to the different soil compartments as well as losses of fertilizer N to the environment.

The third part of this work concentrates on the impact of different inorganic N fertilizers (NH_4^+, NO_3^- or NH_4NO_3) on greenhouse gas emissions. After the development and implementation of special retrofit kit for further elongation of the microcosm test tubes it was possible to reliably measure carbon dioxide (CO_2), nitrous oxide (N_2O) and methane (CH_4) fluxes from two agricultural soils used in the two previous experiments. Head-space gas samples were taken in parallel to all measurements described in part two of the present work, as well as in a separate experiment in another greenhouse at a different time, but under the same controlled conditions. The latter experiment included a set of control samples, which received distilled water instead of N fertilizer. Additionally, samples were taken before fertilization, after fertilization without growing plants, and directly after seedlings were transferred to the test tubes. This setup was chosen to investigate the role of fertilizer N form and of growing plants on greenhouse gas emissions, and to prove the reliable performance of the test tube system for measuring even highly sensitive parameters like gas fluxes. However, the overall aim of this part of the work was to assess fertilizer-induced changes of the net global warming potential of two different agricultural soils and to investigate potential relationships of greenhouse gas emissions with soil properties and microbial population structure determined previously (part two of this work).

Besides being cost effective and easily available, one of the major advantages of the presented microcosm system is that it allows simultaneous handling of several hundreds of replicates requiring only little space. The outstanding performance that can be achieved with this system therefore allows establishing new or improving already existing methodologies to measure a huge variety of soil parameters. One example is given in Appendix A. In this study a suite of rapid and simple methods for measuring total dissolved N, inorganic N and microbial N in soils was established, with the microcosm system presented here contributing to a major part of the experimental setup.

Chapter 1

A cost-effective high-throuput microcosm system for studying nitrogen dynamics at the plant-microbe-soil interface

Erich Inselsbacher[1,2], Katrin Ripka[3], Sylvia Klaubauf[4], Dimitry Fedosoyenko[5], Evelyn Hackl[3], Markus Gorfer[4], Rebecca Hood-Novotny[1], Nico von Wirén[5], Angela Sessitsch[3], Sophie Zechmeister-Boltenstern[2], Wolfgang Wanek[1]*, Joseph Strauss[4]

[1]Department of Chemical Ecology and Ecosystem Research, Faculty Centre for Ecology, Faculty of Life Sciences, University of Vienna, Austria

[2]Department of Forest Ecology and Soils, Unit of Soil Biology, Federal Research and Training Centre for Forests, Natural Hazards and Landscape (BFW), Vienna, Austria

[3]Department of Bioresources, Austrian Research Centers GmbH-ARC, A-2444 Seibersdorf, Austria

[4]Institute for Applied Genetics and Cell Biology, Fungal Genomics Unit, Austrian Research Centers and BOKU University, Vienna, Austria

[5]Molecular Plant Nutrition, Faculty of Agricultural Sciences, University of Hohenheim, Germany

*corresponding author: Wolfgang Wanek

REGULAR ARTICLE

A cost-effective high-throughput microcosm system for studying nitrogen dynamics at the plant-microbe-soil interface

E. Inselsbacher · K. Ripka · S. Klaubauf ·
D. Fedosoyenko · E. Hackl · M. Gorfer ·
R. Hood-Novotny · N. Von Wirén · A. Sessitsch ·
S. Zechmeister-Boltenstern · W. Wanek · J. Strauss

Abstract In the present study a new microcosm system was evaluated for its suitability to investigate nitrogen dynamics between soils, plants and microbes. Five different agricultural soils were homogenized and transferred in the test tubes, and kept under controlled conditions in a climate chamber for 4 weeks. Soils differed clearly in nitrogen pools and microbial population structures but less in their activities. Bacterial and fungal community compositions and soil properties, except gross N transformation rates, remained stable and reproducible during the test period in all soils. [15]N tracer studies showed that N uptake patterns of barley as well as plant growth were linear in the initial growth period. Overall, the presented microcosm system proved to be a powerful tool to elucidate N pathways in soil-plant-microbe systems. In future studies the microcosm system may greatly help generating new insights in the complex processes and controls of nitrogen biogeochemical cycle in agricultural systems.

Katrin Ripka and Sylvia Klaubauf have contributed equally.

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E. Inselsbacher · R. Hood-Novotny · W. Wanek (✉)
Department of Chemical Ecology and Ecosystem Research,
Faculty Center for Ecology,
Faculty of Life Sciences, University of Vienna,
A-1090 Vienna, Austria
e-mail: wolfgang.wanek@univie.ac.at

E. Inselsbacher · S. Zechmeister-Boltenstern
Department of Forest Ecology and Soils,
Unit of Soil Biology,
Federal Research and Training Centre for Forests,
Natural Hazards and Landscape (BFW),
A-1131 Vienna, Austria

K. Ripka · E. Hackl · A. Sessitsch
Department of Bioresources,
Austrian Research Centers GmbH-ARC,
A-2444 Seibersdorf, Austria

S. Klaubauf · M. Gorfer · J. Strauss
Institute for Applied Genetics and Cell Biology,
Fungal Genomics Unit, Austrian Research Centers
and BOKU University Vienna,
A-1190 Vienna, Austria

D. Fedosoyenko · N. Von Wirén
Molecular Plant Nutrition, Faculty of Agricultural
Sciences, University of Hohenheim,
D-70593 Stuttgart, Germany

Introduction

Intensive agricultural plant production depends on the input of mineral fertilizers, which is mainly provided in the form of urea, NH_4^+ or NO_3^-. A major drawback is that only 30–50% of the applied N fertilizer is taken up by the crop plants (Cassman et al. 2002; Smil 1999). A significant amount of the applied N is lost from the agricultural fields, leading to a variety of adverse environmental effects, namely the pollution of ground water by NO_3^- and the emission of the potential greenhouse gases NO and N_2O (reviewed by Tilman et al. 2002). In the last decade a wealth of studies addressing the consequences of intensive N fertilization have been conducted, ranging from field studies (e.g. Chu et al. 2007) to laboratory experiments (e.g. Well et al. 2006).

A major problem of studying N dynamics in soils is that the N cycle in soils is highly complex as it includes several transformation processes of one N form to another (Hayatsu et al. 2008). The results of soil studies are dependent on the characteristics of the specific soil tested, such as pH, soil texture, initial organic matter content and nutrient content (Grigatti et al. 2007). Elucidating N pathways in soils requires not only studying N pools and transfer rates of N among these pools, but also the explicit consideration of N cycling activities by soil microorganisms. The latter mediate a large range of biochemical N transformations in soils and are therefore critical to soil C and N cycling. The importance and complexity of soil microbiology has often been stressed, and many studies have focused on understanding and predicting soil-microbe dynamics in soils, which proved to be challenging due to the immense heterogeneity of soils (reviewed by O'Donnell et al. 2007). Within microbial communities, soil bacteria have received most attention so far but there is increasing evidence for an important role of fungi in these N transformation processes (Hayatsu et al. 2008). Nutrient cycling dynamics are often scale-dependent (Ettema and Wardle 2002), but also the fact that different soil microorganisms may occupy different ecological niches (microsites) and therefore play different roles in the nutrient cycling, increases the difficulty of understanding soil-microbe interactions (Myrold and Posavatz 2007).

Nevertheless, it remains important to elucidate the role and controls of individual processes involved in N turnover, and its response to N fertilization. To accomplish this, approaches that integrate biogeochemical, physiological, microbiological and agronomic studies are essential (Hirel et al. 2007). As it proved difficult to achieve this goal in a highly reproducible and controlled manner in a natural system, laboratory format microcosm systems may provide a solution to this challenge (Copley 2000). Until now a variety of laboratory format studies have been conducted, focusing on greenhouse gas emissions (e.g. Sanchez-Martin et al. 2008), the characterization of microbial community compositions (e.g. Gordon et al. 2008) or plant versus soil microbe N uptake (Harrison et al. 2008). However, we are not aware of any study using a microcosm design which allows the parallel investigation of the major key factors in the N cycle of soil-plant-microbe systems. To turn a laboratory format test system into powerful and reliable tool for soil studies it is necessary to consider and overcome some major obstacles. For example, microcosm studies may generate poorly reproducible results due to soil heterogeneity and because some factors, such as soil bulk density or soil moisture content, cannot be kept constant over a longer period of time (Grigatti et al. 2007; Jensen et al. 1996; McDowell et al. 2006). Additionally, when using microcosm systems the validity of measurements conducted on soils removed from their original field setting is uncertain, due to various manipulations for the experimental setup (Madsen 2005). Therefore, a proper evaluation of microcosm systems regarding their suitability for soil-plant-microbe studies is crucial.

In this study we present a new, simple and cost-effective laboratory format microcosm system, for standardized high-throughput analysis of nutrient dynamics in soil-plant-microbe-atmosphere systems. The main objectives were to evaluate the microcosm system for its reproducibility and reliability to simultaneously study key processes of the N cycle. Therefore a series of experiments was conducted using five different agricultural soils, which were treated in the same, standardized way and were kept in the test tubes under controlled conditions for 1month. During this test period, samples were taken

for analyses of soil N pools, N transformation processes, N losses, as well as bacterial and fungal populations. The main aim of the experimental setup was to evaluate the suitability of the test system for different types of soils, studying the variance of chemical and microbiological soil properties during the incubation period and investigating plant growth and plant N uptake using the model plant barley.

Material and methods

Test tube design

The design of the microcosm system reflects the idea to study several important parameters at the same time. The system is based on 50ml polypropylene centrifuge tubes (Greiner Bio-one, #227261) complemented with two stainless steel sieves above the tube cones. One sieve was placed at the bottom (27mm in diameter, 1mm thick, mesh size 1mm) for stability and a second sieve above the first one (27mm in diameter, 0.2mm thick, mesh size 5µm) to keep the tube cones free from soil particles or from roots when plants are grown in the microcosms. Eight holes (1mm in diameter) were drilled into the tube cones to provide sufficient aeration of the soil from the bottom and to allow the removal of leachates when conducting leaching experiments. Additionally, we designed a retrofit kit for further elongation to meet individual requirements of different experimental setups. This retrofit kit consists of a polypropylene tube (20cm length, 30mm in diameter) which fits exactly onto the centrifuge tubes. To ensure an airtight closure between the two tubes, a 5mm strong rubber seal was used as connecting strap. The whole system can be closed airproof with rubber seals (commercial bottle caps) at the bottom and butyl rubber septa (Suba-Seal 57, Sigma-Aldrich, #Z124680) at the top, allowing sampling of headspace gas with gas tight syringes. A detailed scheme of the microcosm setup is given in Fig. 1.

Site description, soil packing effects and experimental setup

Soil samples were collected in April 2006 from five sites in the vicinity of Vienna, Lower Austria, Austria, representing different bedrocks, soil textures, pH
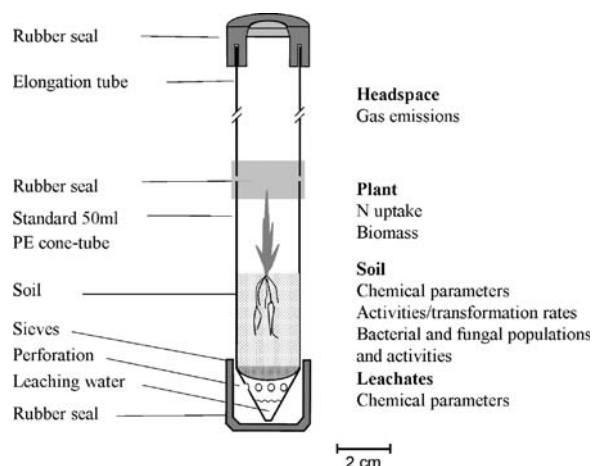
Fig. 1 Setup of the microcosm system to study interactions between soil, plant, atmosphere and microbial communities

values, water, and humus contents (Table 1). All sites were used as agricultural fields with the exception of Riederberg, which was a grassland site. All soil types are widely distributed and are frequently used for barley cultivation in this area. Detailed site characteristics and soil properties are given in Table 1.

Soil samples (each ~25kg) were collected from 0 to 20cm depth from all sites, and immediately stored at 4°C until further analysis. Prior to the start of the experiments soils were homogenized, sieved (< 2mm) and stored at 4°C for 10days. Different amounts of moist soil were weighed into the test tubes and either centrifuged (1min, 187g) or packed by continuously increasing the weight from above up to 17.1 kN m^{-2} to reach a final volume of 30ml. After packing, soils were sampled in 4 equal parts (13mm height increments in the test tubes). Subsequently soil water content, based on oven-dry weight (in % soil DW), and bulk density (in g cm^{-3}) of each increment was observed (Fig. 2). Thus the optimal amount of moist soil for each site was determined (Table 2).

For the following experiments the respective soil aliquots were weighed and centrifuged into the test tubes (1min, 187g). The centrifuge (Sigma 3–15K) was supplemented with a swing-out rotor (Sigma 11133) and additional round carriers for the test tubes (Sigma 17049). The microcosms were kept under controlled conditions in a climate chamber with a 15h/9h day/night cycle at 21/18°C temperature and 55% relative air moisture. During 2weeks of equilibration the soil water content of each soil was

Table 1 Site characteristics and general properties of five soils (0–20cm soil layer) collected in the vicinity of Vienna, Lower Austria, Austria

	Purkersdorf	Riederberg	Maissau	Niederschleinz	Tulln
Soil type	Gleyic Cambisol from sandy loamy flysch	Calcaric Cambisol from clay flysch	Cambisol from silicate material	Chernozem from Loess	Pseudogley on Planosol
Geographic site	48°12′25″ N 16°10′37″ E	48°15′0″ N 16°04′0″ E	48°34′0″ N 15°49′0″ E	48°35′59″ N 15°10′24″ E	48°20′0″ N 16°03′0″ E
Altitude (m. a. sl.)	248	384	341	244	180
Management	Winter barley	Grassland	Arable field	Arable field	Arable field
Water condition	Moist	Changing water conditions	Dry	Moderately dry	Moderately moist
Clay (%)	1.7	29.1	43.2	17.7	0
Silt (%)	64.9	53.8	48.3	74.2	70.4
Sand (%)	33.4	17.2	8.6	8	29.6
pH (KCl)	5.67	6.63	6.99	7.15	6.21
$CaCO_3$ (%)	0.06	2.11	0.2	8.5	0.04
Exchange capacity (mval%)	11.2	33.2	8.9	15.4	37.9
Base saturation (%EC)	81.4	82.1	92.3	98.1	70.4
Bulk density (g DW ml^{-1})	1.06	0.52	1.13	0.96	0.70

adjusted gravimetrically to water contents given in Table 2, which represent 70% of field capacity of homogenised soils after the packing procedure in the microcosms. Thereafter, every 2weeks five replicates of each soil were taken at random and analysed for fluxes of CO_2, N_2O and CH_4, for C and N pools, gross N mineralization and gross nitrification rates, microbial biomass C and N, dehydrogenase and nitrate-reductase activities and bacterial and fungal community patterns. During the test period of 4weeks samples were left untreated, except daily adjusting the soil water content.
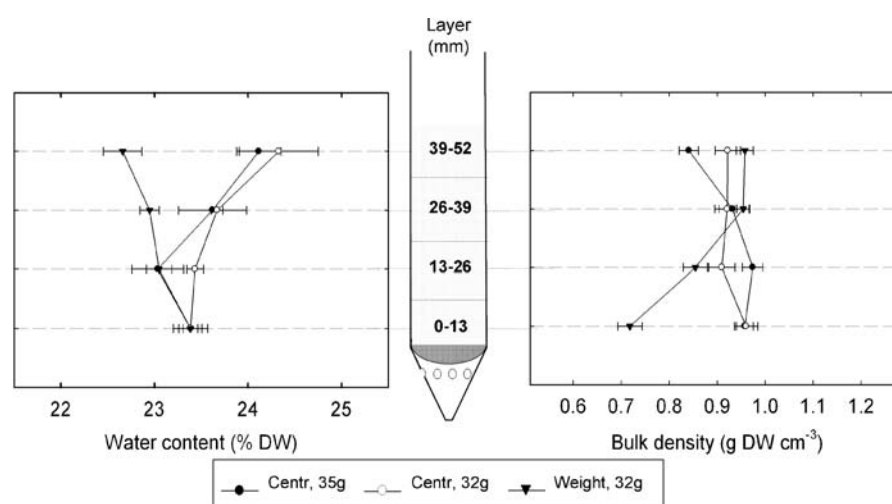
Fig. 2 Vertical gradient of soil water content and soil bulk density of soil Niederschleinz in the microcosm system using three different filling procedures. Soil was centrifuged into the test tubes, using either 35g FW (full circles) or 32g FW (open circles). Triangles represent values where soil (32g FW) was packed by gradually increasing the weight from the top to a final weight of 1kg. Error bars represent standard errors ($n = 5$)

Table 2 Soil fresh weight, water content, nitrogen and carbon pool sizes and transformation rates of five soils collected in the vicinity of Vienna, Lower Austria, Austria

	Purkersdorf42	Riederberg	Maissau	Niederschleinz	Tulln
Fresh weight (g microcosm^{-1})	42	31	40	32	32
Initial water content (% DW)	27.9	42.1	24.2	19.9	33.7
Water content(% DW) at equilibrium	25.2 (0.7)	43.4 (1.0)	24.5 (0.8)	18.0 (0.6)	34.5 (1.1)
Total C (mg C g^{-1} DW)	16.2 (0.5)b	47.7 (0.9)e	13.7 (0.3)a	26.4 (0.2)c	29.3 (0.5)d
Total N (mg N g^{-1} DW)	1.63 (0.05)a	4.78 (0.07)d	1.45 (0.05)a	1.86 (0.02)b	3.27 (0.05)c
C/N	9.96 (0.09)c	9.98 (0.05)c	9.43 (0.12)b	14.17 (0.07)d	8.95 (0.03)a
TDC (μg C g^{-1} DW)	40.2 (1.4)b	61.3 (0.8)c	41.0 (1.5)b	31.2 (1.3)a	33.1 (2.4)a
NO$_3$-N (μg N g^{-1} DW)	18.6 (1.2)a	17.6 (2.4)a	42.3 (1.3)b	19.3 (1.0)a	15.9 (1.4)a
NH$_4$-N (μg N g^{-1} DW)	1.31 (0.23)a	2.15 (0.17)b	1.18 (0.21)a	1.35 (0.18)a	1.28 (0.15)a
Available PO$_4^-$ (μg P g^{-1} DW)	0.66 (0.03)a	0.07 (0.02)a	3.07 (0.10)c	1.33 (0.07)b	5.83 (0.29)d
δ^{15}N	5.28 (0.12)ab	4.72 (0.06)a	5.50 (0.29)b	6.50 (0.10)c	6.65 (0.12)d
δ^{13}C	−27.25 (0.05)a	−26.35 (0.07)b	−26.42 (0.07)b	−19.13 (0.11)d	−23.92 (0.06)c
N$_{mic}$ (μg N g^{-1} DW)	14.6 (0.8)a	179.7 (12.2)c	10.1 (1.1)a	22.9 (2.5)a	52.9 (2.5)b
C$_{mic}$ (mg C g^{-1} DW)	0.37 (0.03)a	2.27 (0.05)c	0.15 (0.008)a	0.30 (0.03)a	1.29 (0.11)b
C/N $_{mic}$	20.1 (6.9)b	9.9 (2.6)a	7.9 (4.8)a	14.1 (2.2)a	24.7 (0.9)b
CO$_2$ (μg C g^{-1}DW d^{-1})	101.1 (22.8)c	74.4 (10.2)bc	42.2 (5.3)b	6.2 (6.1)a	56.8 (14.4)b
N$_2$O (ng N g^{-1}DW d^{-1})	366 (66)b	239 (62)ab	163 (43)a	106 (10)a	152 (56)a
CH$_4$ (μg C g^{-1}DW d^{-1})	0.57 (0.24)ns	−1.37 (1.03)ns	0.49 (0.71)ns	0.55 (0.30)ns	0.03 (0.88)ns
Dehydrogenase (μg TPF g^{-1} DW d^{-1})	7.17 (0.87)b	35.78 (1.96)e	16.51 (1.04)c	20.37 (0.38)d	3.61 (0.11)a
Nitrate reductase (μg N g^{-1}DW d^{-1})	74.7 (6.38)ab	52.7 (19.3)a	153.4 (10.2)b	93.8 (5.6)ab	224.9 (9.72)b
Mineralization (μg N g^{-1}DW d^{-1})	2.28 (0.49)b	0.26 (0.05)a	1.76 (0.44)b	0.40 (0.13)a	0.22 (0.05)a
Nitrification (μg N g^{-1}DW d^{-1})	4.67 (0.37)ns	6.22 (1.89)ns	12.09 (3.51)ns	3.40 (0.61)ns	2.77 (1.16)ns

Values represent means (± standard errors; $n = 5$) at the start of experiment (time 0; after preequilibration in soil microcosms for 10days). Different letters in rows indicate significant differences between sites (Oneway-ANOVA, LSD post-hoc test, $P < 0.05$). TDC Total dissolved carbon, N$_{mic}$ Microbial biomass N, C$_{mic}$ Microbial biomass C, *ns* not significant

Measurement of gas fluxes and chemical analysis

For gas sampling, test tubes were closed at both sides with butyl rubber seals and gas samples were taken immediately after closing and after 1h. With a gas-tight syringe head space air (10ml) was transferred into evacuated headspace vials and kept at 4°C until analysis. After gas sampling, the test tubes were opened again, soils were quantitatively retrieved, homogenized and prepared for further analyses.

Gas samples were analysed within 48h as described by Kitzler et al. (2006b) by automated headspace gas chromatography. Briefly, the GC was equipped with a 63Ni electron capture detector to quantify N$_2$O concentrations and a flame ionization detector and a methanizer to quantify CO$_2$ and CH$_4$ concentrations. Gas emission rates were then assessed by the linear increase of headspace gas concentration over the closure period.

Aliquots (2g) of homogenized soil were extracted in 15ml CaSO$_4$ (10mM) and subsequently anions were determined by ion chromatography (DX 500, Dionex, Vienna, Austria) and conductivity detection. NO$_3^-$ was separated on an anion exchange column (AS11, 250 x 4mm i.d., Dionex, Vienna, Austria) after chemical suppression (ASRS-Ultra, Dionex) and linear NaOH gradient elution (0.5mM to 37.5mM within 10min at a flow rate of 2ml min^{-1}, with a column temperature of 35°C). Ammonium was extracted from aliquots (2g) of homogenized soil with 15ml KCl (1M) and determined by a modified indophenol reaction method (Kandeler and Gerber 1988).

Microbial biomass C and N in soils were analyzed by chloroform fumigation-extraction technique as described by Amato and Ladd (1988) and was calculated as the difference in N concentration between fumigated and non-fumigated soil samples. Briefly, aliquots of fresh soil (2g) were fumigated over chloroform (ethanol-free) for 24h at 22°C. Both, fumigated and non-fumigated soil samples were extracted with 15ml KCl (1M) for 60min before filtering. Total dissolved C

and N in the KCl extracts were determined by an automated C analyzer (Shimadzu, TOC-VCPH, Japan) and a total nitrogen measuring unit (Shimadzu, TNM-1, Japan). A conversion factor for microbial biomass C (K_{EC}) and N (K_{EN}) of 0.45 was applied for incomplete extraction (Jenkinson et al. 2004).

Nitrate reductase activity was measured as described by Kandeler (1996) using 2,4-dinitrophenol as nitrite reductase inhibitor and subsequent colorimetric determination of nitrite at 520nm. Dehydrogenase activity was quantified by the triphenyltetrazolium chloride (TTC) method according to Alef (1995).

An aliquot (4g) of soil was dried at 70°C and weighed to determine soil moisture. Dry soils were ground in a ball mill (Retsch MM2000). Total N and C, as well as natural ^{15}N and ^{13}C abundance of ground soils were then measured by isotope ratio mass spectrometry (IRMS) using an elemental analyser (EA 1110, CE Instruments) connected in continuous flow-mode to a gas isotope ratio mass spectrometer (DELTA^{PLUS}, Finnigan MAT). The natural abundance of ^{15}N and ^{13}C was calculated as follows:

$$\delta^{15}N \, [\permil \text{ vs. at} - \text{air}] = (R_{sample}/R_{standard} - 1) \times 1000$$
$$\delta^{13}C \, [\permil \text{ vs. V} - \text{PDB}] = (R_{sample}/R_{standard} - 1) \times 1000$$

where R is the ratio of $^{15}N / ^{14}N$ for nitrogen and $^{13}C / ^{12}C$ for carbon isotope abundance. The standard deviation of repeated measurements of a laboratory standard was 0.15‰ for $\delta^{15}N$, and 0.10‰ for $\delta^{13}C$.

^{15}N flux measurements

Gross N mineralization and gross nitrification rates were measured by modified ^{15}N pool dilution assays (Barrett and Burke 2000; Bengtson et al. 2006). The soil cores of each tube were labelled uniformly by adding ^{15}N-enriched (99 atom% excess) solutions of $^{15}NH_4Cl$ for mineralization and $K^{15}NO_3$ for nitrification reaching a total of 12μg ^{15}N per test tube. Homogenous distribution of applied N was ensured by inserting a 7-cm long side-hole needle to the bottom of the soil cores in 4 positions and slowly injecting the labelled solution (400μl each injection) while withdrawing the needle (Pörtl et al. 2007). It has been pointed out previously (Murphy et al. 2003), that uniform labelling of soil is essential for ^{15}N pool

dilution essays, therefore a preliminary experiment was conducted, proving that using this technique ^{15}N was indeed homogenously distributed. After the injection of the labelled solution, aliquots of soil were taken from 8 different positions following vertical and horizontal gradients through the total soil core of the microcosm and analysed for their ^{15}N contents, which did not differ significantly ($P < 0,01$; data not shown). After incubation at 22°C for 4h, one set of the samples was homogenized and aliquots (2g) of soil were extracted with 15ml KCl (1M) for 60min to stop the assays. After 48h, the second set of samples was stopped in the same way. All extracts were kept at −20°C until further analysis. NH_4^+ and NO_3^- from the KCl extracts were isolated for ^{15}N analysis by a modified micro diffusion technique (Pörtl et al. 2007). Briefly, the KCl extract was transferred to a glass bottle (50ml) and NH_4^+ was converted to NH_3 by addition of ~200mg MgO. NH_3 was captured in acid traps (glass-fibre filter discs containing 10μl of 2.5M $KHSO_4$, enclosed in PTFE tapes) during 5days of incubation on a shaker at 37°C. Acid traps were removed and dried for 3days over concentrated $H2SO_4$ in a desiccator. A new trap was added to the bottle followed by 0.4g of Devarda's alloy and 200mg MgO to convert NO_3^- to NH_3, and the incubation process was repeated. Dried filter discs were removed from the PTFE sealing and folded into tin capsules for subsequent analysis of ^{15}N contents using IRMS. Gross N mineralization and nitrification rates were calculated using the equations developed by Kirkham and Bartholomew (1954) and as modified by Bengtson et al. (2006).

Plant growth and plant ^{15}N uptake

Sieved soil from the five sites was filled into the microcosms as described above (fresh weight and water contents as given in Table 2, centrifugation for 1min at 187g). Seeds of winter barley (*Hordeum vulgare* L. cv. Morex) were germinated on moist filter paper for 2days and seedlings were then transferred to the microcosms (one plant per microcosm). During the following 13days plants were continuously harvested from each soil in five replicates for determination of plant dry weight. To determine the kinetics of plant soil N uptake, soil from Purkersdorf was labelled with 1mM $^{15}NH_4Cl$ (10 atom% ^{15}N, 25ml kg^{-1} fresh soil). After applying ^{15}N solution the soil

was sieved again, homogenized and equilibrated for 1week at 4°C before packing. Plant seeding and harvests were performed as above. ^{15}N and N concentration was determined in plants grown in Purkersdorf soil by IRMS.

Seed-derived N was calculated by a two-source mixing model:

$$\%N_{seed} = 100*(\delta^{15}N_{max} - \delta^{15}N_{tx})/(\delta^{15}N_{max} - \delta^{15}N_{seed}),$$

where $\delta^{15}N_{max}$ represents $\delta^{15}N$ of plants after 21days, $\delta^{15}N_{tx}$ of plants at time x, and $\delta^{15}N_{seed}$ of dry seeds.

Bacterial and fungal community patterns

For DNA extraction 0.5g of each soil, taken in three replicas from each sampling time, were processed with the FAST DNA Spin kit for soil (Q-Biogene, Germany) as described by the manufacturer. As a functional marker for nitrifying bacteria, a 491 bp fragment of ammonium monooxygenase catalytic subunit A (amoA) was amplified by PCR using primers amoA1F and amoA2R (Rotthauwe et al. 1997). The forward primer was FAM labelled. PCR reactions were performed in 25μl reaction vials containing 1 x buffer, 3mM MgCl$_2$, 0.2mM dNTPs, 1 U of FIREpol Polymerase (Solis BIODYNE) and 0.24μM of primer. To enhance amplification efficiency 1μl DMSO per reaction and 1μg/μl bovine serum albumin (BSA) were added. As target 25 to 100ng of DNA was applied. Amplifications were performed in a Whatman T1 thermocycler using the following program: an initial denaturing step at 95°C for 5min, followed by 35 cycles of 1min at 95°C, 1min at 60°C, 1min at 72°C and a final extension step at 72°C for 10min. PCR products were checked by standard agarose gel electrophoresis on 1% agarose gels.

Terminal restriction fragment length polymorphism (T-RFLP) analysis was applied to obtain a community profile of ammonium oxidizing bacteria. Two PCR products were pooled for each sample to reduce PCR bias. Pooled amplicons were digested with the restriction enzyme AluI in reaction mixtures (10μl) consisting of 7μl PCR product, 1x buffer, and 0.5μl AluI (10U/μl, Promega). Digestions were performed at 37°C for 4h. Digestion batches were purified by passage through DNA Grade Sephadex G50 (GE Healthcare) columns. Purified product (5μl) was mixed with 15μl HiDi-Formamide (Applied Biosys-

tems) and 0.3μl 500 ROX™ Size Standard (Applied Biosystems) and denatured at 95°C for 2min.

Community profiles of fungi were also analyzed using the T-RFLP method. Fungal internal transcribed spacer (ITS) regions were amplified in 20μl reactions under the following conditions: 20–50ng of template DNA, ReddyMix™PCR Master Mix (ABgene), 3mM MgCl$_2$ (final concentration), 4% DMSO, 1μg/μl BSA, 1μM FAM-labelled ITS1F (Gardes and Bruns 1993) and 1μM ITS4 (White et al. 1990). The cycling conditions were as follows: initial denaturation at 95°C for 2.5min, 30 cycles of 94°C for 30s, 54°C for 30s and 72°C for 45s, and a final extension at 72°C for 5min. All PCR reactions were carried out in triplicate. Pooled PCR products were digested with restriction endonuclease BsuRI (Fermentas, isoschizomer of HaeIII) and purified with the QIAquick PCR Purification Kit (Qiagen).

Detection of FAM-labelled terminal restriction fragments was done by capillary electrophoresis using an ABI 3100 automatic DNA sequencer. T-RFLPs were transformed into numerical data using GenoTyper 3.7 NT software. These raw data were normalized and binned according to Abdo et al. (2006).

Statistical analysis

Significant differences ($P < 0.05$) between soils at the start of the experiment were analysed using one-way ANOVA, followed by a Fisher's LSD post-hoc test with Statgraphics 5.0 (Statistical Graphics Inc., Rockville, MD, USA). Differences between soils and sampling times were analysed by repeated measures ANOVA, followed by a Bonferroni post-hoc test with Statistica 6.0 (StatSoft Inc., Tulsa, OK, USA). Repeated measures ANOVA of microbial community patterns was done by analysing peak areas from the electropherograms of T-RFLPs of ammonium oxidizing bacterial communities and of fungal ITS regions (compare Fig. 3). In detail, peak areas of peaks found in >25% of electropherograms of all harvests and soils were used for statistical analyses. If one or more of the peaks showed a significant difference within one soil, it was considered that there was a significant difference in the microbial community patterns over time. Principal component analyses (PCA) of soil N pools, $\delta^{15}N$ and $\delta^{13}C$ values, N transformation rates and gas emissions were performed with SIMCA-P 11.0 (Umetrics, Umeå, Sweden).
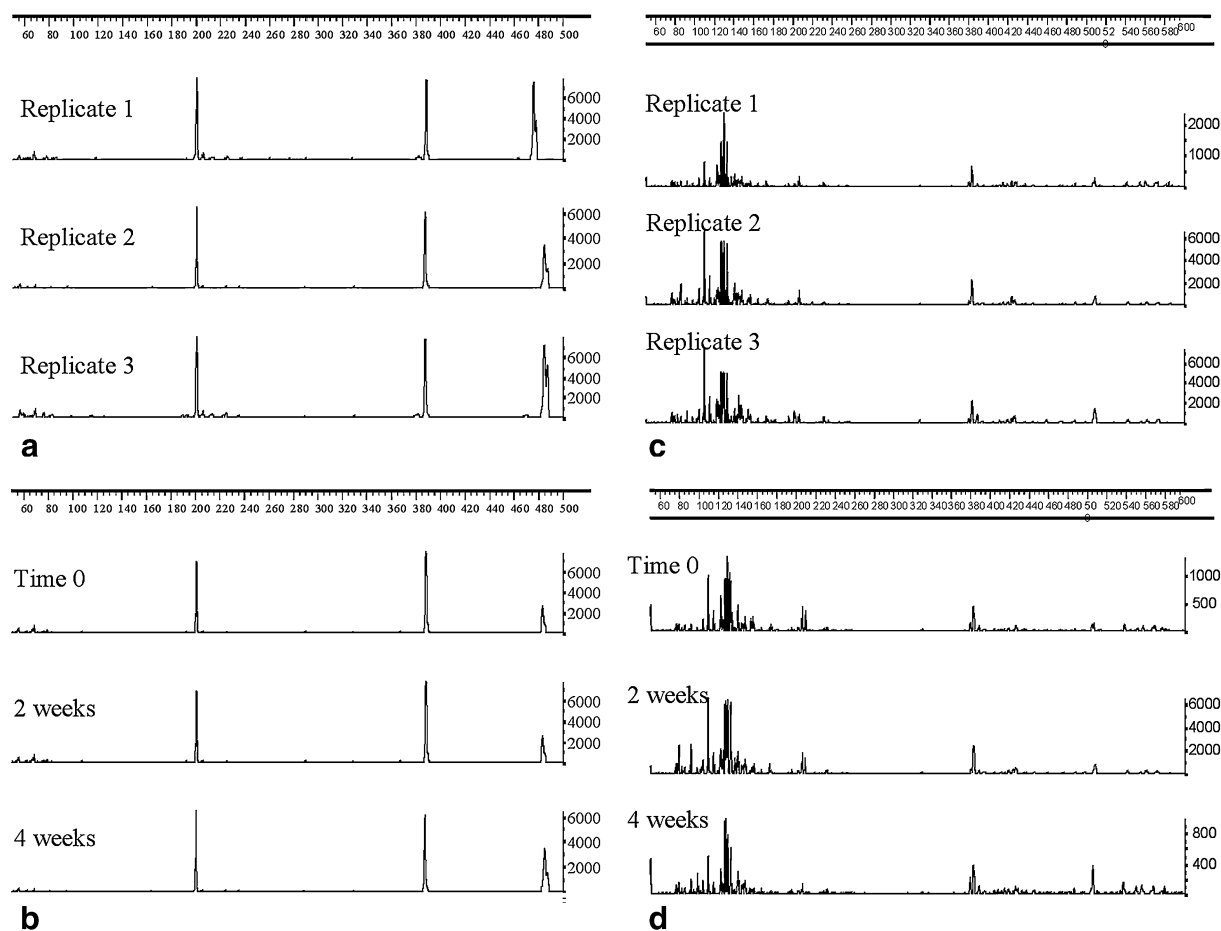
Fig. 3 Microbial community structure of soils collected in the vicinity of Vienna, Lower Austria, Austria and incubated in the microcosm system. Electropherograms of T-RFLPs of (**a**) three replicates of ammonium oxidizing (amoA) bacterial communi-
ties at the first sampling event (time 0), (**b**) of amoA bacterial communities at the three sampling events, (**c**) of three replicates of fungal ITS regions at the first sampling event and (**d**) of fungal ITS regions at the three sampling events at site Maissau

Results

Packing effects

Three different packing procedures were used to test for homogeneity of water content and bulk density of the soil along the vertical gradient within the test tubes. Different amounts of soil fresh weight were either centrifuged or packed by increasing the weight from above into the tubes. Clearly, the way of packing the soil into the test tube had a strong influence on water content and bulk density, as with all packing procedures the formation of vertical gradients in the microcosms could be observed (Fig. 2). When 32g moist soil from Niederschleinz was centrifuged (1min, 187g) into the tubes, the bulk density increased only in the lowest part of the microcosm

by 4.1% ($P < 0.05$). In comparison the bulk density increased by 13.8% ($P < 0.05$) when packing 35g moist soil by centrifugation. When 32g moist soil was packed into the tubes by continuously increasing the weight from the top to 17.1 kN m^{-2}, the observed gradient of bulk density was highest, differing by 25.0% ($P < 0.001$). Vertical gradients in water content due to packing procedures were less pronounced as for bulk density, showing variations of 3–4% in all setups ($P < 0.05$). Similar results in bulk density and water content patterns along a vertical gradient were obtained for the other soil types (data not shown).

Soil N and C pools and activities

Riederberg soil (grassland) differed significantly in most of the measured soil properties from the arable

soils of the other sites (Table 2). The total carbon content of Riederberg soil was nearly double that of the other sites. Additionally, dissolved organic C, total N and NH_4-N contents were significantly higher as well as microbial biomass N and C pools, comprising 3.8% of the total N and 4.8% of the total C pool in the grassland soil. Carbon to nitrogen (C/N) ratios of microbial biomass differed greatly among the five soils, ranging from 7.9 in Maissau soil to 24.7 in Tulln soil. In contrast, soil C/N ratios were similar (between 9.0 and 10.0) with the exception of soil from Niederschleinz having a significantly higher C/N ratio of 14.2 (one-way ANOVA, $P < 0.05$). Further important features of this soil were significantly higher $\delta^{13}C$ and high $\delta^{15}N$ values, being -19.13 ± 0.11 ‰ and 6.50 ± 0.10 ‰ (mean ± SE, $n = 5$), respectively. Niederschleinz was the only Chernozem soil over Loess as bedrock and had much higher carbonate contents (8.5%). At all sites inorganic N pools consisted mainly of NO_3-N while NH_4-N comprised only a small fraction of inorganic N. The proportion of NO_3-N in the inorganic N pool varied between sites, being lowest in Riederberg soil (89.1%) and highest in Maissau soil (97.3%). Maissau soil had the highest NO_3-N contents ($P < 0.05$), comprising 2.91% for the total N pool in the soil, while the total C content was lowest (13.68 ± 0.33 mg C g^{-1} DW, $P < 0.05$). Additionally to high NO_3-N contents, gross nitrification rates tended to be highest in Maissau soil (12.1 µg N g^{-1} DW d^{-1}). Also gross N mineralization rates were significantly higher in Maissau soil compared to the other sites ($P < 0.05$), but were in the same range in soil Purkersdorf. Generally, gross nitrification rates were higher than gross N mineralization rates in all tested soils. Dehydrogenase activity, as an indicator of total microbial metabolic activity, was highest in Riederberg soil ($P < 0.05$), where also high emissions of CO_2 were found (Table 2). Generally, CO_2 emission rates showed marked differences between the five sites. At site Niederschleinz the lowest CO_2 emission was observed ($P < 0.05$), comprising only 0.02% of the total C pool per day. In contrast, the Purkersdorf soil exhibited the highest CO_2 emission, being 16 times higher than CO_2 emission from soil Niederschleinz and comprising a C loss of 0.62% of the total C pool per day. A similar pattern was found for N_2O emissions, where soil from Niederschleinz showed the lowest and soil from Purkersdorf the highest

emission rates, comprising for 0.006% and 0.023% of the total N pools per day, respectively. No significant differences in CH_4 emissions were found between the soils of the different sites ($P > 0.05$).

Stability of the test tube system during the test period

One major prerequisite for the suitability of the presented microcosm system for future studies is that initial soil properties remain constant after equilibration if no further manipulations are carried out. To verify this, all parameters given in Table 2 were measured again after two and 4 weeks and significant changes during this time period were tested by repeated-measures ANOVA (Table 3). None of the measured N and C pools changed significantly ($P > 0.05$). Likewise, $\delta^{13}C$ values of all soils and, with the exception of soil Maissau, $\delta^{15}N$ values also remained stable. With regard to gas emission rates, only N_2O emission from soil Purkersdorf decreased significantly ($P < 0.0001$), while no differences in CO_2 and CH_4 emission rates could be detected in any soil. No significant differences in gross N mineralization and nitrification rates were observed at site Purkersdorf and Riederberg. In soils from Niederschleinz and Tulln, however, gross N mineralization rates increased significantly ($P < 0.0001$), while at site Maissau nitrification rates decreased over time ($P < 0.05$). Nitrate-reductase and dehydrogenase activities did not change significantly during the 4 weeks, except in soil Riederberg, where a significant decrease of dehydrogenase activity was observed ($P < 0.05$). The largest influence of incubation time on soil parameters was observed in soil microbial biomass C, which decreased significantly by 29 to 79% at all sites except in soil Niederschleinz. Microbial biomass N, on the other hand, significantly decreased by 63% in soil Riederberg only ($P < 0.0001$). Contrary to microbial biomass, fungal and bacterial (amoA) community structures did not change significantly ($P > 0.05$) in any soil (Fig. 3, showing results for soil Maissau as representative for the others).

To further evaluate the suitability of the microcosm system the variance of the single parameters during the incubation period was determined; even if the absolute value of a parameter under investigation may change during the test period, it is more important that the variance does not increase. This analysis was done by calculating the ratio of the 95% confidence

Table 3 Changes in soil properties and activities and in microbial community composition of five soils collected in the vicinity of Vienna, Lower Austria, Austria and incubated in the microcosm systems over 4 weeks

	P	R	M	N	T	Ratio *CI* t4 / *CI* t0
Total C	ns	ns	ns	ns	ns	0.7
Total N	ns	ns	ns	ns	ns	0.6
TDC	ns	ns	ns	ns	ns	1.0
NO_3^--N	ns	ns	ns	ns	ns	0.4
NH_4^+-N	ns	ns	ns	ns	ns	0.3
$\delta^{15}N$	ns	ns	***	ns	ns	0.7
$\delta^{13}C$	ns	ns	ns	ns	ns	0.7
CO_2	ns	ns	ns	ns	ns	0.5
N_2O	***	ns	ns	ns	ns	0.7
CH_4	ns	ns	ns	ns	ns	1.3
Mineralization	ns	ns	ns	***	***	1.4
Nitrification	ns	ns	*	ns	ns	1.6
Dehydrogenase	ns	*	ns	ns	ns	0.7
Nitrate reductase	ns	ns	ns	ns	ns	1.0
N_{mic}	ns	***	ns	ns	ns	0.7
C_{mic}	*	***	*	ns	***	0.5
*amo*A	ns	ns	ns	ns	ns	na
Fungal ITS	ns	ns	ns	ns	ns	na

TDC Total dissolved carbon, N_{mic} Microbial biomass N, C_{mic} Microbial biomass C, *amo*A Ammonium oxidising bacterial community. *CI* t4, 95% confidence interval of samples after 4weeks; *CI* t0, 95% confidence interval at the first sampling time; na, not applicable; Time effects were calculated by repeated measures ANOVA and Bonferroni post-hoc test (ns, not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$)

intervals (*CI*) of each parameter at the beginning of the test period (t0; after equilibration) and after 4weeks (t4). Only three of all measured parameters, namely gross N mineralization, nitrification and CH_4 emission rates, showed higher confidence intervals at the end of the test period compared to the beginning (Table 3). The variance of all other investigated parameters decreased or remained constant.
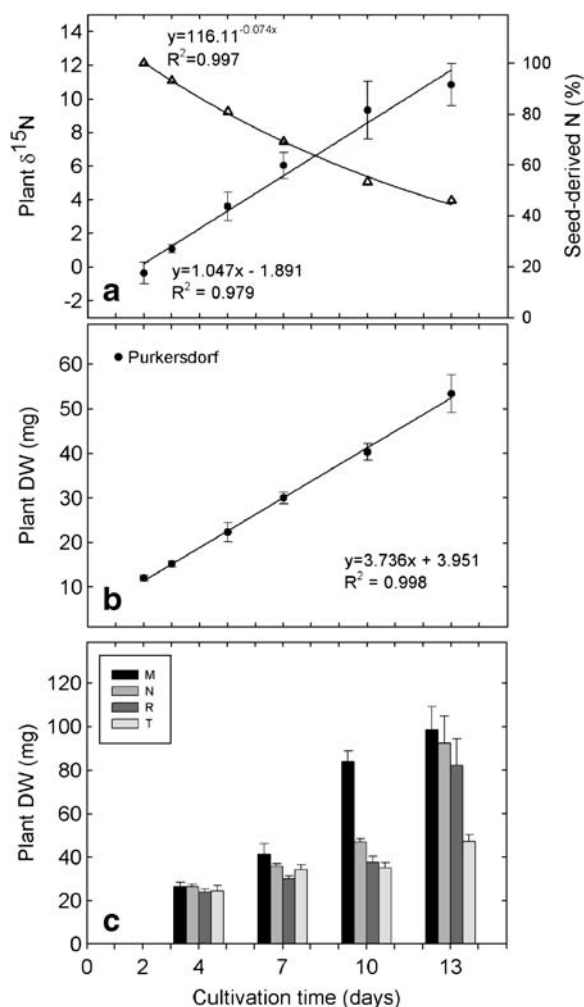
Plant growth and ^{15}N uptake

The $\delta^{15}N$ values of two-day old barley seedlings (−0.36 ‰), which were grown in soil from Purkersdorf labelled with $^{15}NH_4Cl$, increased linearly during the subsequent growth period of 11days and reached a $\delta^{15}N$ value of 10.84 ‰ (Fig. 4a). Simultaneously, the proportion of seed-derived N in the plants decreased almost linearly. When plants were 13days old, 60% of total plant N pool consisted of N taken up from the soil. Plant biomass also increased linearly ($R^2 = 0.998$) during this time, with a gain of 3.7mg dry matter per day (Fig. 4b). When barley was grown in other soils, plant growth rate was similar to Purkersdorf

soil; only in Maissau soil plants showed a faster increase of biomass.

Discussion

Handling guidelines and optimizations

The present study describes a new design for a microcosm system that allows investigating the complexity of physiological processes at the soil-plant-microbe-environment interface with high reproducibility at low costs. The outstanding performance that can be achieved with this system is documented by the low variance and the high reproducibility in chemical properties, bacterial and fungal community structures, gas emissions and plant growth. Besides being cost effective and easily available, this microcosm system allows handling of hundreds of replicates requiring only little space. The simple handling allows three to four people to conduct experiments with 400 microcosms or more. Before starting, however, some general considerations using the tube
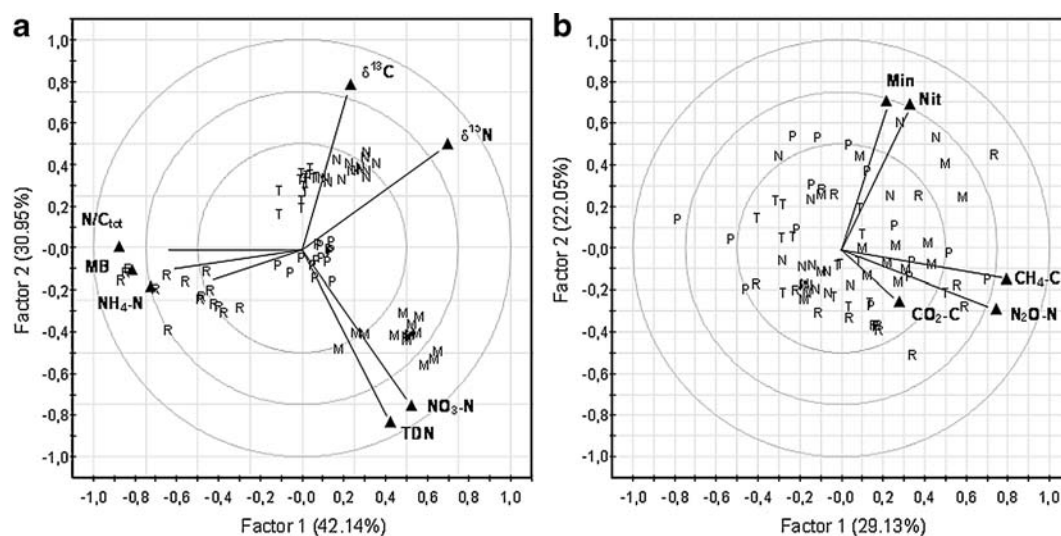
Fig. 4 Time course of barley growth and N uptake in the microcosm system. Increase of plant δ15N (full circles) and simultaneous decrease of seed-derived N (open triangles) in barley plants (a) and increase of plant dry weight (b and c). Seeds of barley were transferred to the microcosms 2 daysafter germination and grown in soil from site Purkersdorf labelled with $^{15}NH_4Cl$ (a and b) or (c) in the unlabelled soils from Maissau (M), Niederschleinz (N), Riederberg (R) and Tulln (T). Soils received no fertilizer before or during plant growth. Symbols and bars represent means, error bars represent standard errors ($n=5$)

reproducibility of the microcosm system hence would decrease. We found out, that slow centrifugation of the tubes (187g) and using 32g of moist soil, represented the optimal conditions in terms of packing for soil Niederschleinz. With this method it was possible to achieve most homogenous soil bulk density and water content in the tubes (Fig. 2). Moreover, packing density in the tubes (0.9–1.0g cm^{-3} soil volume) closely resembled that of soil in situ (0.96g cm^{-3}, Table 1). Changing the amount of soil used for packing had a great influence on the homogeneity of compaction, e.g. soils were significantly less homogenously packed with only a small increase of fresh weight (3g) for the same centrifugation procedure. As these findings were valid for all five tested soils (data not shown), it is therefore essential to test every soil for homogeneity of soil packing before starting an experiment using the microcosm system. Packing the soil by gradually increasing the pressure from the top, or simply compacting the soil by tipping against the lab bench (data not shown), resulted in significantly higher heterogeneity and should not be applied in such a test system. Even if this may sound trivial, achieving a uniform soil distribution in the tubes was found to be one of the crucial steps for all subsequent analyses using this microcosm approach. This is particularly important for processes that are controlled by soil aeration or redox potentials such as N_2O and CH_4 fluxes or nitrification (data not shown). Prior to further analyses soils should be equilibrated for at least one or 2weeks, to avoid priming effects which are likely to occur during the preparation procedures (Madsen 2005).

Between-soil differences in soil N and C pools and activities

To evaluate the microcosm design for its suitability to study soil-plant-atmosphere interfaces we used five representative soils that are commonly used for cultivation of barley in the vicinity of Vienna, Austria but differ in chemical properties (Table 2). Soil N and C pools and microbial biomass differed significantly between soils (Table 2), and based on these parameters it was possible to clearly distinguish the soils by a principal component analysis (PCA) approach (Fig. 5a). Further, the concentrations of C and N pools within each soil did not change significantly ($P > 0.05$)

design need to be addressed. As in other studies (Grigatti et al. 2007), it was one of the major challenges to establish homogenous conditions of the soils in the microcosms in terms of bulk density and water content. Only slight changes of water content, bulk density and other factors, such as light conditions or temperature, might lead to significant differences in soil processes and plant growth, and the

Fig. 5 Principal component analysis of a C and N pools, microbial biomass and $\delta^{15}N$ and $\delta13C$ values and b gas emission and gross N transformation rates of five soils collected in the vicinity of Vienna, Lower Austria, Austria. Data points (indicated as letters) represent soils sampled at time 0, after

2 weeks and 4 weeks (P, Purkersdorf; R, Riederberg; M, Maissau; N, Niederschleinz; T, Tulln). Min, gross mineralization rate; Nit, gross nitrification rate; Ntot, total soil nitrogen; Ctot, total soil carbon; Nmic, microbial biomass N; Cmic, microbial biomass C; TDN, total dissolved nitrogen

during the test period (Table 3), showing that without further treatment such as fertilization or plant growth and under constant conditions these parameters remained stable throughout time in the microcosms. The C content of soil microbial biomass, however, decreased significantly in four of the five tested soils, while the N content in soil microbial biomass only decreased at site Riederberg. This change may be a result of initial priming effects after sieving and homogenizing the soils (Kuzyakov et al. 2000), indicating that 10days of equilibration were yet too short. However, these changes appear to be small compared to treatment effects such as fertilization, cutting or plant growth where massive changes in microbial biomass can be expected.

As the chemical composition of the soils studied differed so clearly, we expected to be able to separate the five soils by PCA based on microbial activities and gross transformation rates. Though soil respiration (in terms of CO_2 production) and production of N_2O differed significantly between (some) soils (Table 3), no clustering of sites was evident in this PCA approach (Fig. 5b). This suggests that after equilibration under standardized conditions overall soil activities (e.g. gas fluxes and N transformations) did not allow a clear separation of soils. We attribute this to the generally high variability in trace gas fluxes

and gross N transformation rates (e.g. 100–150% coefficients of variation, Parsons et al. 1991), hindering PCA separation of soils according to soil activities. Moreover, it is well known that emissions of trace gases, such as N_2O and CH_4, are controlled by a complex interplay of production and consumption processes, being influenced by many factors, such as water filled pore space as a proxy for soil redox status and aeration (Bateman and Baggs 2005; Kitzler et al. 2006a; Well et al. 2006). In our study we equilibrated the soils at 70% water holding capacity. It is therefore likely that in this experiment the occurrence of aerobic and anaerobic microsites increased the variance in trace gas fluxes reflecting the transient and sensible balance between simultaneous trace gas production and consumption.

Reproducibility of the microcosms and changes of variance of data during time

More important than the quantitative changes of pools or activities during the test period per se, is the reproducibility of each parameter. If soil aliquots are not large enough to be representative or homogenous over all microcosms, or if soil environmental factors, such as temperature or moisture vary between single microcosms during the incubation period, this may

lead to a divergent development of single microcosms and their soil pools, microbial communities and activities with time. In detail this means that the coefficient of variation (measured as 95% confidence interval) of each parameter would increase, due to increasing data variability. This was not the case for most parameters in our study, as standard deviations remained constant or decreased during the 4weeks, even if absolute values changed (Table 3). In the case of gross N mineralization, gross nitrification and CH_4 emissions, there were minor increases in data variance between the first and last sampling event by 30 to 60% (Table 3). However, gross transformation rates generally show high variance (e.g. Parsons et al. 1991) and 4weeks represent a long time period for such fluxes to remain constant. Although not uncommon (e.g. Accoe et al. 2004), it was surprising that gross nitrification rates were higher than gross N mineralization rates in all tested soils. This could be due to over- or underestimation of gross N transformation rates, caused by e.g. high heterotrophic nitrification rates or remineralization of $^{15}NH_4^+$ (Watson et al. 2000). To measure gross N transformation rates using the pool dilution assay further studies are underway to address the effects of homogeneity of ^{15}N labelling and timing of sampling on N fluxes in the microcosm system. However, overall the microcosm system allows a wide range of soil-microbe-atmosphere interactions to be studied, as under controlled conditions each replicate sample developed similarly in the microcosm system.

Bacterial and fungal community patterns

To elucidate N pathways in soils it is not sufficient to study chemical pools and transfers between these pools without investigating soil bacterial and fungal communities. Despite the crucial role they play in the cycling of nutrients in soils, the microorganisms that are actually responsible for the key processes have rarely been described (Madsen 2005). As it is not easily possible to manipulate these communities in a natural system, it may be advisable to conduct additional experiments in a laboratory format microcosm system (Copley 2000). To evaluate our microcosm system for its future suitability to study microbial community patterns and their role and response to fertilizer application, it was crucial that the initial community patterns did not change during

the test period in untreated soils and between replicas. T-RFLP analysis of ammonium-oxidising communities (amoA) clearly showed that these community patterns were stable during the 4weeks (Fig. 3b), even though significant changes in microbial biomass carbon could be found (Table 3). Analysis of fungal ITS regions showed that no significant change in fungal communities could be observed as well (Fig. 3d). In the absence of manipulations, e.g. fertilization or plant growth, the bacterial ammonium-oxidizing as well as fungal communities did not change during the test period. Even more importantly, the analysis of replicas from one time point (Fig. 3a and c) showed a consistent community T-RFLP pattern with negligible variations between the replicates. These findings strongly suggest that the microcosm system can also be used to study microbial community structures and functions (e.g. enzyme activities) in soils. Previous studies have shown that bacterial growth rates and activities differ greatly between bulk soil and rhizosphere along barley roots (Soderberg and Baath 1998). These findings were yet not considered in the present study, and will have to be dealt with in follow-up experiments, as here the main aspect was to validate the chemical and microbiological stability of the bulk soils in the microcosms.

Plant growth and N uptake

Since future studies using the test tube system should allow investigations of plant-soil-microbe interactions, plant growth and N uptake were monitored in a time frame of 2–13days after germination (Fig. 4). This time period of 13days proved to be optimal for studying plant N dynamics, as within this time a linear increase of plant biomass as well as ^{15}N uptake was observed. These results demonstrate that, over this time period, model plants like barley can be grown in the test tube system without restriction by soil nutrients and rooting space and the effect of different soil parameter on plant physiology and N uptake can be assessed. When the plants were continuously grown in the microcosms for one more week, no mineral nutrient deficiencies were observed (von Wiren, data not shown) and plants grew vigorously without fertilizer treatment. Nevertheless, experiments using barley should not exceed a time period of 3weeks, as there may occur unexpected and

hardly traceable side effects due to reduced root growth and nutrient uptake in the limited soil volume. Obviously, the increase of plant biomass differed between the different soils (Fig. 4c), most likely because of their different initial physico-chemical conditions.

Already 1day after transferring two-day old barley seedlings to the microcosms the ^{15}N tracer derived from the soil was detected in the plants (Fig. 4a). While the ^{15}N content in the plants increased linearly during the following 11days, the amount of seed-derived N in the plants decreased (Fig. 4a). Plants therefore immediately took up soil inorganic N and N reliance on soil N pools increased continuously over time in the presented microcosms. It therefore appears feasible to investigate plant N uptake patterns and other physiological processes in 3 to 13days-old plants in a highly reproducible way.

Conclusions

This study describes a simple and cost-effective laboratory format microcosm system for high-throughput analyses of N dynamics in soil-plant-microbe-atmosphere systems. Our results show so far unrecorded evidence that several key processes in N dynamics in agricultural soils can be studied in parallel under controlled conditions. The microcosm system is suitable to study soil N cycling or other biogeochemical cycles as well as microbial populations and functions. Further, the microcosm system has the potential to investigate the interplay between plants and microbes in soil N cycle processes. Keeping the microcosms under stable, standardized and reproducible conditions proved to be easily possible, provided a uniform distribution of soil bulk density had been achieved. However, to reliably apply the microcosm system for measurements of gross N transformation rates as well as CH_4 fluxes, further testing is necessary. As pointed out by others, it is important to consider the limitations of such a microcosm system, and that the validity of results has a model character which needs up-scaling at certain points by field studies (Madsen 2005). Nevertheless, this system is a powerful tool to elucidate interactive pathways in soil-plant-microbe-environment systems, and may allow generating new insights and the discovery of new basic mechanisms

in the complex processes and controls of the agricultural soil N cycle.

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Chapter 2

Short-term competition between crop plants and soil microbes for inorganic N fertilizer

Erich Inselsbacher[1,2], Nina Hinko-Najera Umana[1], Florian C. Stange[3], Markus Gorfer[4],

Elisabeth Schüller[4], Katrin Ripka[5], Sophie Zechmeister-Boltenstern[2], Rebecca Hood-

Novotny[1], Joseph Strauss[4], Wolfgang Wanek[1]*

[1]Department of Chemical Ecology and Ecosystem Research, Faculty Centre for Ecology, Faculty of Life Sciences, University of Vienna, Austria

[2]Department of Forest Ecology and Soils, Unit of Soil Biology, Federal Research and Training Centre for Forests, Natural Hazards and Landscape (BFW), Vienna, Austria

[3]UFZ Department Soil Physics, Helmholtz Centre for Environmental Research-UFZ, Halle, Germany

[4]Department for Applied Genetics and Cell Biology, Fungal Genomics Unit, Austrian Institute of Technology and BOKU University Vienna, Vienna, Austria

[5]Austrian Institute of Technology, Department of Health and Environment, A-2444 Seibersdorf, Austria

*corresponding author: Wolfgang Wanek

Abstract

Agricultural systems that receive high amounts of inorganic nitrogen (N) fertilizer in the form of either ammonium (NH_4^+), nitrate (NO_3^-) or a combination thereof are expected to differ in soil N transformation rates and fates of NH_4^+ and NO_3^-. Using ^{15}N tracer techniques this study examines how crop plants and soil microbes vary in their ability to take up and compete for fertilizer N on a short time scale (hours to days). Single plants of barley (*Hordeum vulgare* L. cv. *Morex*) were grown on two agricultural soils in microcosms which received either NH_4^+, NO_3^- or NH_4NO_3. Within each fertilizer treatment traces of $^{15}NH_4^+$ and $^{15}NO_3^-$ were added separately. During 8 days of fertilization the fate of fertilizer ^{15}N into plants, microbial biomass and inorganic soil N pools as well as changes in gross N transformation rates were investigated. One week after fertilization 45-80% of initially applied ^{15}N was recovered in crop plants compared to only 1-10% in soil microbes, proving that plants were the strongest competitors for fertilizer N. In terms of N uptake soil microbes out-competed plants only during the first 4 hours of N application independent of soil and fertilizer N form. Within one day microbial N uptake declined substantially, probably due to carbon limitation. In both soils, plants and soil microbes took up more NO_3^- than NH_4^+ independent of initially applied N form. Surprisingly, no inhibitory effect of NH4+ on the uptake and assimilation of nitrate in both, plants and microbes, was observed, probably because fast nitrification rates led to a swift depletion of the ammonium pool. Compared to plant and microbial NH_4^+ uptake rates, gross nitrification rates were 3 to 75-fold higher, indicating that nitrifiers were the strongest competitors for NH_4^+ in both soils. The rapid conversion of NH_4^+ to NO_3^- and preferential use of NO_3^- by soil microbes suggest that in agricultural systems with high inorganic N fertilizer inputs the soil microbial community could adapt to high concentrations of NO_3^- and shift towards enhanced reliance on NO_3^- for their N supply.

Keywords

Agriculture, ammonium monooxygenase, archaea, bacteria, fungi, gross N transformations, inorganic N fertilizer, qPCR, ^{15}N tracer

1. Introduction

Intensive agricultural crop production in central Europe largely depends on the input of nitrogen fertilizers, mainly provided in the form of NH_4^+ or NO_3^- or a combination thereof. Global estimates indicate that less than ~50% of the applied fertilizer N is used by the crop, while 2-5% are stored in the soil, ~25% are emitted to the atmosphere and ~20% are discharged to aquatic systems (Galloway et al., 2004). In the last decade a wealth of studies have focused on ways to improve fertilizer use efficiency, largely in cereal grain production, and on reducing adverse effects, namely losses of fertilizer N to the environment (Tilman et al., 2002; Mosier et al., 2004). Other studies have focused on fates of N inputs during one or more growing seasons, or on predicting N availability to crops (Jackson et al., 2008).

In agricultural soils plant available N is present in soluble inorganic (NO_3^-, NH_4^+, NO_2^-) and organic N forms. The pool of soluble organic N in unfertilized arable soils can be as large as the mineral N pool and might be strongly involved in mineralization and immobilization processes (reviewed by Murphy et al., 2000). Although dissolved organic N, especially free amino acids, may contribute significantly to plant nutrition in several ecosystems (reviewed by Näsholm et al., 2009), the quantitative importance seems to be negligible (<1%) in agricultural systems that receive high quantities of inorganic N fertilizer (Xu et al., 2008). This indicates that the plant N relations in agricultural soils are predominantly influenced by the rate of mineral N fertilizer application. But not only the amount, also the form of mineral N applied (i.e. NO_3^- vs. NH_4^+ vs. NH_4NO_3) is strongly affecting plant and microbial N metabolism.

In many cultivated soils it has been observed that in plants and microbes NH_4^+ assimilation exceeds NO_3^- assimilation (e.g. Azam et al., 1993), which was explained by higher energy costs associated with biological NO_3^- assimilation (Gutschick, 1981; Smirnoff and Stewart, 1985; Puri and Ashman, 1999). Moreover, it was shown that the presence of NH_4^+ inhibits NO_3^- uptake by fungi (Wang 2007) and by plants (Gessler et al., 1998; Gazzarrini et al., 1999; Siddiqi et al., 2002), further promoting plant NH_4^+ uptake. However, in contrast to these molecular studies, other studies using soils showed in this environment that NH_4^+ utilization by soil microbes and plants was lower or similar to NO_3^- utilization (Burger and Jackson,

2003; Song et al., 2007), presumably due to higher mobility of NO_3^- in soils as compared to NH_4^+ (Hodge et al., 2000).

One factor leading to these differences in inorganic N assimilation might be that the inorganic N pools in agricultural soils are extremely dynamic (e.g. Jackson et al., 1989), caused by high microbial activities and fertilizer application. Although these dynamics are well established, little is still known about the mechanisms and short-term effects (hours to days) of different ratios of $NH_4^+ : NO_3^-$ availabilities on microbial N transformation processes. As some of these processes lead to N-losses to the environment (e.g. leaching of NO_3^- or N gas emissions caused by nitrification or denitrification), even short-term increases in microbial activity can negatively affect the fertilizer use efficiency of crop plants.

Besides the availability of inorganic N, competition with soil microbes is one of the most critical factors affecting the ability of plants to acquire N from soil (Kaye and Hart, 1997). In several short-term ^{15}N tracer studies (up to several days) investigating grassland ecosystems, microbes often assimilated more ^{15}N-labelled inorganic N than plants did, but after an initially rapid N capture, microbial biomass appeared to reach a steady state, probably because of insufficient available C to maintain the fast initial growth rates (Hodge et al., 2000; Harrison et al., 2008). The same studies showed that during the following period, microbial ^{15}N was gradually released by microbial decay and re-mineralization into the soil, eventually becoming available for plant root uptake. After longer time periods (weeks to months), plants contained an increasing proportion of the added ^{15}N, showing that plants indeed utilized this N resource (Harrison et al., 2007). In other words, the turnover rate of plant biomass was much slower than that of microbes, which allowed plants to compete for the same N for extended periods, therefore enhancing plant competitiveness for N.

However, in high N ecosystems, like in agricultural soils, it was suggested that competition could be less severe (Schimel and Bennett, 2004), though plants and microbes are considered to compete for any available N, particularly for NH_4^+ and NO_3^-. Further, besides competition between plants and soil microbes, competition among different groups of soil microbes for fertilizer N is of considerable importance. Burger et al., (2003) found that, due to the high availability of inorganic N after fertilization, competition between plants and heterotrophic microbes for NH_4^+ markedly decreased and nitrification became the major fate of NH_4^+. Over time, the N economy in well-aerated agricultural soils therefore becomes progressively NO_3^- dominated and plants cover their N-demand from mainly NO_3^- (Schimel et al., 2004). However, it still remains unclear to which degree short-term (hours to days) competition for

fertilizer N between plants, heterotrophic and autotrophic microbes is affected by the form and amount of mineral N applied.

The main objectives of the present study therefore were to assess (1) the effect of applied inorganic N form on the degree of competition for fertilizer N between crop plants and soil microbes, (2) the population response of bacteria and fungi to the different N treatments, as determined by genome equivalent measurements (quantitative PCR), (3) preferences in uptake of NH_4^+ or NO_3^- by plant roots and soil microbes as well as possible inhibitory effects of one N form on uptake of the other form and (4) fertilizer induced changes of microbially mediated N transformation rates that control N distribution to the different soil compartments as well as losses of fertilizer N to the environment.

To accomplish this we conducted a greenhouse experiment using a previously established microcosm system and experimental design (Inselsbacher et al., 2009). Within these microcosms barley plants were grown on two previously characterized agricultural soils from the vicinity of Vienna, Austria. Three different forms of N fertilizer (NH_4NO_3, KNO_3, NH_4Cl) were applied, adding trace amounts of $^{15}NH_4^+$ and $^{15}NO_3^-$ within each N treatment. This design allowed us to follow the ^{15}N label into plants and microbial biomass and to calculate gross N transformation rates on an hourly to daily time scale.

2. Material and Methods

2.1. Soil sampling and experimental setup

Soil was collected in April 2006 from two sites in the vicinity of Vienna, Austria, namely from Purkersdorf and Niederschleinz. Soils from both sites are widely distributed and are frequently used for barley cultivation in this area. A detailed summary of site characteristics and soil properties is given in Table 1. Soil samples (each ~25 kg) were collected from 0 to 20 cm depth from both sites and immediately stored at 4°C until further analysis. Prior to the start of the experiments soils were homogenized and sieved (< 2 mm).

Experiments were carried out using a recently developed microcosm system described by (Inselsbacher et al., 2009). Briefly, the microcosms consisted of 50 ml polypropylene centrifuge tubes complemented with two stainless steel sieves above the tube cones. 8 holes were drilled into the tube cones to guarantee sufficient aeration of the soils. Aliquots of sieved and homogenized field-moist soil were centrifuged (1 min, 187 g in a swing out rotor) into the test tubes to reach a final volume of 30 ml. The microcosms were kept under controlled

conditions in a climate chamber with a 15 h/9 h day/night cycle at 21/18°C temperature and 55% relative air moisture. During 14 d of pre-equilibration the soil water content (WC) of both soils was adjusted gravimetrically to 62% water filled pore space (WFPS). In detail 28.8 g DW of soil Purkersdorf were adjusted to 23.6% WC (% of dry weight), and 25.8 g DW of soil Niederschleinz to 19.2%. Supplementary lighting was provided via eight 400 W daylight lamps.

Seeds of barley (*Hordeum vulgare* L. cv. *Morex*) were germinated on moist filter paper for 2 d and seedlings were then planted in the microcosms (one plant per microcosm). During the experimental period the WC of the soils was adjusted gravimetrically twice a day.

2.2. Fertilization and ^{15}N tracing

Three experiments were performed using the same protocol with 3 different sources of N. A solution of 6.25 mM K_2HPO_4 and either 12.5 mM NH_4NO_3, 25 mM NH_4Cl or 25 mM KNO_3 were mixed and used for fertilization. 3 d before planting 1.6 ml of these mixtures were applied to each microcosm and 5 d after planting 1.2 ml, resulting in a total of 1 mg of N, 0.55 mg of P and 1.4 mg of K in all treatments. To determine gross rates and immobilization by microbes ^{15}N was added as $^{15}NH_4Cl$ and $K^{15}NO_3$ separately within each fertilizer treatment. Total amounts of ^{15}N per vial were 22.05 µg $^{15}NH_4^+$-N or $^{15}NO_3^-$-N in the NH_4NO_3 fertilizer treatment, 44.1 µg $^{15}NH_4^+$-N or 8.8 µg $^{15}NO_3^-$-N in the NH_4Cl treatment and 8.8 µg $^{15}NH_4^+$-N or 44.1 µg $^{15}NO_3^-$-N in the KNO_3 treatment. Homogenous distribution of applied ^{15}N was ensured by inserting a 7-cm long side-hole needle to the bottom of the soil cores in 4 positions and slowly injecting the labelled solution (400 µl each injection) while withdrawing the needle (Pörtl et al., 2007). This technique has already been proved to guarantee uniform ^{15}N labelling in the microcosms to meet the assumptions for ^{15}N tracing studies (Inselsbacher et al., 2009). Plant and soil samples were taken 4 h, 1, 2, 3, 6 and 8 d after the second fertilization event.

2.3. Chemical analysis

At each harvest plants were separated into shoots and roots and briefly rinsed with distilled water. Plant material was oven-dried (70°C for 48 h) and weighed. Soil of each microcosm was mixed and homogenized before further analyses. An aliquot (4 g) of soil was dried at 70°C and weighed to determine soil moisture. Dry soils and plants were ground in a ball mill

(Retsch MM2000) for total N and ^{15}N analysis by isotope ratio mass spectrometry (IRMS) using an elemental analyser (EA 1110, CE Instruments) connected in continuous flow-mode to a gas isotope ratio mass spectrometer (DELTA^{PLUS}, Finnigan MAT).

Another aliquot (2 g) of homogenized soil was extracted in 15 ml $CaSO_4$ (10 mM) and subsequently anions were determined by ion chromatography (DX 500, Dionex, Vienna, Austria) and conductivity detection. NO_3^- was separated on an anion exchange column (AS11, 250 x 4 mm i.d., Dionex, Vienna, Austria) after chemical suppression (ASRS-Ultra, Dionex) and linear NaOH gradient elution (0.5 mM to 37.5 mM within 10 min at a flow rate of 2 ml min^{-1}, with a column temperature of 35°C). NH_4^+ was extracted from aliquots (6 g) of homogenized soil with 45 ml KCl (1 M) and determined by a modified indophenol reaction method (Kandeler and Gerber, 1988).

Microbial biomass N in soils was analyzed by chloroform fumigation-extraction technique as described by (Amato and Ladd, 1988) and was calculated as the difference in N concentration between fumigated and non-fumigated soil samples. Briefly, aliquots of fresh soil (6 g) were fumigated over chloroform (ethanol-free) for 24 h at 22°C. Both, fumigated and non-fumigated soil samples were extracted with 45 ml K_2SO_4 (0.5 M) for 60 min before filtering. Total dissolved N and C in the KCl extracts were determined by an automated C analyzer (Shimadzu, TOC-VCPH, Japan) and a total N measuring unit (Shimadzu, TNM-1, Japan). A conversion factor for microbial biomass N (K_{EN}) of 0.45 was applied to take account of incomplete extraction (Jenkinson et al., 2004).

2.4. Soil DNA isolation and genome equivalent measurements by quantitative PCR

DNA was extracted from 0.5 g of soil taken from three replicate test tubes per sampling time by using the FastDNA® Spin Kit for Soil (MP Biomedicals, Solon, Ohio, USA) as described by the manufacturer. Purified DNA was quantified photometrically (Nanodrop ND-1000, Nanodrop Technologies, Wilmington, DE, USA). SYBR-Green quantitative PCR assays were performed in an iCycler iQ5 Multicolor Real Time PCR Detection System (BIO-Rad Laboratories) using primer pair 16S0101F (ACTGGCGGACGGGTGAGTAA)/16S0537R (CGTATTACCGCGGCTGCTGG) (Schmalenberger et al., 2001) for the quantification of bacteria and primer pair NSI1 (GATTGAATGGCTTAGTGAGG) /5,8S (CGCTGCGTTCTTCATCG) (Martin and Rygiewicz, 2005;Vilgalys and Hester, 1990) for the quantification of fungi.

Amplification of genes encoding the ammonium monooxygenase catalytic subunit A (*amoA*) was accomplished using primers amoA1F /amoA2R (Rotthauwe et al., 1997) for bacteria and Arch-amoAF/Arch-amoAR (Francis et al., 2005) for archaea, yielding fragments of 491 bp and 635 bp length, respectively. 25 µl reactions were composed of 12.5 µl 2x IQ ^{TM} SYBR$^{®}$- Green Supermix (BIO-Rad Laboratories), 0.4 µM of each primer and 0.2 mg/ml BSA. Standards and samples were processed in triplicates. The thermocycler program was set on 95°C for 3 min (single time denaturation) followed by 40 cycles of 95°C for 10s, annealing at 64°C for bacteria or 60°C for fungi for 30s, and subsequent 72°C for 30s. Data collection point was at 72°C. *amoA*/Arch-*amoA* PCR was carried out using the following protocol: 95°C for 3 min (single time denaturation), followed by 45 cycles of 95°C for 1 min, 57°C (bacteria) or 53°C (archaea) for 1 min, 72°C for 1 min and data collection at 78°C for 1 min. Melting curve analysis was performed in order to confirm the specificity of the PCR product. A two-times dilution series of one representative of each soil was carried out to test for inhibition. No inhibiting effects were observed for any of the samples. PCR products of each targeted gene were cloned (Strataclone PCR cloning Kit, Stratagene) and sequenced to proof specificity. Plasmids for each functional gene were isolated using the Quantum Prep Plasmid Miniprep Kit (BIO-Rad) and DNA concentrations were determined by spectroscopy to calculate copy numbers. Standard curves from serial dilutions of known amounts of the target genes were generated for each run, showing correlation coefficients (R^2) of 0.996 to 0.999 and PCR efficiencies of 92 to 102%. As standards for bacteria and fungi pure culture genomic DNAs from *Pseudomonas fluorescens* AHN1 and *Cadophora finlandica* PRF15 (Gorfer et al., 2007) were used, respectively. For further calculations, DNA concentrations were converted to genome numbers based on sizes of published genomes.

2.5. Gross transformation and ^{15}N uptake rates

Gross N mineralization, gross nitrification and gross NH_4^+ immobilization rates, as well as total NH_4^+ and NO_3^- consumption rates were calculated using the equations for ^{15}N pool dilution assays developed by Kirkham and Bartholomew (1954) as modified by Wessel and Tietema, (1992) and Bengtson et al. (2006). Soil samples were taken 4 h and 24 h after label application, homogenized and aliquots (6 g) of soil were extracted with 45 mL KCl (1 M) for 1 h. All extracts were kept at -20°C until further analysis. NH_4^+ from the KCl extracts was isolated for ^{15}N analysis by a modified microdiffusion technique (Sorensen and Jensen, 1991). Briefly, the KCl extracts were transferred to glass bottles (50 ml) and NH_4^+ was converted to

NH_3 by addition of ~200 mg MgO. NH_3 was captured in acid traps (glass-fibre filter discs containing 10 µl of 2.5 M $KHSO_4$, enclosed in PTFE tapes) during 5 d of incubation on a shaker at 37°C. Acid traps were removed and dried for 3 d over concentrated H_2SO_4 in a desiccator. Dried filter discs were removed from the PTFE sealing and folded into tin capsules for subsequent analysis of ^{15}N contents using IRMS. Soil microbial biomass ^{15}N was determined by converting total N in K_2SO_4 extracts of non-fumigated and fumigated soils to NO_3^- by alkaline persulfate digestion (Doyle et al., 2004). The ^{15}N abundances in NO_3^- after persulfate digestion as well as NO_3^- in KCl extracts were determined by SPINMAS system (Stange et al., 2007). Briefly, nitrate in aqueous samples was reduced in the SpinMas system (**S**ample **P**reparation for **I**norganic **N**itrogen by **MAS**s Spectrometry) to NO with V(III)Cl_3 solution (Merck, Darmstadt, Germany) in acidic medium (HCl; Merck, Darmstadt, Germany) ($NO_3^- + 3V^{3+} + 4H^+ \rightarrow NO + 3V^{4+} + 2H_2O$). The NO gas produced was transferred in a permanent He carrier stream from the vial via an open split to the inlet capillary of the quadrupole mass spectrometer (GAM 400, InProcess Instruments GmbH, Bremen, Germany). For nitric oxide the ion currents [I] at m/z 30 and 31 were measured to calculate the ^{15}N abundance of the NO_3^-.

Rates of dissimilatory nitrate reduction to ammonium (DNRA) during the first 24 h were calculated using the soil cores that received $^{15}NO_3^-$ according to (Silver et al., 2001). Briefly, DNRA was determined as the difference in the $^{15}NH_4^+$ atom% between samples taken 4 h and 24 h after label application, multiplied by the mean NH_4^+ pool size during this interval, and corrected for the mean residence time in the NH_4^+ pool during the interval. This was then divided by the mean $^{15}NO_3^-$ atom% during the interval to account for the isotopic composition of the source pool. Mean residence times of the $^{15}NH_4^+$ pool were estimated by dividing the initial NH_4^+ pool by the rate of gross NH_4^+ consumption using data from the $^{15}NH_4^+$ additions (Silver et al., 2001). Mean residence times of the $^{15}NO_3^-$ pool were calculated similarly by dividing the initial NO_3^- pool by the rate of gross NO_3^- consumption.

N uptake by plants and by microbial biomass was estimated as the increase of ^{15}N recovered in biomass during the first 4 h and 24 h, divided by the average atom% ^{15}N of available NH_4^+ and NO_3^- pools during these time intervals.

2.6. Calculations and statistical analysis

^{15}N recovery for both plants and microbial biomass as well as in NH_4^+ and NO_3^- pools was calculated as:

^{15}N recovery (%) = (amount ^{15}N in sample / total ^{15}N added per microcosm) * 100

Data were analysed using factorial ANOVA followed by Tukey's HSD post-hoc test with Statistica 6.0 (StatSoft Inc., Tulsa, OK, USA). When necessary, data were either square root- or $\log_{10}$- transformed prior to analysis to meet the assumptions of ANOVA after testing normality using Kolmogorov-Smirnov test and homogeneity of variances using Bartlett's test. The effects of adding different forms of N on plant and microbial biomass was analysed by three-way ANOVA with soil type, N form and harvest time as independent variables. The effects on soil processes were analysed using a two-way ANOVA with soil type and N form as independent variables and a three-way ANOVA with soil type, N form and type of process as independent variables. Differences between plant and microbial uptake of NH_4^+ and NO_3^- were analysed by one-way ANOVA each for 4 h and 24 h after fertilization. The influence of fertilizer N form on plant and microbial uptake rates were analysed by two-way ANOVA with soil type and N form as independent variables and four-way ANOVA with soil type, N form, N source (uptake of NH_4^+ or NO_3^-) and fraction (uptake by plants or microbes). Data of ^{15}N recovery in plants and microbial biomass was analysed by four-way ANOVA with soil type, N form, tracer (applied $^{15}NH_4^+$ or $^{15}NO_3^-$) and harvest time as independent variables.

3. Results

3.1. Plant and microbial biomass and microbial community composition

Plant biomass significantly increased during 8 d in both soils in all fertilizer treatments (Fig. 1). During the first 2 d after N fertilization, however, there was no significant difference in plant biomass between applied N forms and between soils (one-way ANOVA, $P>0.05$). After 3 d plant biomass was significantly lower in both soils in the NH_4Cl treatment compared to NH_4NO_3 and KNO_3 as N source. This difference became stronger until the end of the experimental period and at the last sampling time plant biomass was significantly higher in KNO_3 treatments than in NH_4NO_3 and NH_4Cl treatments in both soils (one-way ANOVA, $P<0.05$). Additionally, results from three-way ANOVA (Table 2) showed that plant biomass accumulation started significantly later when N was solely applied as NH_4^+ in both soils. Within this treatment, plant biomass accumulation was significantly faster in soil Niederschleinz resulting in higher plant biomass after 8 d of plant growth (Fig. 1).

Unlike for plant biomass, the most important factor affecting microbial biomass N was not harvest time (F = 38.3, $P<0.0001$) but soil type (F = 229.6, $P<0.0001$) followed by fertilizer N form (F = 121.3, $P<0.0001$, Table 2). Initial microbial biomass N was higher in soil Niederschleinz than in soil Purkersdorf, and was highest in the NH_4Cl treatment for both soils (Fig. 1). Microbial biomass changed significantly during the following 8 d, with greater shifts in soil Niederschleinz (Table 2). During the first 2 d after N fertilization microbial biomass N declined after NH_4Cl or NH_4NO_3 application in both soils, while no significant change was found in the KNO_3 treatment. Like microbial biomass N, numbers of both, bacterial and fungal genome equivalents were higher in soil Niederschleinz compared to Purkersdorf and were strongly affected by fertilizer N form but were less variable over time than observed for microbial biomass N (Table 3). In both soils bacterial genome equivalents were higher than fungal genome equivalents, with the ratio between bacterial and fungal genome equivalents being in the same range in the two soils. Nevertheless there were different effects of fertilizer N form on fungal genome equivalents between the two soils (Table 3). In soil Niederschleinz fungal genome equivalents were lowest in the NH_4Cl treatment and increased with incubation time in all fertilizer treatments, while in soil Purkersdorf initially highest abundance was found in the NH_4Cl treatment which declined over time, and initially lowest numbers in the NH_4NO_3 treatment were increasing strongest (Table 3). Taken together this resulted in different effects of fertilizer N form on fungal to bacterial genome equivalent ratios in the two soils (Fig. 2).

Bacterial ammonium monooxygenase catalytic subunit A (*amoA*) copies were generally higher in soil Purkersdorf, while archaeal *amoA* copies were markedly higher in soil Niederschleinz (Table 3), indicating a strongly different community composition of NH_3 oxidizers in the two soils. While archaeal *amoA* abundance changed during the time course and was strongly affected by applied fertilizer N form, bacterial *amoA* abundance did not change with time and was hardly influenced by fertilizer N form (Table 3). Both, bacterial and archaeal *amoA* numbers were lowest in the NH_4Cl treatment which was reflected in decreased archaeal/bacterial *amoA* ratios, especially in soil Niederschleinz (Fig. 2).

3.2. N transformation rates

In general, 24 h after fertilization soil Niederschleinz was more active than soil Purkersdorf, exhibiting significantly higher gross N mineralization, consumption and nitrification rates, as

well as shorter mean residence times of soil NH_4^+ and NO_3^- pools (Table 4). With the exception of NH_4^+ immobilization, all investigated processes were significantly affected by fertilizer N form (Table 4, Table 5). In both soils NH_4^+ application significantly increased NH_4^+ consumption rates, mean residence times of NH_4^+ and surprisingly also that of NO_3^-. Additionally, in soil Purkersdorf, but not in soil Niederschleinz, NO_3^- consumption rates strongly decreased after applying NH_4^+ compared to NH_4NO_3 or NO_3^-The effects of NO_3^- fertilization were less pronounced, only increasing NO_3^- consumption rates, though not being significant ($P > 0.05$). Clearly, in both soils the dominating N transformation process was nitrification which was markedly higher than gross N mineralization in all treatments (Table 4). The importance of nitrification was also reflected in proportional ^{15}N recoveries in NH_4^+ and NO_3^- pools (Fig. 3). In all fertilizer treatments the initial $^{15}NH_4^+$ tracer decreased rapidly in the NH_4^+ pool and was concomitantly recovered in the NO_3^- pool. Rates of dissimilatory nitrate reduction to ammonium (DNRA), on the other hand, made up only <3% of nitrification rates (Table 4), which was again reflected in ^{15}N recoveries, as there was no significant increase of ^{15}N in the NH_4^+ pool after applying $^{15}NO_3^-$ tracers (Fig. 3). Gaseous losses of N from the system as N_2O were low and made up less than 0.1% of total dissolved N (data not shown).

3.3. ^{15}N recovery in plants and microbes

Proportional ^{15}N recovery in both, plants and microbes, did not differ significantly between the two soils (Fig. 4, Table 6). Plant ^{15}N content increased continuously during 8 d after fertilization (and ^{15}N tracer application), reaching a total recovery of 45-80% of initially applied ^{15}N. On the contrary, ^{15}N immobilisation in microbes was highest directly (4 h) after ^{15}N application, reaching up to 30% of total applied ^{15}N, and subsequently decreased to less than 10% at the end of the experiment. While ^{15}N recovery in microbes was generally higher or similar compared to plants up to 2 d after fertilization, after 3 d significantly more ^{15}N was recovered in plants in all treatments and both soils (Fig. 4). Independent of fertilizer N form, ^{15}N recovery in plants was significantly higher when ^{15}N tracer was added as $^{15}NO_3^-$ compared to $^{15}NH_4^+$. This difference was even more pronounced in soil Purkersdorf than in soil Niederschleinz. No such difference could be found in microbial ^{15}N recovery. Although there was a trend towards greater ^{15}N recovery in microbes from soils which received $^{15}NH_4^+$ during the whole experimental period, this difference was not significant in any of the cases (four-way ANOVA, Tukey's HSD post-hoc test).

However, the form of applied fertilizer N significantly affected the rate of ^{15}N recovery in plants and microbes (Table 6). Significantly more ^{15}N was recovered in plants when soils were fertilized with KNO_3 or NH_4NO_3, compared to NH_4Cl. Recovery of ^{15}N in microbial biomass, on the other hand, was highest in soils fertilized with NH_4Cl or KNO_3. Further, there were significant differences in ^{15}N recovery in plants, depending on soil type, fertilizer N form and applied ^{15}N tracer. No such dependencies were found for ^{15}N recovery in microbial biomass. Despite these effects, results from four-way ANOVA revealed that harvest time was clearly interacting with all other factors. When harvest time was therefore included in the data analysis as a covariate, N form and ^{15}N tracer still remained the most significant factors affecting ^{15}N recovery. This indicates that the patterns of ^{15}N recovery in plants and microbes significantly differed between fertilizer and ^{15}N tracer form, independent of harvest time.

3.4. Plant and microbial uptake of inorganic N

Uptake rates from either the soil NH_4^+ or NO_3^- pool by plants and microbes were only calculated for the first two sampling times, 4 h and 24 h after fertilization, as, due to the high N transformation rates in the soils, it was not possible to reliably dissect in which form ^{15}N was taken up at later time points (particularly in the NH_4^+ and the NH_4NO_3 treaments). At both times plants took up significantly more NO_3^- than NH_4^+ in both soils (Fig. 5, Table 4). While the rates of NH_4^+ and NO_3^- uptake were independent of applied N form 4 h after fertilization, there was a significant effect after 24 h. Plants took up most NO_3^- after KNO_3 and NH_4NO_3 fertilization and most NH_4^+ after NH_4Cl and NH_4NO_3 fertilization in both soils (two-way ANOVA, $P<0.0001$, Table 4). Surprisingly, microbes preferentially took up NO_3^- at both sampling times, but this difference was only significant 4 h after fertilizing with NH_4NO_3. Further, there was no significant influence of fertilizer N form on microbial NH_4^+ and NO_3^- immobilization rates, except in soil Niederschleinz, which exhibited higher NO_3^- immobilization rates after NH_4^+ fertilization.. In both tested soils no inhibitory effect of NH_4^+ on NO_3^- uptake rates, or of NO_3^- on NH_4^+ uptake rates could be observed at any time for both, plant and microbial uptake.

3.5. Short-term competition for fertilizer N

Microbes immobilized significantly more inorganic N than plants did 4 h after fertilization (Table 7), but due to a significant decrease in microbial N immobilization rates, plants already

took up more N 24 h later, except after fertilization with NH_4Cl. In this N treatment there was no significant difference between plant and microbial uptake of NH_4^+ or NO_3^- in soil Purkersdorf, and in soil Niederschleinz microbial N immobilization still exceeded plant uptake for both NH_4^+ and NO_3^- ($P<0.05$). These findings were also expressed by a significant interaction effect of fertilizer N form with plant vs. microbial N uptake. Further, there was a significant interaction effect between NH_4^+ vs. NO_3^- uptake and plant vs. microbial N uptake 24 h after fertilizer application, which emphasizes that plants preferred NO_3^- for uptake, while microbes took up NH_4^+ and NO_3^- at the same rates.

4. Discussion

A major goal of the present study was to assess the intensity of competition for inorganic N between barley plants and soil microbes in two agricultural soils, and its dependence on the form of applied fertilizer N. We found that 8 d after N application crop plants were the major sink for inorganic fertilizer N in both soils. At this time 45-80% of initially applied ^{15}N fertilizer was recovered in the plants compared to only 1-10% recovered in soil microbial biomass, proving that plants were the strongest competitors for fertilizer N under our experimental conditions. Plants out-competed soil microbes already 3 d after N application and only within the first 4 h of fertilizer application soil microbes recovered significantly more ^{15}N than plants. This effect was due to much higher initial N uptake rates of soil microbes for both NH_4^+ and NO_3^- compared to plants in all fertilizer treatments. During the initial 4 h microbes took up 1.5 – 4.3 times more N than plants. These findings fit well with the general opinion that soil microbes are stronger competitors for N than plants at a short term scale, presumably because they have higher substrate affinities, larger surface area to volume ratios, and faster growth rates than plants (e.g. Lipson and Näsholm, 2001). However, after 24 h N uptake rates of microbes had already decreased substantially and did not differ significantly from plant N uptake rates. Moreover, during the following week microbial biomass as well as microbial ^{15}N recoveries appeared to reach a steady state or even decreased, probably due to insufficient C availability to maintain the fast initial uptake and growth rates, as has been suggested previously (Hodge et al., 2000; Jackson et al., 2008).

At the moment we can not fully exclude that microbial ^{15}N recoveries 4 h after label application may be overestimated resulting from the applied methodological procedures. To calculate microbial biomass N a conversion factor (K_{EN}) of 0.45 was applied for incomplete extraction (Jenkinson et al., 2004) at all measured time points. But as freshly assimilated ^{15}N

label may be more chloroform labile than the total microbial N pool (Schimel and Chapin, 1996; Clemmensen et al., 2008), the applied correction factor may have led to a maximal two-fold overestimation of microbial biomass N conversion.

A multitude of studies, including molecular studies using bacterial and fungal model organisms, showed that soil microbes prefer NH_4^+ over NO_3^- as N source (e.g. Azam et al., 1993; Burger et al., 2003, Berger et al. 2008). Interestingly, we found no evidence that soil microbes preferentially utilize either NH_4^+ or NO_3^-, as uptake rates for both N forms did not differ significantly at any time point (but NO_3^- preference at 4 hours in the NH_4NO_3 treatment).

There was a trend towards uptake rates for NO_3^- being even higher than for NH_4^+, which was unexpected especially in the NH_4^+ treatment. Previous studies argued that higher uptake rates of NO_3^- by microbes compared to NH_4^+ may be related to the higher mobility of NO_3^- in soils resulting in higher availability of NO_3^- for microbial uptake (Jackson et al., 1989; Song et al., 2007). In our study, however, this was probably only of minor importance because 4 h after application of NH_4^+, when NH_4^+ concentrations were in the range of 5-10 mM (data not shown), microbial uptake rates of NH_4^+ were nevertheless not significantly different from NO_3^- uptake rates. The occurrence of microsites of high available C and low NH_4^+ where NO_3^- assimilation is abundant (Schimel et al., 2004; Booth et al., 2005) is unlikely in our system, as the fertilizer was distributed homogenously throughout the microcosm (Inselsbacher et al., 2009), but nevertheless cannot be excluded neither. Additionally, recoveries of ^{15}N in the microbial biomass were similar irrespectively of the form of N applied ($^{15}NH_4^+$ vs. $^{15}NH_4NO_3$ and $^{15}NO_3^-$ vs. $NH_4^{15}NO_3$). Thus the presence of one N form did not affect the uptake of the other. This pattern is somewhat puzzling, as at least higher concentrations of NH_4^+ can inhibit NO_3^- assimilation (Rice and Tiedje, 1989; McCarty and Bremner, 1992; Berger et al. 2008). One explanation to this discrepancy might be that, while NH_4^+ represses the synthesis of assimilatory NO_3^- reductase, this enzyme may remain active in fungal cells, allowing NO_3^- assimilation to continue for some time after NH_4^+ addition (Jackson et al., 1989). Moreover, absence of NH_4^+ repression of NO_3^- uptake was indeed found for phytopathogenic fungi from the genus *Fusarium* (Celar, 2003). The composition of the soil microbial community, i.e. the bacterial/fungal ratio, may therefore be an important factor determining rates of NH_4^+ and NO_3^- immobilization (Bengtson et al., 2006; Myrold and Posavatz, 2007). Based on the results from quantitative PCR, however, around 10 to 20-fold more bacterial compared to fungal genome equivalents were present in both tested soils, indicating that also bacteria were responsible for microbial NO_3^- immobilization. When

taking into account the high rates of nitrification in both soils, it seems likely that the NH_4^+ pool in the soils was oxidized fast enough not to allow negative effects of initially high concentrations of NH_4^+ on NO_3^- assimilation. This possibility is also supported by the fact that plant NO_3^- uptake was higher than NH_4^+ uptake by a factor of three even under NH_4Cl-fertilization conditions. This study therefore suggests that in agricultural systems with high N inputs the soil microbial community structure could become more and more adapted to high concentrations of NO_3^- over longer time periods and could shift towards enhanced reliance on NO_3^- for their N supply.

The presence of microbial preferences towards NO_3^- as N source could reflect an N limitation of the heterotrophic microbes due to strong competition with nitrifiers and plants, as demonstrated for temperate grasslands (Harrison et al., 2007; Harrison et al., 2008) and agricultural soils (Burger et al., 2003). However, in this study only the form of fertilizer N was changed and not the concentration of N, therefore it still has to be tested, if N limitation of heterotrophic microbes occurred and decreases with higher amounts of fertilizer applied. However, the fast decline in microbial N immobilization after 4 hours points to C rather than N limitation of microbes. Unpublished observations from our laboratories support this hypothesis: the expression of both bacterial (nas) and fungal (niaD) nitrate reductase genes in the two agricultural soils tested here is dramatically increased by the addition of different carbon sources.

Taking into account the ^{15}N recoveries of plants during the whole incubation period, it becomes clear that they were the major sink of fertilizer N. There is a considerable body of published evidence that in various ecosystems plants gain most of the added N in the long term, as they often acquire N which has initially been immobilized by soil microbes and thereafter was gradually released into the soil during microbial turnover (e.g. Hodge et al., 2000; Schimel et al., 2004; Harrison et al., 2007). In our study this was not tested, as the incubation time of 8 d would be too short to allow soil microbes to incorporate and release a substantial fraction of fertilizer ^{15}N through turnover and remineralization. Further, if ^{15}N taken up as $^{15}NO_3^-$ by soil microbes would have been assimilated and remineralized (or exuded as NH_4^+ produced by DNRA) this ^{15}N would have been detectable in the NH_4^+ pool. As this was not the case we assume that remineralisation (and DNRA) was not significantly contributing to the NH_4^+ pool during this short time period. Therefore, plants were directly and more efficiently competing for fertilizer N than microbes within a few days. Plants, unlike soil microbes, are not limited by carbon, and could therefore incorporate more N than microbes, while the microbial N sink "saturated" after a few hours due to insufficient

available carbon. The actual rate of ^{15}N recovery in plants was dependent on the form of applied fertilizer N, with lowest rates after NH_4^+ addition. After 8 d of growth, plant biomass was significantly lower in the NH_4^+ treatment compared to the NH_4NO_3 and KNO_3 treatments. This is in accordance with previous studies, showing that plants grown on NO_3^- are frequently superior to those grown on NH_4^+, e.g. the shoot length of potted barley following NH_4^+ application was significantly lower compared to plants grown on NO_3^- or NH_4NO_3 fertilized soil after 6 weeks of plant growth (Söderberg and Baath, 2004). NH_4^+ nutrition is known to reduce growth of plants due to effects related to rhizosphere acidification (von Wiren 2000). In addition, in our system, we can expect a considerable degree of competition for NH_4^+ between plants and soil microbes, especially NH_3 oxidizers.

Besides the positive effects of NO_3^- on plant growth, NO_3^- was also the preferred N source for plant uptake, independent of the applied fertilizer N form. This was again unexpected in the NH_4^+ treatment as we assumed that at high availabilities of NH_4^+, plants would prefer NH_4^+ over NO_3^-. One likely explanation for this could be that both soils had high background concentrations of NO_3^- to meet plant N demands. Soil pre-treatment (ca. half of total N fertilizer was applied 3 d before planting) elevated soil NO_3^- by nitrification of NH_4^+ deriving from this first fertilization dose. The fact that NO_3^- is generally much more mobile in soil compared to NH_4^+ (Hodge et al., 2000), combined with increased mass-flow of NO_3^- driven by plant transpiration (Jackson et al., 1989) may have further contributed to the strong preference for NO_3^- uptake by plants in situ. Additionally, the plant available NH_4^+ pool could have been further depleted by ion-exchange binding to soil organic matter or soil particles (Murphy et al., 2003; Song et al., 2007).

Even if a small proportion of NH_4^+ was bound to soil particles, and plants as well as heterotrophic microbes took up NH_4^+, the major fate of NH_4^+ in both soils was clearly nitrification. Our results indicate that NH_3 oxidizers were the strongest competitors for NH_4^+. During the first day of N application gross nitrification was 5 to 75-fold higher than plant and microbial NH_4^+ uptake in soil Niederschleinz and 3 to 23-times higher in soil Purkersdorf. Our results further indicate that the rate of gross nitrification was not affecting NH_4^+ uptake rates by plants or microbes *per se*, as these were similar in both soils, although gross nitrification rates were significantly higher in soil Niederschleinz. However, gross nitrification had a marked influence on N cycling in both soils, by rapidly decreasing the NH_4^+ pool and increasing the amount of the more mobile NO_3^-. On the one hand, in natural systems this NO_3^- could be subject to hydrological and gaseous losses by leaching and denitrification, and therefore the rate of nitrification is a major factor controlling N losses in

agriculture. Moreover, a small fraction of N can be directly lost in gaseous form as NH_3 at alkaline soil conditions or as NO, N_2O and N_2 during the nitrification process. On the other hand, in our study NO_3^- was the preferred N form for plant uptake. Due to higher nitrification rates in soil Niederschleinz, the ^{15}N label deriving from the NH_4^+ pool was rapidly transformed to NO_3^- and recovered in plants. Therefore, while in both soils ^{15}N recovery in plants was significantly higher when the label was applied as $^{15}NO_3^-$ compared to $^{15}NH_4^+$, in soil Niederschleinz this difference was less pronounced, because the initially applied $^{15}NH_4^+$ was faster nitrified to $^{15}NO_3^-$. The high rates of nitrification were also responsible for the low mean residence time of the NH_4^+ pool (2.7 ± 0.5 d; mean ± SE, n=30), which was turning over significantly faster than the NO_3^- pool (10.5 ± 1.8 d; mean ± SE, n=30) in all treatments. This lends support that in agricultural soils nitrification is the major fate of NH_4^+, and due to the strong depletion of the NH_4^+ pool, the system becomes more and more NO_3^- dominated (e.g. Chen and Stark, 2000; Schimel et al., 2004).

In both soils gross nitrification increased significantly with NH_4^+ amendment, indicating that the activity of autotrophic NH_3 oxidizers was limited by NH_4^+, while the population size of autotrophic nitrifiers was not affected or decreased in the NH_4^+ treatment. In a recent study five unfertilized arable soils (including soil Niederschleinz and Purkersdorf) exhibited higher gross nitrification rates than gross mineralization rates (Inselsbacher et al., 2009) as also evident in this experiment in non-NH_4^+ amended soils. These findings provide evidence that in agricultural soils heterotrophic nitrification may account for a significant part of total gross nitrification, akin to forest or grassland soils (e.g. Pedersen et al., 1999; De Boer and Kowalchuk, 2001; Islam et al., 2007). Until now heterotrophic nitrification was assumed to be of little significance in arable soils (De Boer et al., 2001), as also indicated by a kinetic isotope model approach (Stange and Döhling, 2005). However, the latter authors debated that during soil pre-treatment, especially during drying, the microbial community could shift in favour of heterotrophic fungi, increasing the ratio of heterotrophic to autotrophic nitrifiers. In our study, however, soils were always kept field-moist at 4°C until the start of experiments. Nevertheless, sieving and packing the soils into the microcosms might have changed microbial communities compared to natural conditions leading to a possible overestimation of heterotrophic nitrification. Additionally to the potential contribution of heterotrophic microbes to nitrification, another significant part of total nitrification may have derived from archaea as in soil Niederschleinz which had highest nitrification rates archaeal-to-bacterial *amoA* gene copy numbers were significantly higher as in soil Purkersdorf. The role and especially the significance of archaea in soil N transformation processes is yet not well

understood, but our results indicate that a high abundance of archaeal *amoA* is positively related to gross nitrification rate. Recently AOA were found to numerically dominate the ammonium oxidizing bacteria in various soil types by up to 3,000 -fold (Leininger et al., 2006; Nicol and Schleper, 2006; Nicol et al., 2008; Wang et al., 2009) pointing to a major role in nitrification in arable soil habitats. DNA stable isotope probing and net nitrification measurements in a NH_4^+ fertilized arable maize cropped soil indicated the dominance of bacterial above archaeal ammonium oxidation (Jia and Conrad, 2009). In contrast, in an antibiotic inhibition study in two arable soils a functional redundancy of AOA and AOB was postulated (Schauss et al., 2009). AOA showed a higher stress resistance upon antibiotic application than AOB and lower turnover rates were suggested to be compensated by higher copy numbers assigning a `backup`function to AOA to insure ammonium oxidation under different, more unfavourable conditions (Schauss et al., 2009). To get better insight into the real relevance of AOA, studies on the level of gene expression would be necessary, since abundance is only a surrogate for potential activiy but is not necessarily directly related to it. Autotrophic nitrifiers very likely live in close association with mineralizers and immediately use the NH_4^+ released in agricultural soils. This would explain why NO_3^- became the dominant N form in both soils, and why plants and heterotrophic microbes preferably took up NO_3^- instead of NH_4^+. Clearly, in the heterogeneous soil matrix manifold N transformations, including gross N mineralization and nitrification, are running simultaneously at varying rates over time. Many of these processes are responsible for losses of N to the atmosphere (e.g. denitrification, nitrification, nitrifier-denitrification, anaerobic NH_4^+ oxidation), while others (e.g. DNRA) have the potential to reduce gaseous N losses (Huygens et al., 2007). In our study 8 d after fertilization total recovery of ^{15}N was 84.8 ± 9.9% (mean ± SD, n = 60) of initially applied ^{15}N on average. This means that 15% of the ^{15}N label applied was not recovered in plants and soils (including microbial biomass) and consequently was lost from the system which is in the general range of agricultural soils where up to 25% of applied fertilizer is lost to the atmosphere (Galloway et al., 2004). Due to methodological restrictions no gaseous losses other than N_2O emissions from the soils were measured which accounted for <1% of applied fertilizer N (data not shown). Soils in the microcosms were kept at 62% WFPS which allowed nitrification and denitrification to occur simultaneously. It is likely that NO_3^- was reduced at anaerobic microsites resulting in production and emission of N_2O and N_2. Even though N_2O emissions were low, it is well known that, compared to N_2 (or NO) emissions, N_2O often accounts for only a very small proportion of total gaseous losses (e.g.Spott et al., 2006; Scheer et al., 2009). Another major risk of fertilizer N loss is NO_3^-

leaching to aquatic systems (~20% of applied fertilizer N (Galloway et al., 2004)). Due to the fast N sequestration by plants the risk of N loss by NO_3^- leaching was decreasing rapidly as NO_3^- concentrations in the soils levelled off to less than 10% during three days time. Moreover, to meet the needs of the present study of constant soil moisture soil water contents were strictly controlled effectively preventing leaching. Therefore N losses (15% of applied fertilizer N during 8 days) must have been caused by emissions of nitrogenous gases. As it was not possible to measure N_2 (and NO) emissions during this experiment, additional mathematical approaches may help. ^{15}N tracing models have proved to be a powerful tool to gain further knowledge about the complex network of N transformation processes as well as microbial N immobilization in soils (e.g. Stange and Döhling, 2005; Müller et al., 2007; Rütting and Müller, 2008), but are still lacking the plant N uptake component. Our study, however, shows that plant N sequestration was markedly depleting the inorganic N pools in the soils. Therefore, incorporation of plant N uptake into existing soil ^{15}N models and subsequent combination with ^{15}N tracing studies could be of potential importance for providing valuable insights in fates of N in agricultural soil-plant-microbe systems. Reducing gaseous (and hydrological) losses from agricultural system still remains a major challenge and will require intense interdisciplinary research. To gain essential insights into mechanisms involved in the N cycle in agriculture and to further increase N fertilizer use efficiency of crop plants it will be necessary to combine biochemical and isotope techniques with molecular based analyses and modeling to elucidate fertilizer effects on the bacterial and fungal community structure and function.

Acknowledgements

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	Purkersdorf	Niederschleinz
Soil type	Gleyic Cambisol from sandy loamy flysch	Chernozem from Loess
Geographic location	48°12'25'' N 16°10'37'' E	48°35'59'' N 15°10'24'' E
Altitude (m. a. sl.)	248	244
Water condition	Moist	Moderately dry
Clay (%)	2	18
Silt (%)	65	74
Sand (%)	33	8
pH (H_2O)	6.6	7.7
$CaCO_3$ (%)	0.06	8.5
Exchange capacity (mval%)	11.2	15.4
Base saturation (%EC)	81.4	98.1
Bulk density (g DW cm^{-3})	1.06	0.96
Total C (mg C g^{-1} DW)	16.2	26.4
Total N (mg N g^{-1} DW)	1.6	1.9
C/N	10.0	14.2

Table 1 Physical and chemical characteristics of the top layer (0 – 15 cm) of two agricultural soils, Purkersdorf and Niederschleinz, collected from the vicinity of Vienna, Austria.

Source of variation	Plant DW		N mic	
	df	F	df	F
N form	2	194.2***	2	121.3***
Soil	1	28.2***	1	229.6***
Harvest time	5	392.1***	5	38.3***
N form x soil	2	0.3	2	15.4***
N form x time	10	29.4***	10	28.7***
Soil x time	5	4.9***	5	16.7***
N form x soil x time	10	0.9	10	18.7***

Table 2 F-statistics of three-way ANOVA of the effects of applied N form (NH_4NO_3, NH_4Cl, KNO_3), soil (Purkersdorf, Niederschleinz) and harvest time (4h, 1d, 2d, 3d, 6d, 8d after N application) on plant dry weight and microbial N content (n = 15) in soil microcosms. Sub: * $P<0.05$, ** $P<0.01$, *** $P<0.001$

Source of variation	Bacterial genome equivalents		Fungal genome equivalents		Archaeal AmoA		Bacterial AmoA	
	df	F	df	F	df	F	df	F
N form	2	45.6***	2	8.4***	2	41.5***	2	4.2*
Soil	1	122.0***	1	284.7***	1	220.5***	1	19.5***
Harvest time	4	6.3*	4	9.5***	5	5.0***	5	2.0
N form x soil	2	0.7	2	47.8***	2	24.3***	2	3.4*
N form x time	8	3.1**	8	5.0***	10	4.2***	10	2.2*
Soil x time	4	7.6***	4	6.0***	5	4.8***	5	0.4
N form x soil x time	8	3.0**	8	7.4***	10	4.5***	10	2.6**

Table 3 F-statistics of three-way ANOVA of the effects of applied N form (NH_4NO_3, NH_4Cl, KNO_3), soil (Purkersdorf, Niederschleinz) and harvest time (1d, 2d, 3d, 6d, 8d after N application) on bacterial and fungal genome equivalents and archaeal and bacterial ammonium monooxygenase catalytic subunit A (amoA) copies in soil microcosms (3 biological replicates in three technical repetitions).
Sub: * $P<0.05$, ** $P<0.01$, *** $P<0.001$

Gross rates	Purkersdorf Applied N form			Niederschleinz Applied N form			
	NH_4NO_3	NH_4Cl	KNO_3	NH_4NO_3	NH_4Cl	KNO_3	
Mineralization	10 (15)	19 (9)	4 (27)	124 (17) B	46 (3) A	12 (3) A	Soil *** N form *** Soil x N form **
Nitrification	61 (29)	104 (16)	23 (50)	171 (44) B	285 (82) C	50 (25) A	Soil *** N form ***
NH_4^+ consumption	78 (13) ab	114 (23) b	46 (18) a	193 (15) B	308 (17) C	63 (8) A	Soil *** N form *** Soil x N form ***
NO_3^- consumption	178 (25) b	45 (22) a	173 (32) b	147 (70)	146 (41)	167 (24)	Soil x N form *
NH_4^+ immobilization	17 (21)	10 (24)	23 (56)	22 (58)	23 (17)	14 (32)	
MRT NH_4^+	3.8 (0.6) ab	7.9 (1.7) b	1.8 (0.6) a	0.6 (0.1) A	1.2 (0.1) B	0.8 (0.2) AB	Soil *** N form *** Soil x N form *
MRT NO_3^-	5.7 (0.9) a	25.7 (8.4) b	9.4 (1.9) ab	1.1 (2.1) A	10.4 (2.6) B	11.1 (0.5) B	Soil * N form **
DNRA	0.7	3.6	0.1	0.5	6.8	0.2	
N uptake rates							
Plant NH_4^+ uptake	24 (2) ab	38 (8) b	4 (8) a	32 (5)	21 (2)	3 (33)	N form ***
Plant NO_3^- uptake	189 (18) b	81 (11) a	200 (25) b	120 (39)	58 (12)	176 (44)	Soil * N form ***
Microbial NH_4^+ uptake	11 (12)	14 (24)	1 (11)	13 (15)	58 (20)	1 (20)	
Microbial NO_3^- uptake	66 (38)	67 (11)	55 (43)	31 (46) A	145 (21) B	29 (7) A	

Table 4 Gross N transformation rates and plant and microbial inorganic N uptake rates 24 h after application of 3 different N fertilizers to soils Purkersdorf and Niederschleinz in microcosms. Gross transformation rates and plant and microbial N uptake rates are expressed as µg N microcosm^{-1} d^{-1}, mean residence time (MRT) is expressed in days. Values represent means of 5 microcosms. Significant effects were tested by two-way ANOVA followed by Tukey's HSD test (*** $P<0.001$, **$P<0.01$, *$P<0.05$, n = 5).

Source of variation	N transformation rate	
	df	F
N form	2	13.5^{***}
Soil	1	26.4^{***}
Process	3	17.2^{***}
N form x soil	2	11.2^{***}
N form x process	6	8.2^{***}
Soil x process	3	8.7^{***}
N form x soil x process	6	3.1^{**}

Table 5 F-statistics of three-way ANOVA of the effects of applied N form (NH_4NO_3, NH_4Cl, KNO_3), soil (Purkersdorf, Niederschleinz) and type of gross process (N mineralization, nitrification, NH_4^+ consumption, NO_3^- consumption) on transformation rates (n = 5) in soil microcosms.
Sub: * $P<0.05$, ** $P<0.01$, *** $P<0.001$

Source of variation	Plant ^{15}N recovery		Microbial ^{15}N recovery	
	df	F	df	F
N form	2	207.0***	2	6.5**
Soil	1	2.2	1	1.0
Tracer	1	304.2***	1	4.6*
Time	5	1532.9***	5	7.1***
N form x soil	2	41.3***	2	0.4
N form x tracer	2	0.6	2	13.0***
Soil x tracer	1	63.9***	1	0.5
N form x time	10	10.0***	10	6.6***
Soil x time	5	8.4***	5	4.9***
Tracer x time	5	4.1**	5	6.1***
N form x soil x time	10	4.8***	10	2.1*
N form x tracer x time	10	3.2***	10	4.0***
Soil x tracer x time	5	3.2***	5	2.8*
N form x soil x tracer x time	10	0.9**	10	2.7**

Table 6 F-statistics of four-way ANOVA of the effects of applied N form (NH_4NO_3, NH_4Cl, KNO_3), soil (Purkersdorf, Niederschleinz), applied ^{15}N tracers ($^{15}NH_4^+$, $^{15}NO_3^-$) and harvest time (4h, 1d, 2d, 3d, 6d, 8d after N application) on relative ^{15}N recovery (n=5). Non-significant interaction terms were excluded from the table.
Sub: * $P<0.05$, ** $P<0.01$, *** $P<0.001$

Source of variation	N uptake (4h)		N uptake (24h)	
	df	F	df	F
N form	2	0.5	2	0.02
Soil	1	0.3	1	0.3
N source (for uptake)	1	15.6^{***}	1	71.8^{***}
Fraction	1	5.3^{*}	1	14.9^{***}
N form x fraction	2	0.1	2	9.4^{***}
N source x fraction	1	2.9	1	11.9^{***}
N form x N source x fraction	2	0.3	2	6.9^{**}

Table 7 F-statistics of four-way ANOVA of the effects of applied N form (NH_4NO_3, NH_4Cl, KNO_3), soil (Purkersdorf, Niederschleinz), N source for uptake (NH_4 uptake, NO_3 uptake) and fraction (plant or microbial N uptake) on N uptake rates 4h and 24h after N addition in soil microcosms (n=5). Non-significant interaction terms were excluded from the table. Sub: $^{*} P<0.05$, $^{**} P<0.01$, $^{***} P<0.001$

Fig. 1. Plant biomass and microbial biomass N in soils Purkersdorf and Niederschleinz within 8 days of addition of three different inorganic N fertilizers (NH_4Cl, NH_4NO_3 and KNO_3). Soils were preincubated in microcosms for 14 days and seedlings of barley grown for 5 days before fertilization. Symbols and bars represent means ± SE, n = 15.

Fig. 2. Ratios of bacterial to fungal genome equivalents and archaeal to bacterial ammonium monooxygenase catalytic subunit A (amoA) copies in soils Purkersdorf and Niederschleinz within 8 days of addition of three different inorganic N fertilizers (NH_4Cl, NH_4NO_3 and KNO_3). Soils were preincubated in microcosms for 14 days and seedlings of barley grown for 5 days before fertilization. Symbols and bars represent means ± SE, n=9 (3 biological replicates with 3 technical repetitions).

Fig. 3. Proportional recovery of ^{15}N in soil pools of NO_3^- (circles) and NH_4^+ (triangles) in soils Niederschleinz (Nied, full symbols, solid lines) and Purkersdorf (Purk, open symbols, dotted lines) within 8 days of applying different N fertilizers. For each N fertilizer treatment (NH_4Cl, NH_4NO_3 and KNO_3) two experiments were conducted in parallel, applying traces of either $^{15}N\text{-}NH_4^+$ or $^{15}N\text{-}NO_3^-$ (given in brackets). Symbols and bars represent means ± SE, n = 5.

Fig. 4. Proportional recovery of ^{15}N from ^{15}N-labelled NH_4^+ (circles, solid lines) or NO_3^- (triangles, dotted lines) in soil microbial biomass (full symbols) and barley plants (open symbols) in soils Purkersdorf and Niederschleinz within 8 days of N fertilization. For each N fertilizer treatment two experiments were conducted in parallel, applying traces of either $^{15}N\text{-}NH_4^+$ or $^{15}N\text{-}NO_3^-$. Different N fertilization treatments are given in brackets. Symbols and bars represent means ± SE, n = 5.

Fig. 5. N uptake rates of NH_4^+ (black bars) and NO_3^- (grey bars) by plants and soil microbes four hours after addition of three different N fertilizers (NH_4Cl, NH_4NO_3 and KNO_3) to soils Purkersdorf and Niederschleinz. N uptake rates are expressed for total plant and microbial biomass per microcosm. Significant differences between NH_4^+ and NO_3^- uptake rates are indicated as *** $P<0.001$, **$P<0.01$, *$P<0.05$ (one-way ANOVA, Tukey's HSD test). Bars represent means + SE, n = 5.
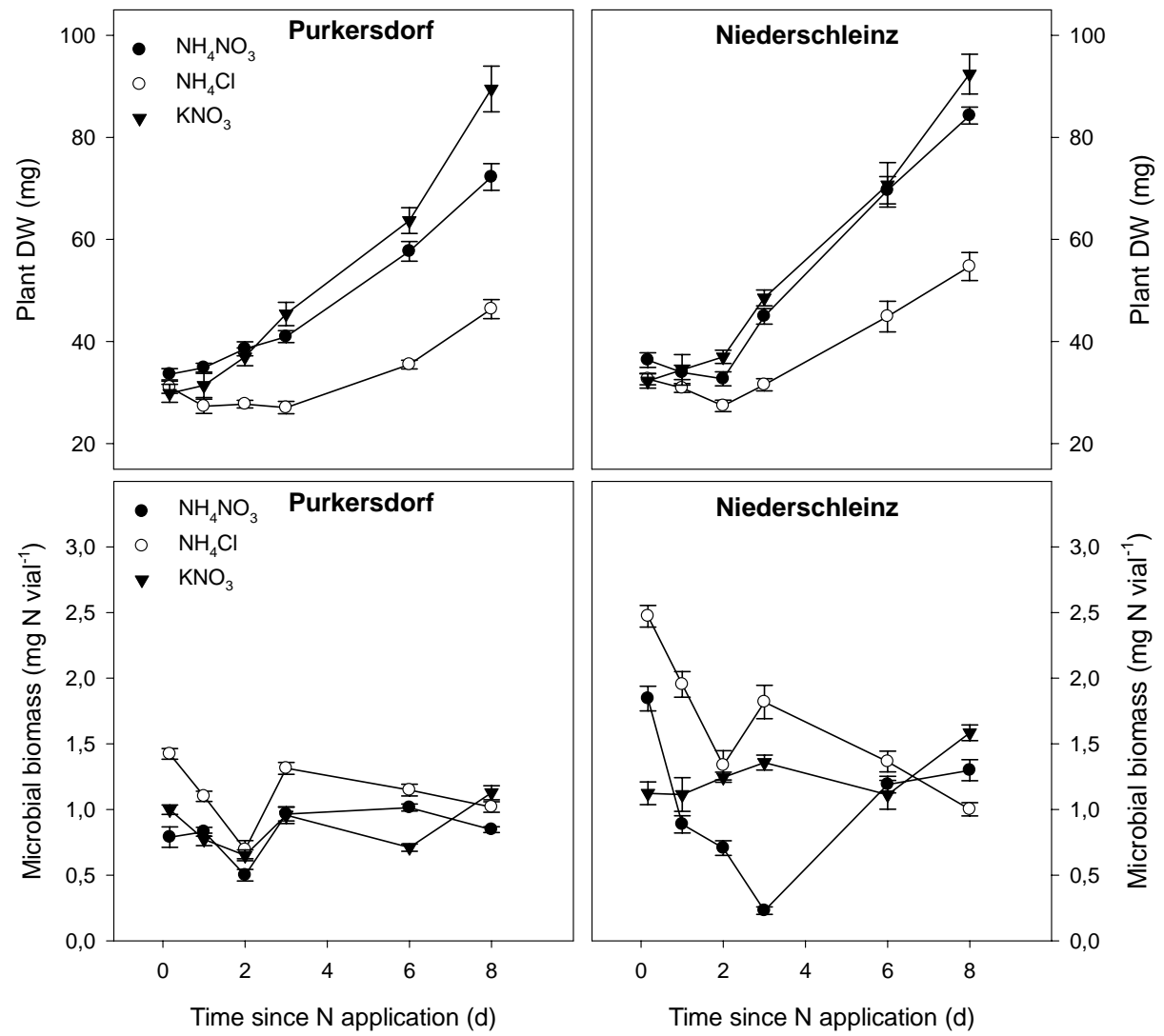
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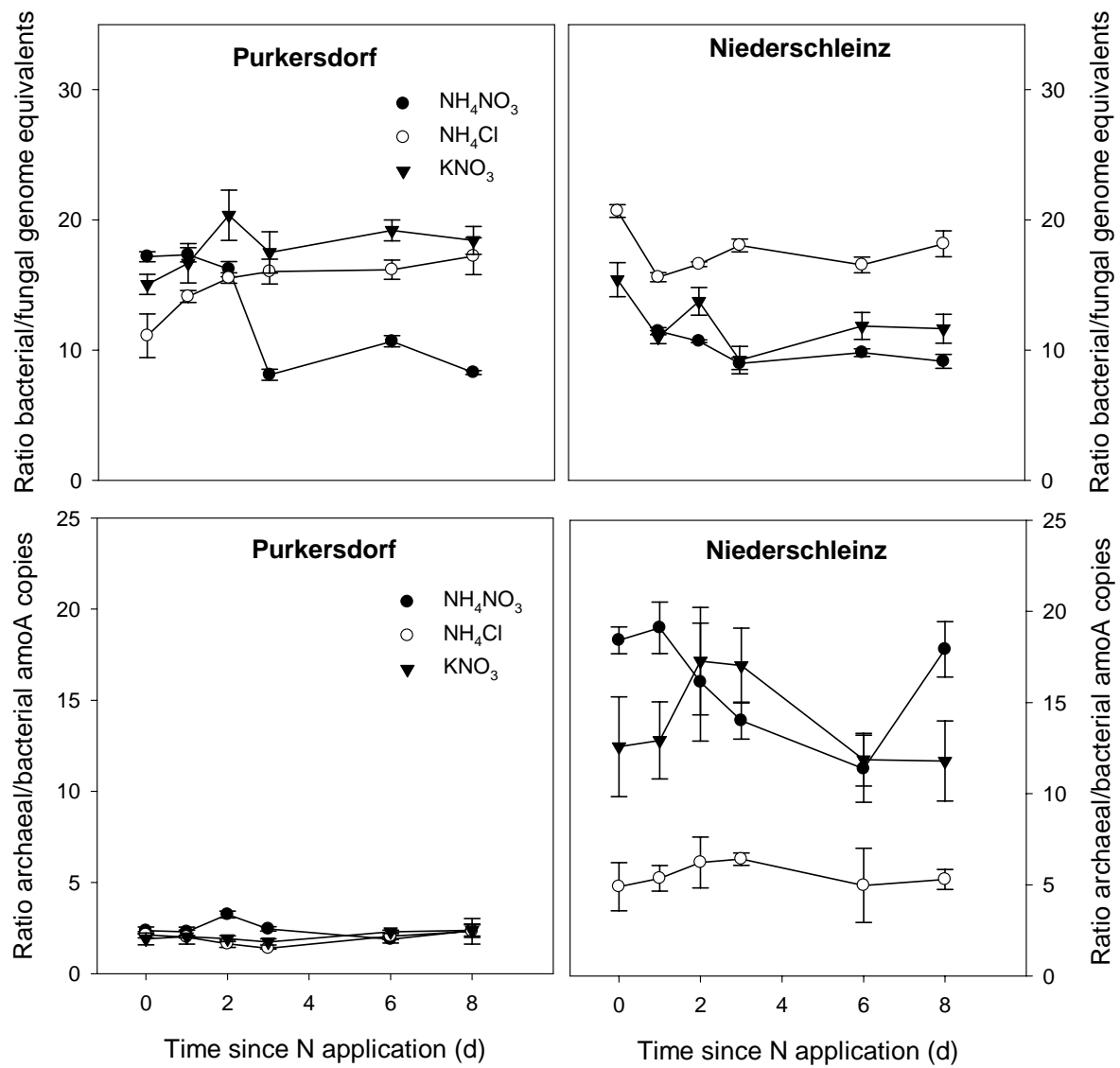
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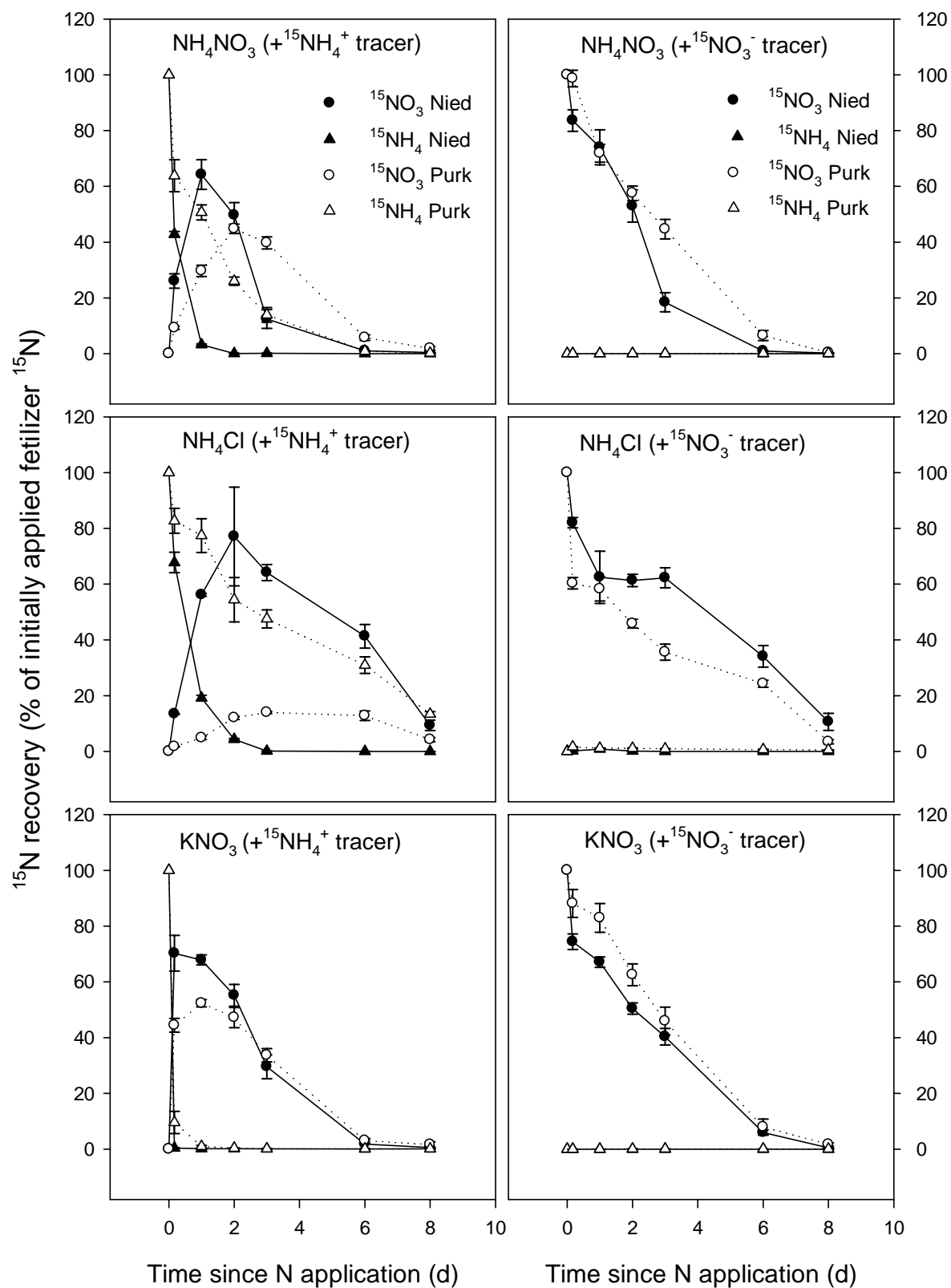
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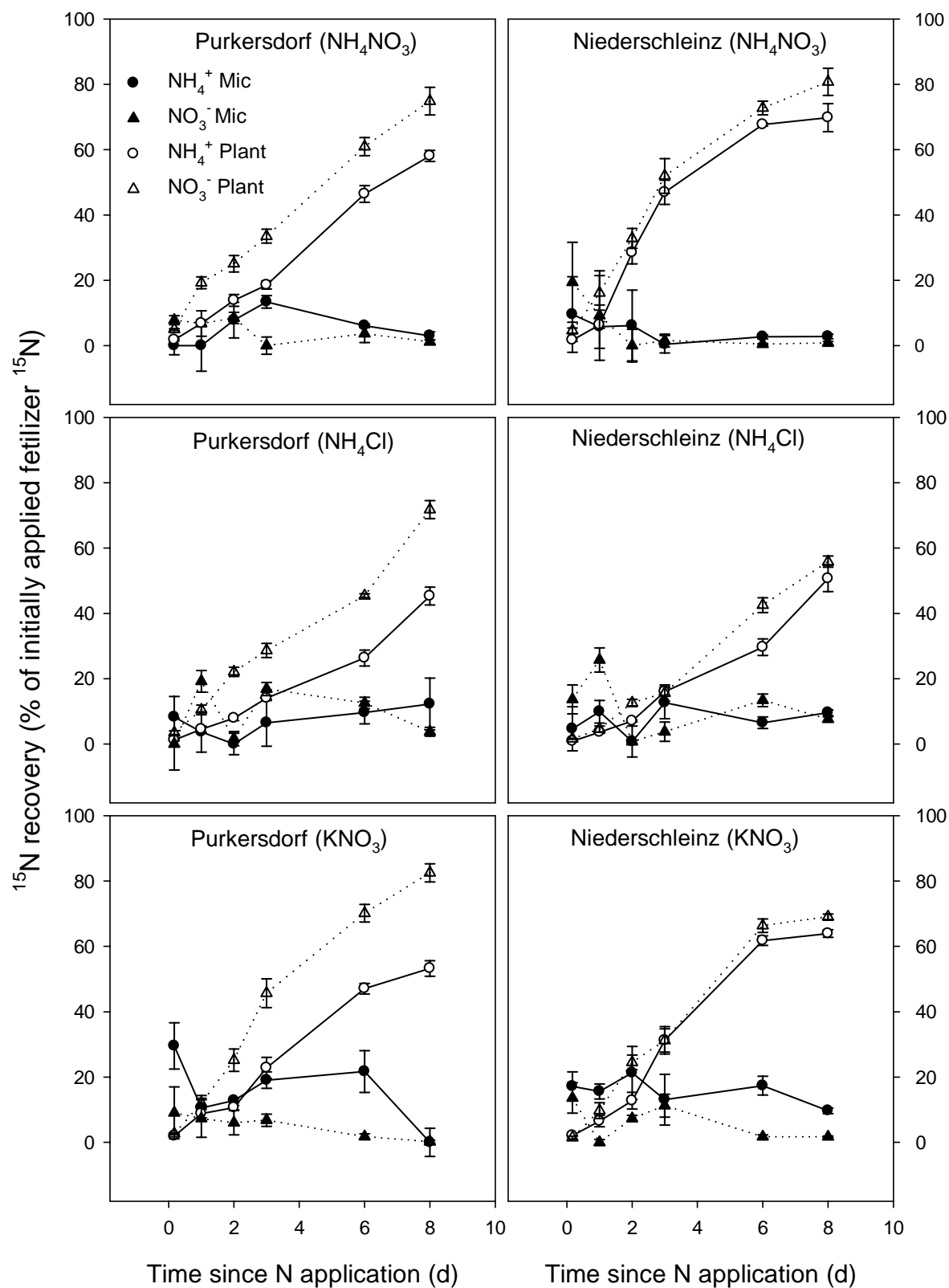
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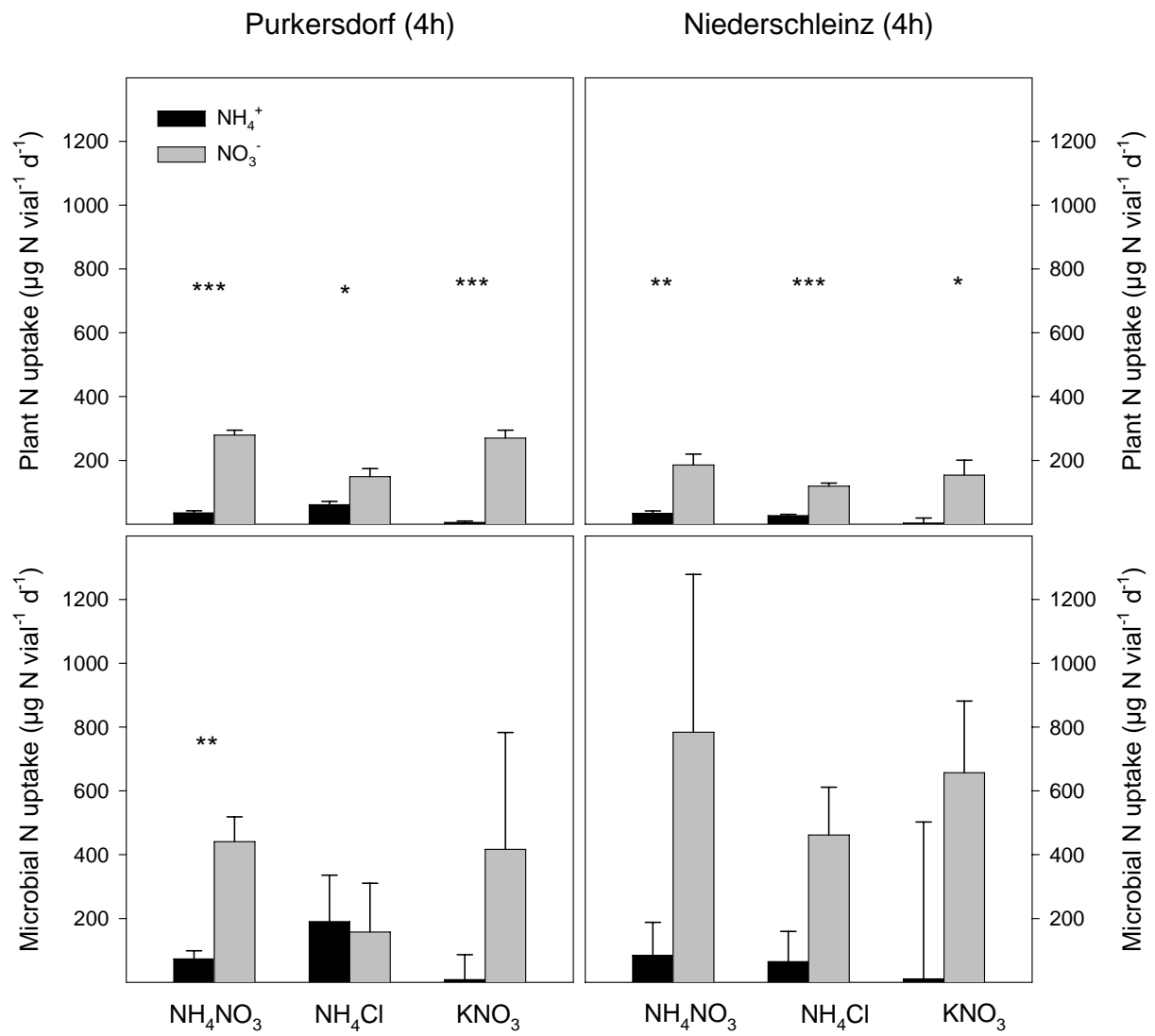
Figure 5.

Chapter 3

Influence of different inorganic N fertilizers on greenhouse gas fluxes in agricultural soil-plant microcosms

In preparation for submission

Erich Inselsbacher[1,2], Wolfgang Wanek[1], Florian Stange[3], Joseph Strauss[4], Sophie Zechmeister-Boltenstern[2]*

[1]Department of Chemical Ecology and Ecosystem Research, Faculty Centre for Ecology, Faculty of Life Sciences, University of Vienna, Austria

[2]Department of Forest Ecology and Soils, Unit of Soil Biology, Federal Research and Training Centre for Forests, Natural Hazards and Landscape (BFW), Vienna, Austria

[3]UFZ Department Soil Physics, Helmholtz Centre for Environmental Research-UFZ, Halle, Germany

[4]Department for Applied Genetics and Cell Biology, Fungal Genomics Unit, Austrian Institute of Technology and BOKU University Vienna, Vienna, Austria

*corresponding author: Sophie Zechmeister-Boltenstern

1. Introduction

N_2O, CH_4 and CO_2 are the three most important greenhouse gases, which cause radiative forcing of climate change (IPCC, 2001). While the global increases in CO_2 concentration are primarily due to fossil fuel use and land-use change, agricultural practice is assumed to be one of the major sources of N_2O and CH_4 emissions (IPCC, 2007). N_2O emissions from agriculture are estimated to account for more than 75% of the total global anthropogenic emission (Duxbury et al., 1993; Isermann, 1994). Therefore, the contribution of agricultural soils to the global warming potential has received increasing attention, with a major research focus on the influence of the form and quantity of N fertilizers on greenhouse gas fluxes (e.g., Steudler et al., 1989; Bouwman, 1990; Granli and Bockman, 1994; Matson et al., 1998; Mosier, 1998; Verma et al., 2006; Jones et al., 2007). As the major part of N_2O emitted from soils is produced during nitrification and denitrification (Hutchinson and Davidson, 1993), increased N inputs by mineral fertilizers lead to a surplus of NH_4^+ and NO_3^-, the substrates of these processes, and consequently lead to enhanced N_2O emissions (Matson et al., 1998; Hall and Matson, 1999; Chu et al., 2004). However, besides the availability of NH_4^+ and NO_3^-, these processes are regulated by a number of edaphic factors, including soil water content, soil temperature, aeration, the amount of soluble organic C and pH (Bouwman, 1990; Granli et al., 1994). Thus, large variability is typical for these emissions and therefore until now it was not possible to draw a general conclusion about the impact of fertilizer N form on N_2O emissions. Increased N_2O emissions also lead to losses of fertilizer N from the agricultural system and reduce N availability for crop plants. For calculating N_2O emissions from fertilized soils the IPCC report (1997) recommends a constant emission factor of 1.25% for the amount of N applied to agricultural land. However, literature emission factor values for cereal crops are extremely variable, ranging from 0.2 to 8% (Abdalla et al., 2009).

Fertilization not only potentially increases N_2O emissions but also strongly influences CO_2 emissions. Raich and Tufekcioglu (2000) estimated that plant root respiration, including microbial respiration in the rhizosphere, contributed 12–38% to the total soil respiration in agricultural fields and N fertilization generally increases root respiration due to increased plant growth leading to higher total CO_2 emissions (Chu et al., 2007). Thereby, the form of inorganic N applied strongly affects rates of root-derived respiration, as high energy demands of NO_3^- reduction in roots result in a stronger increase of respiration compared to NH_4^+ assimilation (Gavrichkova and Kuzyakov, 2008). However, this effect differs between crop species, depending on the contribution of shoots and roots to the NO_3^- reduction process. Until

now identification of the mechanisms contributing to N fertilizer-induced changes in CO_2 efflux from soil under agricultural crops remains challenging because of difficulties in separating root and microbial contribution to total CO_2 efflux.

Not only N_2O and CO_2 fluxes are controlled by a multitude of controlling factors but CH_4 fluxes as well. Aerated soils act as sinks of CH_4, and their sink strength has been estimated to be 3-9% of the global annual removal of CH_4 from the atmosphere (Smith et al., 2000). Application of mineral N fertilizers has been shown to reduce the natural CH_4 oxidation capacity of agricultural soils and may therefore lead to increased CH_4 emissions (e.g., Hütsch, 2001; Hu et al., 2002). This increase has mainly been attributed to the competitive inhibition of the enzyme methane monooxygenase, and to the resulting decrease in pH when NH_4^+ is applied to soil (Bedard and Knowles, 1989; Hütsch, 1998). However, there is contradictory evidence about the effect of different inorganic N forms on CH_4 emissions, and the underlying mechanisms are still poorly understood, especially in the field.

The main difficulties in assessing the effect of different N fertilizers on greenhouse gas fluxes are the interactions of fertilizer effects with various conditions of the agricultural system (soil, crop species, moisture, temperature, etc.) and therefore are hard to define. In the field it is not possible to control most of these factors, and often unwanted side effects (e.g., flooding or draught stress) are masking actual influences of fertilization. To investigate the effects of different inorganic N fertilizers it is necessary to control as many other factors as possible. To achieve this, microcosm studies proved to be a helpful tool (Inselsbacher et al., 2009b), when keeping in mind that results gained from such studies need to be validated in the field (Madsen, 2005). This approach also allows comparing fertilizer effects in soils with barely to completely different chemical, physical or microbial properties. For a better comparability all measured gas emissions should be converted to CO_2 equivalents using the global warming potential (GWP), which is defined as the cumulative radiative forcing between the present and a selected time in the future, caused by a unit mass of gas emitted now. The GWP (with a time span of 100 years) of CO_2, CH_4 and N_2O is 1, 25 and 298, respectively (IPCC, 2007).

Despite the magnitude of studies on greenhouse gas emissions in agriculture, there is still a lack of knowledge about the effects of different mineral N fertilizers on soil-derived GWP from different soil types. With more data, it could be possible to account for these effects in greenhouse gas inventories, and thus make the achieved emission reductions visible in the official inventories as well.

Therefore, the aim of this study was to investigate the effect of different inorganic N fertilizers (NH_4NO_3, NH_4Cl, KNO_3) on fluxes of CO_2, N_2O and CH_4 from soil microcosms

with or without growing young barley (*Hordeum vulgare* L. cv. Morex) plants and on soil-derived GWP in two different, but equally treated soils.

2. Material and Methods

2.1. Soil sampling and experimental setup

Soil was collected in April 2006 from two sites, Purkersdorf and Niederschleinz, in the vicinity of Vienna, Austria. Soils from both sites are widely distributed and are frequently used for barley cultivation in this area. Both soils are well characterized and have been used in previous studies (Hood-Novotny et al., 2009; Inselsbacher et al., 2009a; Inselsbacher et al., 2009b). A brief summary of site characteristics and soil properties is given in Table 1. Soil samples were collected from 0 to 20 cm depth from both sites and immediately stored at 4°C until further analysis. Prior to the start of the experiments soils were thoroughly mixed, homogenized and sieved (< 2 mm).

Experiments were carried out using a recently developed microcosm system described by (Inselsbacher et al., 2009b). Briefly, the microcosms consisted of 50 ml polypropylene centrifuge tubes complemented with two stainless steel sieves above the tube cones. 8 holes were drilled into the tube cones to guarantee sufficient aeration of the soils. Aliquots of sieved and homogenized field-moist soil were centrifuged (1 min, 187 *g* in a swing out rotor) into the test tubes to reach a final volume of 30 ml. The microcosms were kept under controlled conditions in a climate chamber with a 15 h/9 h day/night cycle at 21/18°C temperature and 55% relative air moisture. Supplementary lighting was provided via eight 400 W daylight lamps. During 14 d of pre-equilibration the soil water content (WC) of both soils was adjusted gravimetrically to 62% water filled pore space (WFPS). To achieve this, 28.8 g DW of soil Purkersdorf were adjusted to 23.6% WC (% dry weight) and 25.8 g DW of soil Niederschleinz to 19.2% WC.

Seeds of barley (*Hordeum vulgare* L. cv. *Morex*) were germinated on moist filter paper for 2 d and one seedling per microcosm was then planted. During the experimental period the WC of the soils was adjusted gravimetrically twice a day.

2.2. Fertilizer application and sampling events

To investigate the influence of fertilizer N form on greenhouse gas emissions four experiments were conducted in parallel, modified after Inselsbacher et al. (2009a). A solution of 6.25 mM K_2HPO_4 and either 12.5 mM NH_4NO_3, 25 mM NH_4Cl, 25 mM KNO_3 or distilled water were mixed and used for fertilization. These solutions were applied to the soils two times: 3 d before planting 1.6 ml and 5 d after planting 1.2 ml of the mixtures were applied to each microcosm, resulting in a total of 1 mg of N (except in control samples), 0.55 mg of P and 1.4 mg of K in all treatments. Homogenous distribution of fertilizer N was ensured by inserting a 7-cm long side-hole needle to the bottom of the soil cores in 4 positions and slowly injecting the solution (400 µl each injection) while withdrawing the needle (Pörtl et al., 2007). Samples were taken 2 h before and 4 h, 1 d and 3 d after the first fertilization (the last sampling being equal with 2 h before the second fertilization) and 4 h, 1 d, 2 d, 3 d, 6 d and 8 d after the second fertilization. Seedlings of barley were planted into the microcosms 2 d after the first fertilizer application.

2.3. Gas fluxes measurements

For gas sampling, test tubes were supplemented with gas tight retrofit kits described by Inselsbacher et al. (2009b) to increase head space volume in order to prevent significant under-pressure during gas sampling. Test tubes were closed at both sides with butyl rubber seals and gas samples were taken immediately after closing, after 30 min and after 1h. With a gastight syringe head space air (10 ml) was transferred into evacuated headspace vials and kept at 4°C until analysis. Immediately after taking gas samples the same volume of standard air was injected into the microcosms to compensate air pressure. Gas samples were analysed within 48 h as described by (Kitzler et al., 2006) by automated headspace gas chromatography. Briefly, the GC was equipped with a 63Ni electron capture detector to quantify N_2O concentrations and a flame ionization detector and a methanizer to quantify CO_2 and CH_4 concentrations. Gas emission rates were then assessed by the linear increase of headspace gas concentration over the closure period. The correction for dilution effects resulting from injecting standard air into microcosms was included into these calculations.

2.4. Soil properties measurement

After gas sampling, the test tubes were opened again, soils were quantitatively retrieved, homogenized and prepared for further analyses. Soil chemical properties were analyzed as

described by Inselsbacher et al. (2009a). An aliquot (4 g) of homogenized soil was dried at 70°C and weighed to determine soil moisture. Another aliquot (2 g) of homogenized soil was extracted in 15 ml $CaSO_4$ (10 mM) and subsequently anions were determined by ion chromatography (DX 500, Dionex, Vienna, Austria) and conductivity detection. NO_3^- was separated on an anion exchange column (AS11, 250 x 4 mm i.d., Dionex, Vienna, Austria) after chemical suppression (ASRS-Ultra, Dionex) and linear NaOH gradient elution (0.5 mM to 37.5 mM within 10 min at a flow rate of 2 ml min^{-1}, with a column temperature of 35°C). NH_4^+ was extracted from aliquots (6 g) of homogenized soil with 45 ml KCl (1 M) and determined by a modified indophenol reaction method (Kandeler and Gerber, 1988). Total dissolved C and N in the same KCl extracts were determined by an automated C analyzer (Shimadzu, TOC-VCPH, Japan) and a total N measuring unit (Shimadzu, TNM-1, Japan).

2.6. Statistical analysis

Data were analysed using one-way and multi-factorial ANOVA followed by Tukey's HSD post-hoc test using Statistica 6.0 (StatSoft Inc., Tulsa, OK, USA). When necessary, data were either square root- or log_{10}- transformed prior to analysis to meet the assumptions of ANOVA after testing normality using Kolmogorov-Smirnov test and homogeneity of variances using Bartlett's test. The effects of adding different forms of N on emissions of CO_2, CH_4 and N_2O was analysed by three-way ANOVA with soil type, N form and harvest time as independent variables. Multiple regression analyses of greenhouse gas fluxes in soils Purkersdorf and Niederschleinz were performed with Statgraphics 5.0 (Statistical Graphics Inc., Rockville, MD, USA). Within this analyses data of microbial biomass, root and shoot DW, total plant C contents, numbers of fungal and bacterial genome equivalents as well as archaeal and bacterial ammonium monooxygenase catalytic subunit A (amoA) copies were taken from a previously published set of experiments which were conducted in parallel to the present study (Inselsbacher et al., 2009a).

Results

3.1. Soil physical and chemical properties

Dissolved inorganic nitrogen (DIN) pools in both soils decreased rapidly during 8 d after the second fertilization event mainly due to severe plant N uptake during this period (Fig. 1). 8 d

after the second fertilization event NH_4^+ and NO_3^- pools have reached background levels in both soils again (1.3 ± 0.2 and 19 ± 1.5 µg N g^{-1} DW, respectively). NH_4^+ was depleted significantly faster in soil Niederschleinz, whereas NO_3^- concentrations remained longer in this soil compared with soil Purkersdorf.

In both soils total dissolved carbon (TDC) contents were markedly higher after the second fertilization compared to untreated soils (40 ± 2 and 31 ± 2 µg C g^{-1} DW in soil Purkersdorf and Niederschleinz, respectively). Higher amounts of TDC, but also higher shifts of TDC during the test period and stronger effects of different forms of fertilizer N were found in soil Niederschleinz (Fig. 1, three-way ANOVA, $P<0.0001$). In both soils TDC contents increased after addition of NH_4NO_3, while after applying either NH_4Cl or KNO_3 TDC contents decreased. Compared to background levels, TDC concentrations increased least in the KNO_3 treatment in both soils. After 8 d TDC contents reached a near-steady state, showing no difference in concentration between different fertilizer N forms in soil Purkersdorf (one-way ANOVA, $P>0.05$), but still slightly lower contents after KNO_3 addition in soil Niederschleinz ($P<0.05$).

Microcosms were kept under strictly controlled conditions in a climate chamber to guarantee stable conditions throughout the test period. As intended, after equilibration no shifts in water content, and therefore also not in water filled pore space (WFPS), were observed in the soils (data not shown, $P>0.05$). However, on a daily basis some minor, but nevertheless significant shifts of WFPS occurred in both soils (data not shown, $P<0.05$). Decreases of WFPS got stronger and less controllable in the end of the test period, which could be attributed to increased water losses via plant evaporation. On the other hand, directly after watering the samples, the WFPS of the soil samples was slightly increased.

Independent of N form and soil, N application did not change pH values of bulk soils in planted and unplanted microcosms (one-way ANOVA, $P>0.05$, n = 10). However, due to insufficient root lengths and biomass at the time of fertilization, shifts in rhizosphere pH could not be measured.

3.2. Carbon dioxide fluxes

Initial emissions of CO_2 from soil Purkersdorf were low (4.0 ± 2.1 mg C kg^{-1} DW d^{-1}) and similar to CO_2 emissions from soil Niederschleinz (3.2 ± 0.8 mg C kg^{-1} DW d^{-1}). The first fertilization event did not affect CO_2 emissions but after seedlings were transferred to the microcosms, CO_2 emissions increased rapidly in both soils, with higher emissions from soil

Purkersdorf (Fig. 2, Table 2). No direct influence of N application was observed, as CO_2 emissions did not differ between fertilizer N forms and the water control ($P>0.05$). After the second fertilization CO_2 emissions from soil Purkersdorf were markedly higher than from soil Niederschleinz and were highest in the NH_4NO_3 treatment and lowest in the KNO_3 treatment. Interestingly, in soil Niederschleinz the opposite effects of fertilizer N forms were found, although much less pronounced (Fig. 2, Table 3). Integrated over the whole test period (11 days) this resulted in 2.6, 2.1 and 1.6-fold higher cumulative CO_2 emissions (calculated by linear interpolation between sampling occasions) from soil Purkersdorf than from soil Niederschleinz in the NH_4NO_3, NH_4Cl and KNO_3 treatments, respectively (Fig. 2). Multiple regression analysis was performed to determine the most significant determinant factors on gas fluxes (Table 4). Accepting a threshold probability of 95%, in soil Purkersdorf concentrations of soil NH_4^+ and NO_3^- and the bacterial to fungal genome equivalent ratio were found to be significant factors determining CO_2 fluxes, explaining 77% of the variability in CO_2 emissions. In soil Niederschleinz the bacterial to fungal genome equivalent ratio was likewise significant, but instead of DIN concentrations root biomass was the second main factor, together explaining 39% of CO_2 emission variability.

3.3. Methane fluxes

After equilibration in the microcosms for 14 d at 62% WFPS and before fertilization and planting, both soils were net sources of CH_4. Initial emission rates did not differ between the two soils (one-way ANOVA, $P>0.05$) and were 2.4 ± 3.2 and 0.6 ± 2.2 µg C kg^{-1} DW d^{-1} from soils Purkersdorf and Niederschleinz, respectively (Fig. 2). After the first fertilizer application both soils became sinks of CH_4 with soil Niederschleinz exhibiting higher consumption rates than soil Purkersdorf (Table 2). No effect of fertilizer N forms was observed as net CH_4 consumption rates were equal in the fertilized and in the water control samples (Fig. 2). 3 d after the first fertilization net CH_4 fluxes have reached background levels again ($P>0.05$) and therefore, on average, both soils have become net sources of CH_4 again. The second fertilizer application was followed by a stronger increase of CH_4 consumption compared to the first fertilization event, and again soil Niederschleinz showed higher CH_4 consumption rates as soil Purkersdorf (Fig. 2). As revealed by three-way ANOVA fertilizer N form did not affect CH_4 consumption rates directly, but a significant interaction effect between fertilizer N form and harvest time was observed (Table 3). This effect indicates that decreases of net CH_4 consumption rates during the monitored time period depended on

fertilizer N form. While CH_4 consumption rates decreased in both soils in the NH_4NO_3 and NH_4Cl treatments to near-background levels 8 d after fertilization, CH_4 consumption rates of soils treated with KNO_3 reached an equilibrium already 3 d after fertilization, but at significantly higher levels of CH_4 consumption (-1.9 $\pm$ 0.2 and -3.7 $\pm$ 0.2 µg C kg^{-1} DW d^{-1} in soils Purkersdorf and Niederschleinz, respectively). Total amounts of CH_4 consumed over the whole time period of 11 d were higher in soil Niederschleinz than in soil Purkersdorf only after KNO_3 application (44 and 24 µg C kg-1 DW, respectively, $P<0.05$), but were not different in the other N treatments (between 17 and 33 µg C kg^{-1} DW, $P>0.05$, Fig. 2).

Multiple regression analysis revealed that, accepting a threshold probability of 95%, in soil Purkersdorf concentrations of soil NO_3^- and TDC were the only significant factors determining CH_4 fluxes and together explained 57% of the variability in CH_4 fluxes (Table 5). Unexpectedly, no correlations between CH_4 fluxes and WFPS or NH_4^+ concentrations were found ($P>0.05$). On the contrary, in soil Niederschleinz WFPS, soil NH_4^+ concentrations and additionally the ratio of archaeal to bacterial ammonium monooxygenase catalytic subunit A (amoA) copies were the determining factors, together explaining 58% of CH_4 fluxes.

3.4. Nitrous oxide fluxes

Both soils were sources of N_2O at all measured time points. After equilibration initial N_2O emission rates from soil Purkersdorf were significantly ($P<0.01$) lower (1 $\pm$ 0.8 µg N kg^{-1} DW d^{-1}) than from soil Niederschleinz (3.2 $\pm$ 1 µg N kg^{-1} DW d^{-1}). 4 h after the first application of fertilizer, N_2O emissions had increased significantly in both soils, with higher emissions from soil Niederschleinz (Fig. 2, Table 2). Thereby, in both soils fertilizer N form did not affect N_2O emission rates, but addition of N generally resulted in higher N_2O emissions compared to water control samples (one-way ANOVA, $P<0.05$). Peaks of N_2O emissions rapidly decreased again to background levels during the following 3 d in both soils. Directly (4 h) after the second fertilization, N_2O emissions were markedly higher than after the first fertilization event (Fig. 2), although less N fertilizer was applied compared to the first fertilization. In the control samples of both soils no increase of N_2O emission was found ($P>0.05$). Unlike observed after the first fertilizer application, the form of applied N fertilizer had a strong influence on N_2O emissions at the second fertilization in both soils (Table 3), with highest peaks of N_2O emission after NH_4NO_3 application and lowest peaks after KNO_3 application (one-way ANOVA, $P<0.01$).

In the cases of NH_4Cl and NH_4NO_3 treatments, N_2O emissions from soil Niederschleinz were again higher than from soil Purkersdorf ($P<0.05$, Table 3), but not after KNO_3 application, which resulted in similar N_2O emissions from both soils ($P>0.05$).

During the subsequent 8 d N_2O emissions decreased in both soils, following different patterns depending on fertilizer N form applied (Fig. 2, Table 3). N_2O emissions from the NH_4NO_3 treatment decreased almost linearly during this time period, but did not reach background values after 8 d (one-way ANOVA, $P<0.05$). On the other hand, N_2O emissions from the NH_4Cl treated soils decreased more rapidly and to background levels again ($P>0.05$). The slowest decrease of N_2O emissions was found after KNO_3 application in both soils, and still clearly remained above background levels after 8 days ($P<0.05$).

Total emissions of N_2O (during an 8 d period following the second fertilization) from soil Niederschleinz treated with NH_4Cl and KNO_3 were as low as 39% and 56% of the NH_4NO_3 treatment, respectively. Cumulative N_2O emissions over this 8 d period therefore resulted in nearly equal emissions from the NH_4NO_3 treatment (85 ± 10 µg N kg^{-1} DW) and the sum of emissions from the NH_4Cl and the KNO_3 treatments (81 ± 12 µg N kg^{-1} DW, Fig. 2). This was not the case in soil Purkersdorf, where N_2O emissions from the NH_4Cl and KNO_3 treatment each were 65% of the NH_4NO_3 treatment, respectively. Consequently, cumulative N_2O emissions from the NH_4NO_3 treatment (58 ± 7 µg N kg^{-1} DW) made up only 77% of the sum of the other treatments (75 ± 10 µg N kg^{-1} DW, Fig. 2).

Like it was the case for CH_4 fluxes, in soil Niederschleinz the most important factors determining the variation in N_2O fluxes were again soil NH_4^+ concentrations and the ratio of archaeal to bacterial amoA copies. Additionally, resulting from multiple regression analysis, soil NO_3^- concentrations and root biomass were found to be significant factors as well (Table 6). Together these four factors explained 88% of the variability of N_2O fluxes. Similarly, in soil Purkersdorf the same factors determining CH_4 flux variations, namely soil NO_3^- and TDC contents, were significant factors determining N_2O fluxes and explained 64% of the variation.

3.5. Fertilizer N lost as N_2O

Taken the first fertilization event alone, average losses of applied N via N_2O emission during the subsequent 3 d were as little as 0.04% in both soils (Table 7). As after 3 d N_2O emissions have reached background levels again, it is unlikely that under the present laboratory conditions additional severe losses would have occurred at later time points. The proportional loss of fertilizer N as N_2O from the second fertilizer, on the other hand, was significantly

higher, with highest losses in the NH_4NO_3 treatments from both soils. While N losses after NH_4Cl and KNO_3 application were similar and in the same range in the two soils, N losses after NH_4NO_3 fertilization were higher in soil Niederschleinz. Taken together, during the monitored 11 d N losses from total applied fertilizer N via N_2O emissions were below 0.3% in both soils. By extrapolating the cumulative curves of N_2O emissions (shown in Fig. 2), it was possible to calculate potential N_2O emissions over a time period of 1 month after fertilization (Fig. 3, Table. 7). The results thereof indicate that the major N losses occurred during the first week after fertilization while additional potential N losses during the following 3 weeks would have accounted for a much smaller part of total N losses. Calculated for the amount of applied N from the second fertilization alone, this resulted in average N losses of 0.62% and 0.88% in the NH_4NO_3 treatment during one month after fertilization in soils Purkersdorf and Niederschleinz, respectively.

3.6. Greenhouse gas balance

N_2O and CH_4 have 298 and 25 times higher global warming potentials (GWP) than CO_2, respectively, in a time horizon of 100 years (IPCC, 2007). In our study the GWP calculated as total CO_2 equivalents over an 11-day period after fertilization was markedly higher in soil Purkersdorf than in soil Niederschleinz. In detail, total CO_2 equivalents were 2.45, 2.09 and 1.53 times higher in the NH_4NO_3, NH_4Cl and the KNO_3 treatment, respectively (Table 8). While the GWP of soil Purkersdorf was highest after NH_4NO_3 and lowest after KNO_3 fertilization, the opposite was the case in soil Niederschleinz. This was related to the same opposite pattern of CO_2 emissions from the two soils (Fig. 2). In soil Purkersdorf CO_2 contributed $98 \pm 6\%$ to GWP in all treatments, while N_2O contributed only approximately $2 \pm 0.5\%$. Even if the contribution of N_2O was highest in the NH_4NO_3 treatment ($2.4 \pm 0.4\%$), this difference was probably not significant. On the contrary, the contribution of N_2O to total GWP in soil Niederschleinz was clearly higher, ranging between 3.7 and 8.6%. Additionally this soil showed a stronger influence of fertilizer N form, as the contribution of N_2O after NH_4NO_3 application was approximately 2-fold higher than in the other treatments (Table 5). Independent of fertilizer N form, soil CH_4 consumption had only little influence on total GWP decreasing it by $0.1 \pm 0.03\%$ and $0.2 \pm 0.1\%$ in soil Purkersdorf and Niederschleinz, respectively.

4. Discussion

One major aim of this study was to emphasize the influence of different inorganic N fertilizer forms on N_2O emissions and in detail on relative amounts of fertilizer N lost from agricultural soils as N_2O. In both soils, N_2O emissions were highest after applying a mixture of NH_4NO_3 compared to application of NH_4^+ or NO_3^- alone. These findings indicate that both nitrification and denitrification were contributing simultaneously to N_2O losses in the mixed fertilizer treatment, which provided N sources for both processes. Additionally, soil moisture, another important controlling factor for N_2O emissions (e.g., Dobbie and Smith, 2001), was steadily kept at 62% WFPS allowing both nitrification and denitrification to occur (Davidson, 1993). When only NO_3^- was applied, concentrations of NH_4^+ in both soils were very low (0.5 to 1.5 µg N g^{-1} DW) and might have been limiting for nitrification (Garrido et al., 2002). On the other hand, when only NH_4^+ was applied during one week after fertilization NO_3^- concentrations were nevertheless higher than 20 µg N g^{-1} DW, which was found to be the threshold concentration above which denitrification is not limited (Myrold and Tiedje, 1985b; Myrold and Tiedje, 1985a). In the end of the experimental period (11 days after the second fertilization) however, concentrations of both, available NH_4^+ and NO_3^- were low enough to potentially limit nitrification and denitrification. Together with the fact that other controlling factors (soil moisture, temperature, pH) were kept at a steady state, these findings indicate that in our study the availabilities of NH_4^+ and NO_3^- clearly were the main determining factors for rates of nitrification and denitrification and consequently directly influencing N_2O emissions as reported previously (Mosier et al., 1986; Baggs and Blum, 2004).

Gross nitrification rates, which were estimated within parallel ^{15}N labelling experiments (Inselsbacher et al., 2009a), were significantly higher in soil Niederschleinz compared to soil Purkersdorf. This fits well with the fact that N_2O emissions from soil Niederschleinz were significantly higher directly after applying either NH_4NO_3 or NH_4^+. However, high nitrification rates also led to a rapid depletion of NH_4^+, consequently limiting nitrification. The marked influence of soil NH_4^+ concentrations on N_2O emissions in soil Niederschleinz was also reflected in a strong correlation between these factors. Interestingly, in soil Niederschleinz N_2O emissions were also correlated with the ratio of archaeal to bacterial amoA copies. Although it is known that archaea might numerically be more abundant than bacteria in soils (Leininger et al., 2006) and might also contribute significantly to nitrification (e.g., Inselsbacher et al., 2009a), until now their ecological role still remains uncertain (Francis et al., 2007; Hayatsu et al., 2008). The strong correlation between N_2O emissions and

archaeal amoA abundance in this study indicates that archaea might have the potential to significantly contribute to N_2O emissions and therefore to soil derived GWP. In soil Purkersdorf, on the other hand, gross nitrification rates were found to be 2 to 3 times lower and archaeal amoA abundance up to 10 times lower than in soil Niederschleinz (Inselsbacher et al., 2009a). Consequently, after application of NH_4NO_3 or NH_4^+ immediate N_2O emissions derived from nitrification were lower than from soil Niederschleinz but due to a longer lasting availability of the substrate NH_4^+, nitrification-derived N_2O losses contributed to total N_2O emissions for a longer time period compared to soil Niederschleinz.

High nitrification rates also led to an increase of the soil NO_3^- pool, which subsequently was subject to denitrification. Over the whole test period of 11 days, as well as estimated over one month, denitrification was therefore contributing stronger to total N_2O emissions than nitrification, due to substrate limitation of nitrification but high availability of NO_3^-. The importance of denitrification for N_2O losses was also reflected by significant correlations between N_2O emissions and soil NO_3^- and TDC concentrations in soil Purkersdorf, two factors known to control denitrification (Tiedje, 1988). Nevertheless, the contribution of each process to total N_2O emissions remains hard to reliably estimate, in part due to technical restrictions. The use of nitrification inhibitors (acetylene, nitrapyrin) might give further insights into the role of nitrification versus denitrification, but there are still no experimental methods to distinguish archaeal and bacterial nitrification activity in soil. Therefore, and due to the diversity of N_2O producing processes and microbial communities mediating both processes (Wrage et al., 2001), it is recommended for future studies to combine highly specific molecular biological approaches with advanced ^{15}N tracer experiments, allowing reliable analyses of trace concentrations of $^{15}N_2O$.

However, besides the importance of nitrification and denitrification, also plants strongly influenced N_2O emissions. It has been shown that barley plants grown on both soils in the same microcosms preferentially took up NO_3^- over NH_4^+ and were the strongest sink of fertilizer N (45 to 80%) after 8 days (Inselsbacher et al., 2009a). However, plant N uptake became obviously stronger with continuing plant growth and was rather low during the first 5 days. If N uptake by plants is low, then there is more N available for nitrification and denitrification (e.g., Maljanen et al., 2004) and therefore for N_2O production. This was reflected by the decrease of N_2O emissions after an initial pulse directly after the second fertilization, due to N depletion during continuous plant N uptake and by a strong negative correlation of N_2O emissions and root biomass in soil Niederschleinz. Additionally to reducing the substrates for nitrification and denitrification, plants may be controlling N_2O

emissions by increasing the transportation of N_2O from the rhizosphere to the troposphere (Verma et al., 2006), by providing an additional C source via root exudation allowing increased denitrifier activity (Philippot et al., 2008), or by a combination of both effects. Additionally, root penetration into the soil decreases soil compaction and creates channels for gas transfer and may therefore be responsible for a faster diffusion of N_2O from the soil to the atmosphere (Philippot et al., 2008). Both soils in the present study exhibited higher N_2O emissions after fertilizer N application in the presence of growing plants than from soils without plants, which supports these suggestions and is in agreement with several other studies (e.g., Klemedtsson et al., 1987; Kilian and Werner, 1996). However, it is hard to tell which of the aforementioned effects was most responsible for the increased N_2O emissions. It is likely that root exudation played a major role, as soil TDC contents increased significantly after planting in both soils. Plant-mediated N_2O transport was probably low but, as it was not estimated directly, cannot be fully excluded either.

Further, our study underlines previous findings that increases in N_2O emission follow N-fertilization for a short time only (8 to 14 days) and are then typically reduced to fluctuate around a low base line level (Mosier, 1998; Ambus, 2005; Mosier, 1994; Bouwman, 1996; Jones et al., 2007; Abdalla et al., 2009). The highest peaks of N_2O emissions were observed directly after the second application, despite the fact that the first application of fertilizer represented more than half of the total N applied. A similar result was found by (Abdalla et al., 2009) who proposed that changing water conditions were responsible for this effect. In our study soil moisture and temperature were kept stable and therefore could not have been responsible for this increase, but were probably attributed to increased denitrification rates induced by root exudation. A raised denitrifier N_2O to N_2 product ratio following N fertilizer application may have further increased N_2O emissions, since NO_3^- is preferred over N_2O as an electron acceptor for denitrifiers at concentrations of > 10 µg g^{-1} (Blackmer and Bremner, 1978; Baggs et al., 2003). However, N_2 emissions were not assessed in the present study, and therefore this still needs to be validated for the tested soils.

In terms of relative amount of fertilizer N emitted as N_2O the influence of plants was leading to a marked increase of N losses. While independent of fertilizer N form only 0.03% of applied N was lost as N_2O after the first fertilization, the amount of N lost from the second fertilization was markedly higher and dependent on fertilizer N form. In both soils cumulative N_2O losses over 11 days were more or less equal when only one N source (either NH_4^+ or NO_3^-) was supplied (0.11-1.14%), but significantly higher in the NH_4NO_3 treatment. These findings indicate that providing sources for nitrification and denitrification at the same time

led to significantly highest N_2O emissions from both soils and therefore should be avoided in order to decrease N_2O-derived GWP. Estimation of proportional N_2O losses from total fertilizer N added over one month showed that N_2O emission factors in both soils were lower (0.14-0.37%) than the default value (1.25%) recommended by the IPCC (1997), but still in the range of previous studies (e.g., Abdalla et al., 2009). Our results also stress that N_2O emissions during the first 11 days already contributed 67-85% of total N_2O emissions during one month. Therefore, the correct timing of fertilizer application in regard to weather conditions and plant N demands is of uttermost importance. Further, when setting up future studies on greenhouse gas emissions, gas flux measurements should be conducted directly after fertilizer application and are recommend to be repeated subsequently in short time intervals to reliably quantify N_2O emissions. However, while the emission factors calculated in the present microcosm study are valuable for short-term laboratory studies, annual site-specific N_2O emission factors still need to be validated directly in the field.

In contrast to N_2O emissions, in both soils neither the application of N fertilizer nor the form of inorganic N was influencing net CH_4 fluxes. This was unexpected, as NH_4^+ is known to potentially inhibit CH_4 oxidation and has been observed in several previous studies (e.g., Steudler et al., 1989; Mosier et al., 1991; Hütsch, 2001; Hu et al., 2002). On the other hand, other studies in landfill and forest soils reported that NO_3^-, rather than NO_2^- or NH_4^+ proved to be the strongest inhibitor of CH_4 oxidation (Kightley et al., 1995; Wang and Ineson, 2003; Reay and Nedwell, 2004) and also a lack of inhibition or even stimulation of CH_4 oxidation following N addition has been reported (e.g., Bodelier et al., 2000; Hilger et al., 2000; Sitaula et al., 2000; De Visscher and Van Cleemput, 2003). In the present study pH values of bulk soils did not change in response to N fertilization, excluding the possibility of pH-induced inhibition of CH_4 oxidation. Additionally, due to high nitrification rates NH_4^+ was depleted rapidly in both soils, therefore excluding potential NH_4^+-induced inhibition of CH_4 oxidation as well. Generally, the results from this study indicate that fertilizer N application was not influencing CH_4 fluxes, as changes of net CH_4 fluxes were identical between the different fertilizer and the control treatments. Clearly, the major control for CH_4 fluxes in both soils was soil moisture. It is a well known fact that WFPS is a controlling factor for CH_4 fluxes, as it is assumed that methanogens are obligate anaerobes whereas CH_4 oxidizers are obligate aerobes (Le Mer and Rogert, 2001). In both soils the slight increase of WFPS (from 60 to 65%) due to fertilizer injection resulted in a strong shift in CH_4 fluxes resulting in soils becoming sinks of CH_4. These findings were contrary to previous findings showing that an increase of WFPS led to higher net CH_4 emissions, due to higher activities of CH_4 producers

(e.g., Yavitt et al., 1990; Key et al., 2008). However, the net CH_4 flux in terrestrial ecosystems is the result of simultaneous gross CH_4 production and gross CH_4 consumption rates (Chu et al., 2007; Kammann et al., 2009), and therefore it is difficult to know whether the observed increase in CH_4 consumption resulted from a stimulation of CH_4 oxidation or an inhibition of CH4 production. Moreover, the site-specific methanotrophic community may have been stimulated by increased CH_4 production (Kammann et al., 2009), leading to oxidation of a significant proportion of the CH_4 produced in anaerobic layers before it reaches the atmosphere. The increase in WFPS during fertilizer application probably also led to reduced diffusion rates of CH_4 in both soils.

In wetland plants, it was shown that passive transport of CH_4 from the anoxic soil to the atmosphere through the plant aerenchyma can represent up to 90% of the total CH_4 flux (Cicerone and Shetter, 1981; Holtzapfel-Pschorn et al., 1986; Nouchi et al., 1990; Butterbach-Bahl et al., 1997). This mechanism was not observed in the present study, as similar CH_4 flux patterns were found in unplanted and planted soils, indicating that upland crop plants are not significantly increasing CH_4 emissions by potentially transporting CH_4 to the atmosphere. We thus hypothesize that the net CH_4 flux may mostly depend on gas diffusion rates in the soil and therefore on gas exchange rates of the soil surface with the atmosphere, as well as the spatial distribution of the methanotrophic and methanogenic communies along aerobic/anaerobic microsites which might further lead to a small-scale stimulation of produced CH_4 on the methantrophic community.

CO_2 production was clearly regulated by plants as well as the soil microbial community composition in both soils. In soil Niederschleinz CO_2 emission was positively correlated with root biomass, indicating that root respiration, including microbial respiration in the rhizosphere, contributed significantly to soil total CO_2 emissions. A similar strong, but less significant correlation was found in soil Purkersdorf (correlation coefficient 0.45, P=0.011) indicating that root respiration was the main source of CO_2 in this soil as well. Further, no fertilizer-induced increase of CO_2 emissions was observed from unplanted control samples in both soils. These findings are in agreement with previous studies who found that root respiration contributed 12-38% to the total soil respiration in crop lands (Raich et al., 2000), and might be even enhanced at the onset of plant growth due to enhanced root activity (Chu et al., 2005).

The negative correlation between DIN pool sizes and CO_2 emission rates in soil Purkersdorf also rather reflect the increasing depletion of DIN pools due to root N together with a simultaneous increase in root respiration then a direct influence of DIN concentrations on CO_2

emissions per se. Nevertheless, CO_2 emissions were influenced by the form of fertilizer N applied, surprisingly with opposing effects in the two soils. One reason for these findings was that the two soils exhibited different soil microbial communities which were affected differently depending on fertilizer N form applied. In a parallel study using the same soils and the same setup, it has been shown that bacterial genome equivalents did not change during time in both soils, but that fungal genome equivalents increased in all fertilizer treatments in soil Niederschleinz, and partly decreased in soil Purkersdorf (Inselsbacher et al., 2009a). However, compared with root respiration, it is likely that microbial respiration in the bulk soil contributed only little to total CO_2 production. Still, as has been pointed out previously, the role of microbial respiration in the rhizosphere remains unclear and hard to distinguish (Gavrichkova et al., 2008).

In order to estimate the soil-derived global warming potential (GWP), which determines the relative contribution of a gas to the greenhouse effect, all gas emissions were converted to CO_2 equivalents. Soil derived GWP was much higher in soil Purkersdorf than in soil Niederschleinz, due to higher CO_2 emissions from this soil. However, CO_2 emissions were the strongest contributors to GWP in both soils while CH_4 uptake decreased the GWP only slightly after N application. This is in agreement with previous findings that CH_4 consumption or emissions from non-flooded temperate soils contributed only negligible to total soil-derived GWP (Flessa et al., 2002; Maljanen et al., 2004; Chu et al., 2007; Regina et al., 2007; Soussana et al., 2007). The contribution of N_2O to the soil-derived GWP became significant in both soils after fertilization, with a much stronger effect in soil Niederschleinz. This suggests that soil-derived GWP was strongly influenced by rates of nitrification and denitrification, and therefore potentially by the soil microbial community. However, to calculate net GWP it is necessary to include C fixation by plants. Due to slower plant growth in the NH_4^+ treatment (Inselsbacher et al., 2009a) less C was fixed in plants over the experimental period of 11 days in this treatment, consequently increasing the net GWP in both soils. Our results indicate that in the initial growth period of barley, both soils were net sinks of CO_2, as CO_2, N_2O and CH_4 emissions could not compensate the C fixation by plants. Nevertheless, due to weight-based calculations within this study and the small scale of the microcosms used, it was not possible to exactly estimate net GWP. To accomplish this it will be necessary to validate these findings in the field together with area-based calculations which are more suitable for this purpose.

Overall, the results of our study indicate that with the right choice of inorganic N fertilizer the soil-derived GWP can potentially be decreased. The optimal ratio of NH_4^+ to NO_3^- contained in fertilizers to get minimum N_2O emissions strongly depends on the soil microbial composition. Our study suggests that archaea have the potential to significantly contribute to N_2O emissions and therefore should be quantitative assessed in future studies on N_2O fluxes from soils. Fertilizer N fertilization additionally increased root respiration leading to higher CO_2 emissions and to higher denitrification rates induced by enhanced root exudation. However, the specific importance of organic carbon in the regulation of N_2O to N_2 ratios still remains unclear and especially the importance of root-derived carbon flow in the rhizosphere against that of soil organic matter carbon requires further studies. The short-lived pulses of N_2O occurring after application of surplus N suggest that a refinement of mineral N fertilizer application could have the potential to reduce losses of applied N via N_2O emissions. At low levels of soil N, competition between plant uptake and soil microbes favours plant assimilation such that proportionally less N_2O is produced than at higher fertilizer concentrations (Abdalla et al., 2009). Therefore, a better match of N supply with crop demand by multiple smaller N applications, as suggested previously (Velthof et al., 1998; Jones et al., 2007), might lead to decreased nitrification- and denitrification-mediated N_2O losses.

CO_2 emissions from the soil contributed most to soil-derived GWP, while CH_4 consumption decreased the GWP only slightly. The contribution of N_2O to the soil-derived GWP was dependent on the soil microbial community composition and was increased by plant growth in both soils. Therefore, for a detailed understanding of mechanisms responsible for greenhouse gas emissions future research should cover an interdisciplinary field, combining biochemical techniques with molecular based analyses, as well as [15]N (and ^{18}O) tracer studies to follow the fertilizer into the different compartments of soil-plant-atmosphere systems.

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	Purkersdorf	**Niederschleinz**
Soil type	Gleyic Cambisol from sandy loamy flysch	Chernozem from Loess
Geographic location	48°12'25'' N 16°10'37'' E	48°35'59'' N 15°10'24'' E
Altitude (m. a. sl.)	248	244
Water condition	Moist	Moderately dry
Clay (%)	2	18
Silt (%)	65	74
Sand (%)	33	8
pH (H_2O)	6.6	7.7
$CaCO_3$ (%)	0.06	8.5
Exchange capacity (mval%)	11.2	15.4
Base saturation (%EC)	81.4	98.1
Bulk density (g DW cm^{-3})	1.06	0.96
Total C (mg C g^{-1} DW)	16.2	26.4
Total N (mg N g^{-1} DW)	1.6	1.9
C/N	10.0	14.2

Table 1 Physical and chemical characteristics of the top layer (0 – 15 cm) of two agricultural soils, Purkersdorf and Niederschleinz, collected from the vicinity of Vienna, Austria.

Source of variation	CO$_2$		CH$_4$		N$_2$O	
	df	F	df	F	df	F
Soil	1	28.1***	1	4.6*	1	5.4*
N form	3	1.8	3	2.4	3	0.1
Harvest time	3	290.0***	3	4.5**	3	8.4***
Soil x N form	*3*	*1.1*	*3*	*0.4*	*3*	*0.14*
Soil x time	3	30.6***	3	1	3	1
N form x time	9	2.5*	9	0.9	9	0.1
Soil x N form x time	9	0.3	9	0.1	9	0.1

Table 2 F-statistics from three-way ANOVA of the effects of soil type (Purkersdorf, Niederschleinz), applied N form (NH$_4$NO$_3$, NH$_4$Cl, KNO$_3$) and harvest time (2h before, 4h, 1d and 3d after N application) on emissions of CO$_2$, CH$_4$ and N$_2$O (n = 7) from soil microcosms after the first fertilization event. * $P<0.05$, ** $P<0.01$, *** $P<0.001$

Source of variation	CO$_2$		CH$_4$		N$_2$O	
	df	F	df	F	df	F
Soil	1	723***	1	4.6*	1	2.9
N form	2	7.7***	2	2.3	2	43.6***
Harvest time	5	60.4***	5	29.3***	5	81.4***
Soil x N form	2	150.9***	2	1.1	2	11.3***
Soil x time	5	38.6***	5	1.3	5	0.5
N form x time	10	8.5***	10	2.7**	10	2.9**
Soil x N form x time	10	3.7***	10	0.4	10	0.4

Table 3 F-statistics from three-way ANOVA of the effects of soil type (Purkersdorf, Niederschleinz), applied N form (NH$_4$NO$_3$, NH$_4$Cl, KNO$_3$) and harvest time (4h, 1d, 2d, 3d, 6d and 8d after N application) on emissions of CO$_2$, CH$_4$ and N$_2$O (n = 7) from soil microcosms after the second fertilization event. * $P<0.05$, ** $P<0.01$, *** $P<0.001$

	Purkersdorf					**Niederschleinz**			
	Sum of square	*df*	Mean square	*F*-ratio		Sum of square	*df*	Mean square	*F*-ratio
Regression	787.39	3	262.46	20.43	Regression	132.0	2	66.0	6.17
Residual	179.82	14	12.84		Residual	150.0	14	10.69	
Variable	Coefficient	SD of coefficient	*t*-ratio	*P*-Value	Variable	Coefficient	SD of coefficient	*t*-ratio	*P*-Value
Constant	51.23	4.20	12.18	<0.0001	Constant	-0.07	4.36	-0.02	>0.05
Ratio Bact/Fung	-0.87	0.27	-3.28	0.005	Ratio Bact/Fung	0.52	0.24	2.13	0.049
NH_4^+	-0.30	0.11	2-60	0.021	Root biomass	0.53	0.15	3.47	0.004
NO_3^-	-0.29	0.05	-6.05	<0.0001					

Table 4 Results for the multiple regression analysis of CO_2 fluxes in soils Purkersdorf and Niederschleinz. Ratio Bact/Fung is the ratio of bacterial to fungal genome equivalents.

	Purkersdorf					**Niederschleinz**			
	Sum of square	*df*	Mean square	*F*-ratio		Sum of square	*df*	Mean square	*F*-ratio
Regression	20.95	2	10.47	12.44	Regression	51.36	3	17.12	8.72
Residual	12.63	15	0.84		Residual	27.48	14	1.96	
Variable	Coefficient	SD of coefficient	*t*-ratio	*P*-Value	Variable	Coefficient	SD of coefficient	*t*-ratio	*P*-Value
Constant	3.95	1.77	2.23	0.041	Constant	3.83	2.21	1.73	>0.05
TDC	-0.05	0.02	-3.32	0.005	WFPS	-0.10	0.05	-2.31	0.037
NO_3^-	-0.07	0.01	4.82	0.0002	Ratio Arch/Bact	-0.20	0.07	-2.71	0.017
					NH_4^+	-0.39	0.12	-3.36	0.005

Table 5 Results for the multiple regression analysis of CH_4 fluxes in soils Purkersdorf and Niederschleinz. Ratio Arch/Bact is the ratio of archaeal to bacterial ammonium monooxygenase catalytic subunit A (amoA) copies.

	Purkersdorf					**Niederschleinz**			
	Sum of square	*df*	Mean square	*F*-ratio		Sum of square	*df*	Mean square	*F*-ratio
Regression	272.85	2	136.42	16.05	Regression	554.21	4	138.56	31.29
Residual	127.50	15	8.50		Residual	57.56	13	4.43	

Variable	Coefficient	SD of coefficient	*t*-ratio	*P*-Value	Variable	Coefficient	SD of coefficient	*t*-ratio	*P*-Value
Constant	-20.35	5.63	-3.62	0.0025	Constant	8.29	3.31	2.51	0.026
TDC	0.22	0.05	4.40	0.0005	NO3-	-0.08	0.03	-2.44	0.029
NO3-	0.22	0.04	5.16	<0.0001	Root biomass	-0.76	0.15	-5.23	0.0002
					NH4+	1.09	0.17	6.35	<0.0001
					Ratio Arch/Bact	0.78	0.12	6.67	<0.0001

Table 6 Results for the multiple regression analysis of N_2O fluxes in soils Purkersdorf and Niederschleinz. Ratio Arch/Bact is the ratio of archaeal to bacterial ammonium monooxygenase catalytic subunit A (amoA) copies.

N lost from (%):	Purkersdorf			Niederschleinz		
	NH_4NO_3	NH_4Cl	KNO_3	NH_4NO_3	NH_4Cl	KNO_3
1st fertilization	0.03 (0.02)	0.04 (0.02)	0.03 (0.02)	0.04 (0.03)	0.03 (0.02)	0.04 (0.03)
2nd fertilization	0.51 (0.06)	0.24 (0.06)	0.24 (0.02)	0.68 (0.08)	0.20 (0.05)	0.27 (0.03)
Total fertilization	0.22 (0.04)	0.13 (0.04)	0.12 (0.02)	0.29 (0.05)	0.11 (0.04)	0.14 (0.02)
2nd fertilization (1 month)	0.62	0.38	0.37	0.88	0.29	0.43
Total fertilization (1 month)	0.26	0.19	0.18	0.37	0.14	0.21

Table 7 Loss of fertilizer N via N_2O emissions from soils Purkersdorf and Niederschleinz after applying 3 different forms of inorganic N (NH_4NO_3, NH_4Cl, KNO_3). Values represent total losses of N (in %) from the first fertilization during the 3 following days, from the second fertilization during 8 following days and from total applied fertilizer during the whole time period (11 days). Total losses of N from the 2nd fertilization and from total N input over a 1-month period were calculated by extrapolating cumulative curves of N_2O as shown in Figure 3. Values represent means ($\pm$ SE), n=7.

	Purkersdorf			Niederschleinz		
	NH_4NO_3	NH_4Cl	KNO_3	NH_4NO_3	NH_4Cl	KNO_3
Total CO_2 equivalents	351.5 (15.5)	306.1 (19.7)	265.9 (14.0)	143.2 (14.9)	146.5 (15.4)	174.1 (13.8)
Contribution of CO_2 (% of total)	97.7 (4.0)	98.0 (5.9)	98.0 (4.9)	91.6 (8.9)	96.5 (9.2)	96.0 (7.1)
Contribution of CH_4 (% of total)	-0.1 (0.02)	-0.1 (0.04)	-0.1 (0.03)	-0.2 (0.1)	-0.2 (0.1)	-0.2 (0.1)
Contribution of N_2O (% of total)	2.4 (0.4)	2.0 (0.5)	2.1 (0.3)	8.6 (1.5)	3.7 (1.2)	4.3 (0.7)
C fixed in plants	25.1 (1.7)	18.0 (0.7)	33.5 (1.7)	31.7 (2.1)	21.4 (1.2)	35.7 (1.4)

Table 8 Total gas emissions from two agricultural soils under different fertilizer treatment (expressed as total CO_2 equivalents kg^{-1} DW) and the relative contribution of CO_2, CH_4 and N_2O to total CO_2 equivalents. Values represent mean cumulative CO_2 equivalents calculated over an 11-day period. Negative values represent CH_4 oxidation. Net CO_2 fixation by plants is given as mg CO_2-C per single plant (n = 7; SE in parentheses).

Figure 1: Concentration of NH_4^+, NO_3^- and total dissolved C in soils Purkersdorf and Niederschleinz during 8 days after application of 3 different inorganic N fertilizers (NH_4NO_3, NH_4Cl or KNO_3). Soils were preincubated in the microcosms for 14 days before fertilization. The first samples were taken at day 3, 4 hours after the second fertilization event. Symbols and bars represent means $\pm$ SE, n = 15.

Figure 2: Cumulative CO_2 emissions, CH_4 consumptions and N_2O emissions from microcosms filled with soils Purkersdorf and Niederschleinz during 11 days. Soils were preincubated in the microcosms for 14 days before fertilization. Dashed arrows indicate date of planting barley seedlings. Full arrows indicate dates of inorganic N fertilization (NH_4NO_3, NH_4Cl or KNO_3). Control samples received distilled water instead of fertilizer and were measured only until day 4. Symbols represent cumulative means $\pm$ SE, n = 7.

Figure 3: Cumulative N_2O emission from microcosms filled with soils Purkersdorf and Niederschleinz during one month. Soils were preincubated in the microcosms for 14 days before fertilization. Full arrows indicate dates of inorganic N fertilization (NH_4NO_3, NH_4Cl or KNO_3). The dotted line at day 11 indicates the end of direct measurements. Values from day 11 onwards were calculated with best fitting exponential growth equations.
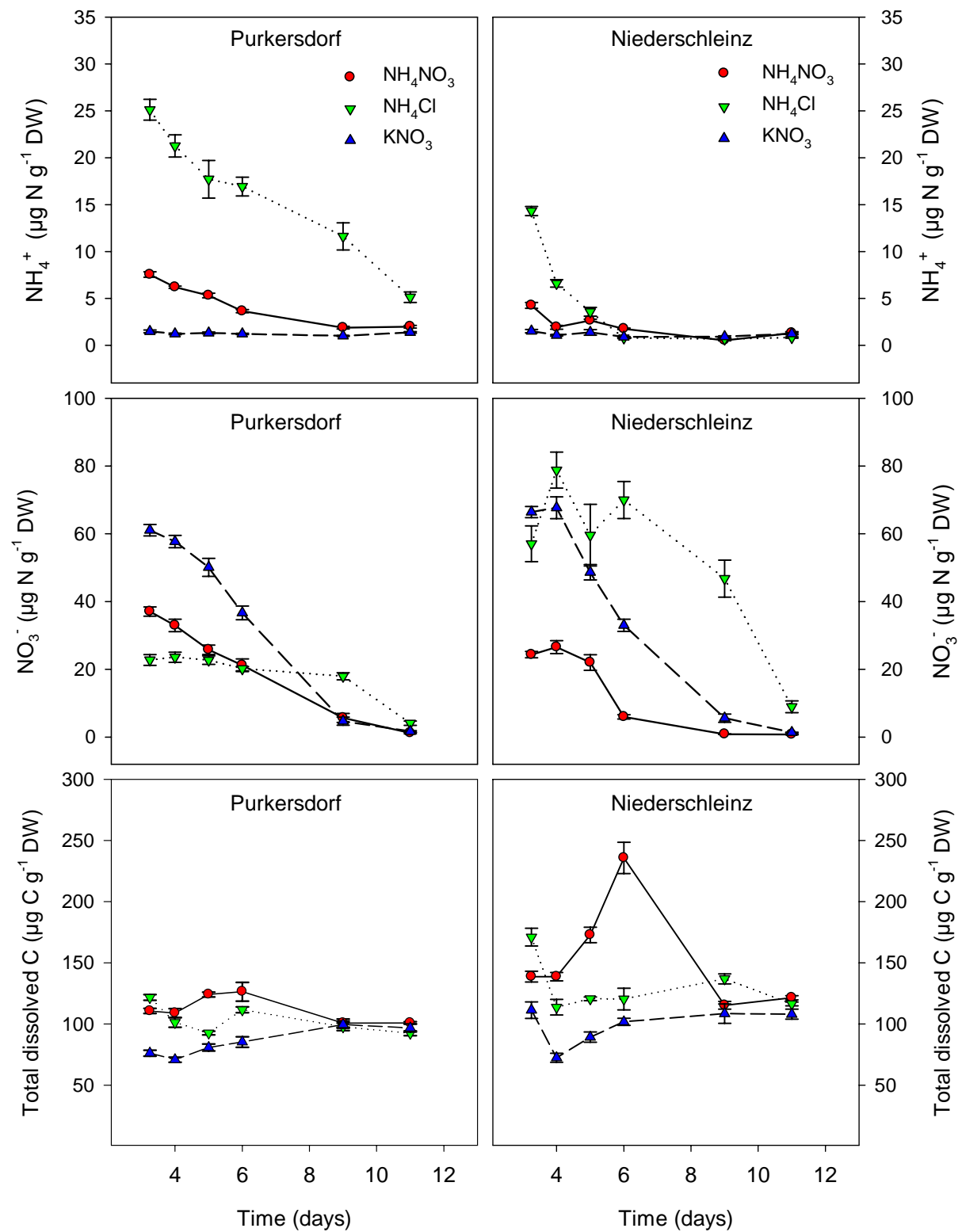
Figure 1
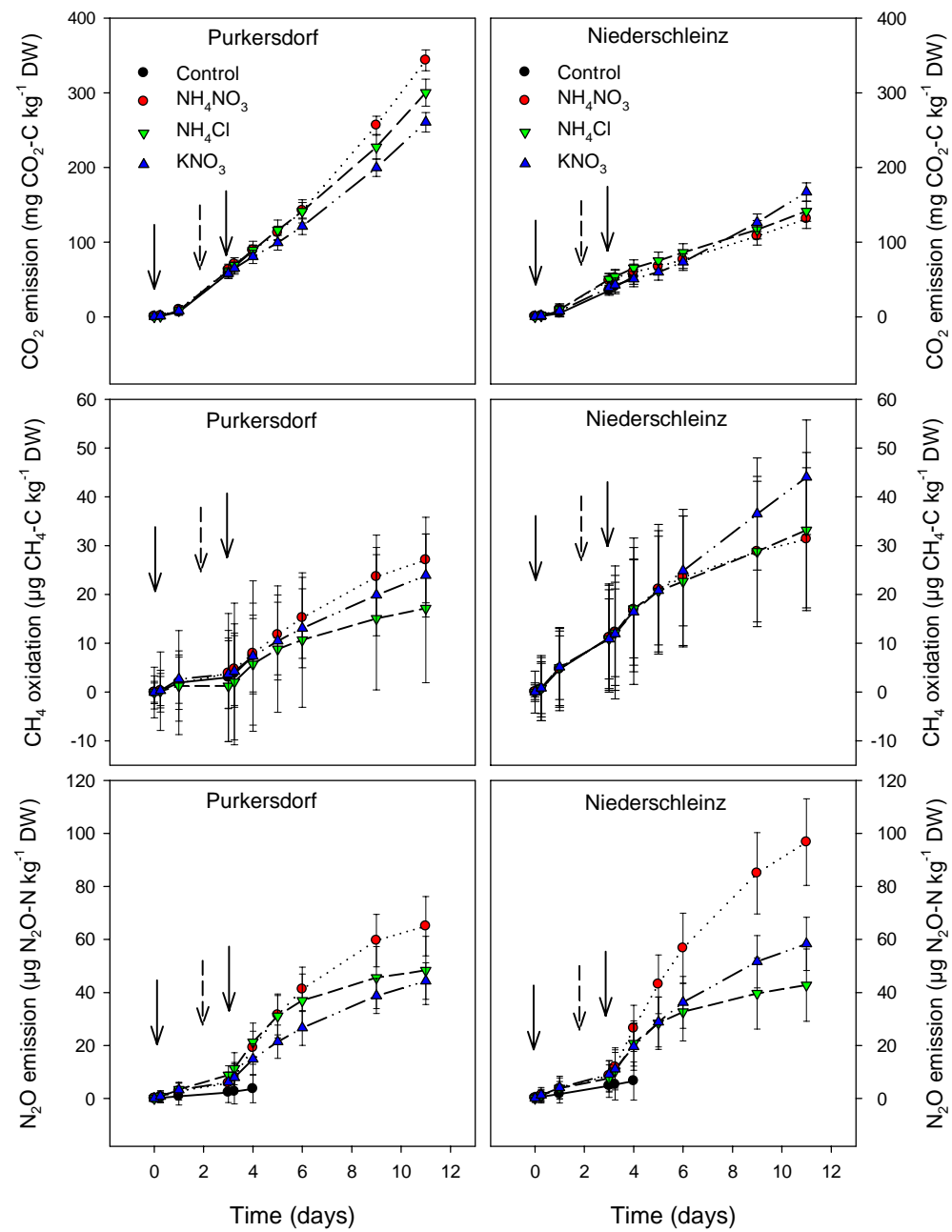
Figure 2
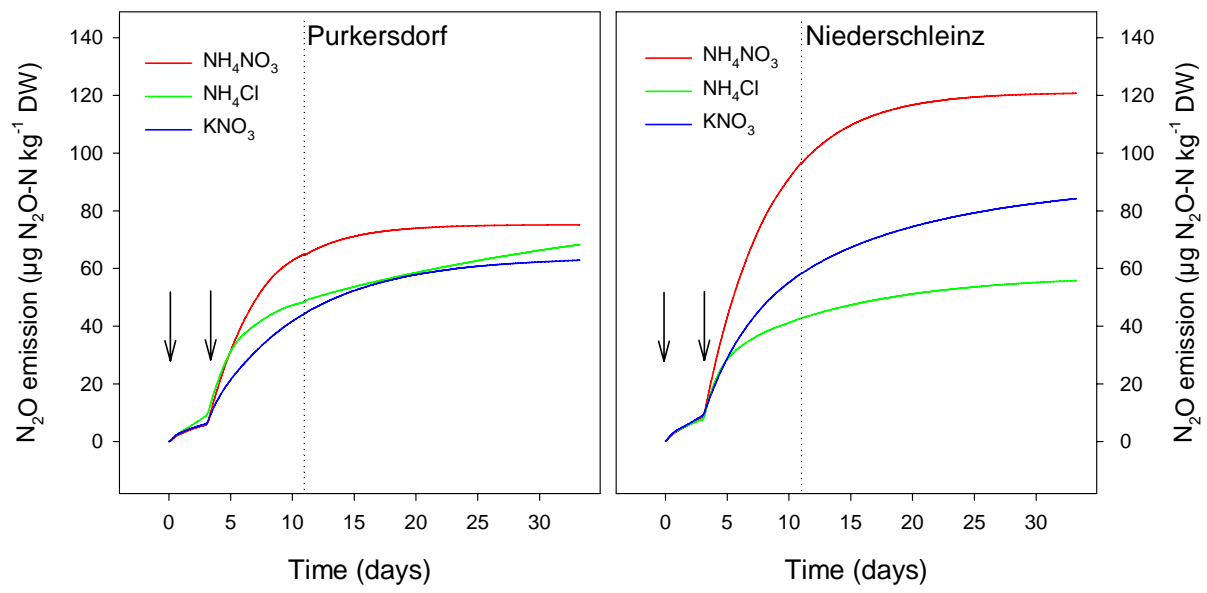
Figure 3

Appendix A

A Suite of Rapid and Simple Methods for Measuring Total Dissolved Nitrogen, Inorganic Nitrogen and Microbial Nitrogen in Soils

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Rebecca Hood-Nowotny, Nina Hinko-Najera Umana, Erich Inselsbacher, Petra Oswald, Wolfgang Wanek*

Department of Chemical Ecology and Ecosystem Research, University of Vienna, Althanstrasse 14, A-1090 Vienna, Austria

* corresponding author: Wolfgang Wanek

14 **ABSTRACT**

There are numerous methods for measuring inorganic, soluble organic and microbial nitrogen

16 in soils, although many of these are complex and/or require expensive equipment. We have

modified a number of methods for measurement of ammonium, nitrate, total dissolved N and

18 soil microbial biomass N in soils. The methods are based on a micro-titer plate format and are

rapid and simple to perform. Ammonium is quantified by a colorimetric method based on the

20 Berthelot reaction. Total dissolved N and soil microbial biomass N (released by chloroform

fumigation) are quantified as nitrate after alkaline persulfate oxidation. Nitrate is estimated

22 directly or after persulphate oxidation by reduction of nitrate to nitrite by vanadium (III)

chloride and subsequent colorimetric determination of nitrite by acidic Griess reaction. The

24 new suite of methods was compared with conventional methods such as anion

chromatography (nitrate) and high temperature catalytic oxidation (total dissolved N) and

26 produced comparable detection limits, linearities and precisions compared to conventional

methods. The simplicity, rapidity and low cost of this suite of methods therefore allow an

28 expansion of the scope and range of nitrogen cycle studies in agricultural and natural settings

where high tech instrumentation is not available.

30

KEY WORDS: ammonium, dissolved organic nitrogen, microbial biomass, nitrate, persulfate

32 digestion, soil, total dissolved nitrogen, vanadium chloride.

34   Plant available nitrogen is one of the main limiting constraints to productivity in agro-ecosystems and an important parameter in defining the structure and function of natural

36   ecosystems. In the soil there are many soluble nitrogen pools, e.g. dissolved organic N, ammonium, nitrate and nitrite which are interdependent on the size and fluxes from other N

38   pools (total soil N, microbial biomass N), as well as environmental and physicochemical factors. Studies of isolated pools or processes rarely reveal the subtle interactions which drive

40   globally important ecosystem functions and processes, such as the loss of greenhouse gases, nitrate leaching or the terrestrial carbon cycle. For example low net fluxes of N may be the

42   result of highly dynamic gross N fluxes, leading to false assumptions about the role of these processes controlling the terrestrial N cycle. Therefore methods which trace the dominant

44   gross nitrogen processes i.e. ammonification, the breakdown of organic N to inorganic plant available ammonium, and nitrification, the oxidative conversion of ammonium to nitrate, are

46   required to assess their role and to allow us to predict and model their impacts. The complex nature of the nitrogen cycle therefore calls for a holistic approach to the understanding of the

48   system.

       One of the constraints of a holistic approach is the range and number of parameters

50   that must be simultaneously measured. Many of these measurements are laborious, time consuming or require expensive specialised equipment. This is especially an issue in field

52   settings remote of state-of-the-art laboratories and in developing countries where a better understanding and management of the nitrogen cycle could yield the greatest benefits, not

54   only from an agro-ecological perspective, but also through the generation of validated experimental data for predictive N cycle and climate models.

56   A number of techniques exist for the measurement of inorganic nitrogen in soil extracts. These include cation and anion chromatography, flow injection analysis

58   (photometry) and chromatographic analysis with pre-column derivatisation (fluorescence). Nitrate is usually measured using either ion chromatography or colorimetric determination

60   after reduction of nitrate to nitrite and addition of Griess reagent. Reduction of nitrate to nitrite has been achieved using either cadmium or biological reduction by bacterial nitrate

62   reductase (Hevel and Marletta, 1994). The health and environmental hazards posed by cadmium and its non specific nature at low nitrate concentrations (Marzinzig et al., 1997)

64   render cadmium an unsuitable reduction agent for routine nitrate analysis. An alternative reduction agent, vanadium (III) chloride (VCl_3), has been shown to adequately reduce nitrate

66   in medical and water samples (Cemek et al., 2007; Miranda et al., 2001). Additionally there have been reports of using vanadium reduction for nitrate determination in soil extracts

68   (Cavagnaro et al., 2008). However, there has been no thorough systematic validation of the

vanadium (III) chloride reduction method for soil nitrate analysis and for nitrate quantification

70   following persulphate digestion of total dissolved N. This paper will attempt to fill this gap

and thereby investigate the suitability of this simple method.

72   The role of soil microbial biomass (SMB) is fundamental in soil processes such as organic

matter decomposition, nutrient cycling and plant growth. It is its role in these globally

74   important biogeochemical processes that has led to extensive research into its size and

function in the soil ecosystem. Soil microbial biomass N is routinely measured in aerobic soils

76   using the chloroform fumigation extraction method (Brookes et al., 1985a; Brookes et al.,

1985b). Although there are some criticisms of this technique (Müller et al., 2003) it remains

78   the standard technique in many laboratories. Basically chloroform fumigated (24 hours) and

non-fumigated soils are extracted with 0.5 M K_2SO_4 and the difference in total soluble N

80   measured in the digested extracts is attributed to the release of N from the lysed microbial N

fraction. This is then multiplied by an extraction coefficient (K_{EN}) to obtain a microbial

82   biomass N value.

Recently there has been an increased interest in the role of dissolved organic nitrogen

84   (DON) in the soil nitrogen cycle which was reviewed by (Murphy et al., 2000). It has been

shown that in many soils there is a pool of soluble organic N which is of comparable size to

86   the mineral N pool being about 0.3-1% of the total N pool in arable soils. It is thought that this

pool is composed of easily mineralisable N and that it has a significant impact on ecologically

88   and agronomically important processes such as N mineralization and nitrification. Previous

studies have demonstrated that the best way to extract soluble organic N is by salt solutions,

90   such as KCl or K_2SO_4 (Jones and Willett, 2006), as the use of water alone causes dispersion

of clays. The DON pool is not measured directly but is obtained by subtraction of the mineral

92   N concentration from the total soluble N concentration (TSN).

Nitrogen in TSN and SMB extracts can be measured as ammonium following Kjeldahl

94   digestion, but more recently this method has be superseded by automated methods (high-

temperature catalytic oxidation and chemiluminescence detection of NO) or by persulphate

96   ($K_2S_2O_8$) oxidation. During persulphate oxidation inorganic N i.e. NH_4^+ (and NO_2^-) and DON

are converted to NO_3^- through complete oxidation following autoclaving of the soil extract or

98   ultraviolet enhanced digestion (Cabrera and Beare, 1993). The coupling of a simple nitrate

test to this rapid digestion procedure could allow for more holistic studies and advance our

100   understanding of SMB and dissolved organic nitrogen (DON) in the global nitrogen cycle.

Here we present a suite of nitrogen pool measurement methods which are simple,

102   rapid and are carried out with standard laboratory equipment. By simplifying and modifying

these soil nitrogen measurement techniques the scope and range of experiments can be greatly

104   expanded.

106   **METHODS**

108   **Soils**

Soil extracts were prepared from a set of experiments in which the soils had received

110   different rates and forms of nitrogen fertilizer to produce soils with a range of inorganic and

organic nitrogen values (Inselsbacher et al., 2009). For comparison of the proposed suite of

112   methods with conventional protocols for nitrate and total dissolved N analysis the following

soils were used: Purkersdorf, a gleyic cambisol from sandy loamy flysch and Niederschleinz,

114   a chernozem soil derived from flysch. To test for precision of the proposed set of methods soil

extracts (0.5 M K_2SO_4) were produced in five replicates from four un-amended soils and from

116   the same soils 24 hours after amendment with ammonium nitrate (0.2 mg g^{-1} soil f.w.): Tulln

(a pseuogley on planosol), Riederberg (a calcaric cambisol from clay flysch), Purkersdorf and

118   Niederschleinz. For a full description of soils see (Inselsbacher et al., 2009).

120   **Chemicals**

All chemicals for colorimetric determinations as well as standards and salts were

122   purchased in Biochimica Select quality from Fluka (Sigma Aldrich) or of highest quality from

Sigma. Solutions were prepared in deionised ultrapure water (>18.2 MOhm cm^{-1}, MilliQ,

124   Millipore, Vienna, Austria).

126   **Soil extractions**

Two gram aliquots of fresh, sieved soil were weighed into 20 mL HDPE vials

128   (scintillation vials, screw cap) using a three figure balance and weights noted. Soil samples

were extracted with 15 ml $CaSO_4$ (10 mM), KCl (1 M) or K_2SO_4 (0.5 M) for inorganic N

130   analysis, shaken for 60 minutes and filtered gravimetrically through ashless Whatman filter

paper. For determination of total soluble N and soil microbial biomass N soil samples (2 g)

132   were extracted with 15 mL K_2SO_4 (0.5 M) as given above.

134   **Ammonium**

Ammonium was quantified in 1 M KCl or 0.5 M K_2SO_4 extracts by a modified

136   indophenol method based on the classical Berthelot reaction (Kandeler and Gerber, 1988).

During this process ammonium is oxidized to monochloroamine by sodium

138   dichloroisocyanuric acid and subsequently forms a green indophenol compound in the

presence of phenolics with an unsubstituted paraposition in an alkaline medium. Nitroprusside

140   serves as a catalyst. The colour reagent was prepared by mixing equal volumes (1:1:1, v:v:v)

of salicylate-nitroprusside solution, NaOH solution and deionised water. The salicylate-

142   nitroprusside solution was prepared fresh daily by dissolving 8.5 g sodium salicylate and 63.9

mg sodium nitroprusside dihydrate in 50 mL deionised distilled water, the 0.3 M NaOH

144   solution by diluting 785 µl of 50% NaOH in 50 mL deionised water. The oxidation reagent

was prepared by dissolving 0.1 g of dichloroisocyanuric acid sodium salt dihydrate in 100 ml

146   of deionised water. For colour reaction, 100 µL sample, standard or blank (extraction

solution) were pipetted into a microtitre plate, followed by 50 µL colour reagent and 20 µL

148   oxidation reagent. Mixtures were left at room temperature (21°C) for 30 minutes for colour

development and the absorbance was subsequently measured photometrically at 660 nm on a

150   microtiter plate reader (Tecan Infinite M200, Tecan Austria GmbH). Alternatively the

volumes can be scaled up for use of plastic semi-micro cuvettes (1.5 mL) in a standard

152   photometer. Standards are prepared fresh daily from a NH_4Cl stock solution (1000 mg N L^{-1},

stable at 4° C for several weeks) in the respective extractant, by serial dilution ranging from 5

154   mg N L^{-1} to ca. 0.02 mg N L^{-1}.

156   **Nitrate**

For ion chromatographic (HPLC) analysis nitrate is usually measured in water or

158   $CaSO_4$ extracts due to technical difficulties and overloading of columns when running

concentrated KCl extracts. It is however also possible to extract both ammonium and nitrate

160   simultaneously in 1 or 2 M KCl or 0.5 M K_2SO_4 and measure nitrate concentration based on

the vanadium chloride (VCl_3) method as described below, thus saving an additional

162   extraction. For direct comparison of methods nitrate concentrations were quantified in $CaSO_4$

extracts by anion chromatography and photometry based on the VCl_3–Griess method.

164   Moreover, nitrate concentrations were compared in KCl and $CaSO_4$ extracts, as measured by

VCl_3–Griess method and HPLC respectively.

166   Nitrate was determined in $CaSO_4$ extracts by ion chromatography (DX 500, Dionex,

Vienna, Austria) after separation on an anion exchange column (AS11, 250 x 4 mm i.d.,

168   Dionex, Vienna, Austria) by linear NaOH gradient elution (0.5 mM to 37.5 mM within 10

min at a flow rate of 2 ml min^{-1}, with a column temperature of 35° C) and after chemical

170   suppression (ASRS-Ultra, Dionex). Nitrate standards were prepared fresh daily from a stock

solution (1000 mg NO_3^- L^{-1}) in the range of 10 to ca. 0.01 mg N L^{-1} by serial 1:2 dilution. The

172   stock solution is stable for several months when kept at 4° C.

The vanadium chloride (VCl$_3$-Griess) method is based on (Miranda et al., 2001) for

174   biological samples. Nitrate is converted to nitrite in an acidic vanadium (III) chloride (VCl$_3$)

medium, and nitrite concentration measured by direct coupling with the Griess reaction.

176   Absorbance is then measured photometrically at 540 nm. A saturated vanadium reagent

solution was prepared fresh daily by dissolving 400 mg vanadium (III) chloride in 50 mL 1 M

178   HCl and excess solids were filtered through ashless Whatman filter paper. Griess reagent 1

was made up by dissolving 50 mg N-napthylethylenediamine dihydrochloride in 250 mL of

180   deionised water. Griess reagent 2 was prepared by dissolving 5 g of sulfanilamide in 500 mL

3 M HCl. The latter solutions are stable for several months when stored in the dark at 4° C.

182   For colour reaction each 100 µL sample, standard or blank (extractant) were pipetted into the

microtitre plates and 100 µL VCl$_3$ was added, rapidly followed by each 50 µL Griess reagent

184   1 and 2 to give a total volume of 300µL. The Griess reagents can also be mixed in equal

volumes directly before addition (100 µL) to the assays. The plates were incubated at 37° C

186   for 60 minutes and measured in a microtiter plate reader (Tecan Infinite M200) at 540 nm.

Reagents were added using a hand-held manual dispenser (Multipette, Eppendorf, Vienna,

188   Austria) allowing fast and highly reproducible adding of reagents. Nitrate standards were

prepared as above but ranging between 5 and 0.02 mg N L^{-1}.

190

Total soluble nitrogen and dissolved organic nitrogen

192   Concentrations of TSN were measured in 1 M KCl or 0.5 M K$_2$SO$_4$ extracts using an

automated TC/TN analyzer based on high temperature catalytic oxidation (HTCO) or by

194   determination of nitrate following persulphate digestion of the K$_2$SO$_4$ extracts. Dissolved

organic N was determined by subtraction of inorganic N (NH_4^+ and NO_3^-) from total soluble

196   N concentrations (TSN).

TSN i.e. the sum of soluble organic and inorganic N in KCl extracts was measured

198   after HTCO of N to nitrogen monoxide (NO) by the TNM-1 unit (Shimadzu TOC-VCPH with

TNM-1 unit and ASI-V autosampler, Shimadzu, Japan). Sample aliquots (0.1 to 2 mL out of a

200   total of 10 mL sample volume) are injected onto a hot reactor (720° C) filled with oxidation

catalyst (Pt supported on alumina beads) where TSN thermally decomposes to create nitrogen

202   monoxide. The carrier gas (synthetic air) carrying nitrogen monoxide from the combustion

204   tube is cooled and dehumidified before passing into a chemiluminescence detector, where the nitrogen monoxide is reacted with ozone to produce nitrogen dioxide (NO_2) in an excited state that when returning to the ground state emits chemiluminescence.

206   Alternatively, TSN was quantified by alkaline persulphate digestion (oxidation) (Cabrera and Beare, 1993; Doyle et al., 2004) of ammonium and organic N to nitrate which

208   was subsequently quantified by VCl_3-Griess method (see above) or by UV absorption at 210 nm (Hagedorn and Schleppi, 2000). Persulphate oxidation depends on peroxodisulphate

210   decomposition to persulphate radicals which represents the oxidizing agent. Persulphate has a half-life of ca. 30 s at 125°C and 4 h at 75°C (Peyton, 1993). It is this decomposition step that

212   is rate-limiting while further oxidation steps are rapid relative to free radical production (Peyton, 1993). The persulphate reagent was prepared by dissolving 50 g $K_2S_2O_8$, 16.8 g

214   NaOH and 30 g H_3BO_3 and making up to 1 L deionised water. For digestion each 2.5 mL K_2SO_4 extract was mixed with 2.5 mL persulphate reagent in 6 mL GC headspace vials

216   (Perkin Elmer N930-2134, with PTFE butyl septa) which were immediately crimped thereafter. Samples were autoclaved for 40 min at 120° C. Septa were re-used three times,

218   thereafter materials corroded and became leaky. Amino acid standards (glycine, alanine and histidine, 0.16-5 mg N L^{-1}) were prepared in 0.5 M K_2SO_4 and digested as above to test

220   digestion efficiency. Nitrate standards (0.078 to 5 mg N L^{-1}) were prepared in 0.5 M K_2SO_4 but calibration was performed with glycine standards, ranging from 0.078 to 5 mg N L^{-1}.

222   Nitrate was consequently quantified by VCl_3-Griess method (see above) or by UV absorption at 210 nm (Hagedorn and Schleppi, 2000). For the UV method, 0.8 mL digested sample was

224   mixed with 0.4 mL 30% H_2SO_4 to give a pH <1. Nitrate was then measured by UV absorption at 210 nm on a spectrophotometer (Beckman DU 7400 diode array spectrophotometer) using

226   semi-micro quartz cuvettes. Nitrate standards were prepared in 0.5 M K_2SO_4 and acidified as above.

228

Microbial biomass extraction

230   Soil microbial biomass N was determined by chloroform fumigation-extraction technique as described previously (Amato and Ladd, 1988; Brookes et al., 1985a; Brookes et

232   al., 1985b). Briefly, aliquots of fresh soil (2 g) were fumigated in a glass desiccator with ethanol free chloroform vapour for 24 h at 22° C. Both, fumigated and non-fumigated soil

234   samples were extracted with 15 mL K_2SO_4 (0.5 M) for 60 min prior to gravimetric filtration through ashless Whatman filter paper. Total soluble N in the extracts was measured by HTCO

236   or by persulphate digestion followed by nitrate determination (see TSN analysis above). The

difference in TSN in extracts of fumigated and non-fumigated soils is attributed to the release

238   (flush) of N from lysed microbial cells. A correction factor for microbial biomass N (K_{EN} =

0.45) was applied for incomplete extraction of microbial N (Jenkinson et al., 2004).

240

Calculations

242   Analyses of standards by the different methods were performed independently on three

to six occasions, each with new reagents and standards. Data for method evaluation are

244   presented combined for all data and for single day data separately. Calibration was performed

by Model I regression analysis of standard concentration versus raw peak area or raw

246   absorbance. Blank samples were run in three to five replicates with each batch. Blank

concentrations were determined from absorbance values of direct blank measurements or

248   from the y-intercept of regression lines, divided by the slope of the standard curve. The limit

of linearity was set by a threshold of the coefficient of regression (R^2) >0.99. Concentrations

250   that caused R^2 to decline below 0.95 were considered outside the linear working range. The

limit of detection (LOD) of each method was estimated as three times the standard deviation

252   of the concentration of blanks (Taylor, 1987). Where blanks yielded negative y-intercepts

and/or undetectable peak areas (HTCO, anion chromatography) the standard deviation of the

254   lowest standards was used for calculation of LOD. The limit of quantification (LOQ) was

calculated as ten times the standard deviation of the concentration of blanks. Precision, the

256   degree of agreement between repeated measurements, was evaluated based on the coefficient

of variation (CV = SD/mean * 100). CV was examined for eight soil samples and N

258   concentrations were measured in five replicates. Moreover, LOD is not only given for N

concentrations in solution (mg N L^{-1}) but was also converted to N content of soil samples (µg

260   N g^{-1} soil d.w.) as analysed by the presented protocol (2 g soil fresh weight, extracted by 15

mL salt solution, ca. 25% water content). Comparison of independent methods e.g. HTCO

262   and persulphate digestion was carried out by Model II correlation analysis. All statistical

analyses were performed with Stat Graphics 5.0.

264

RESULTS AND DISCUSSION

266

Ammonium analysis

268   The colorimetric ammonium method is described here for completeness. The

absorbance of standards was highly linear (r^2 =0.999) in the 0.078-5 mg N L^{-1} range (ca. 5-

270   350 µmol L^{-1}, Figure 1). Blanks were about 0.14 mg N L^{-1} (Table 1) and the precision (SD) of

blanks was greater between-day (±0.005) than in single-day measurements (±0.0006). The

272 long-term precision of blank measurements allowed a low LOD for ammonium-N of 0.016

mg L^{-1} allowing soil ammonium to be measured accurately in soil KCl or K$_2$SO$_4$ extracts at

274 very low concentrations (corresponding to ~0.16 µg N g^{-1} soil d.w.). This overall LOD was

10-fold lower compared to similar photometric methods based on the Berthelot reaction

276 published elsewhere (Husted et al., 2000; Rhine et al., 1998), and was 8-fold lower on a

single-day basis compared to long-term LOD. The mean precision (CV) for a set of eight soil

278 samples was 2.1 (range 1.1-2.7, Table 2) which is comparable to or better than other methods

of soil N analysis e.g. HTCO of TSN ranging between 2.9 and 12.6 (Bronk et al., 2000).

280   There has been much concern with interfering substances such as amino acids, amines

and amides in plants (Husted et al., 2000) and soils (Rhine et al., 1998) since the Berthelot

282 reaction is not entirely specific for ammonium. We did not test this but in agricultural soils

soluble organic N is of the same order of magnitude as mineral N and of equal size in many

284 cases (Murphy et al., 2000), and this consists mainly of high molecular weight forms that do

not interfere with the Berthelot reaction. Moreover, substitution of salicylate or phenol in the

286 Berthelot reaction by 2-phenylphenol sodium salt tetrahydrate (PPS) as a chromogenic

substrate can be used in problematic soils with low ammonium and high organic N contents.

288 Use of PPS revealed a striking improvement in the specificity for ammonium, and

interferences by amino acids decreased by ~10-fold or were eliminated (Rhine et al., 1998).

290

Nitrate analysis

292   The absorbance of nitrate standards by the VCl$_3$/Griess method was highly linear with

concentration (r^2 =0.992) for the range of 0.02-5 mg N L^{-1} (ca. 1.4-350 µmol L^{-1}) suggesting a

294 large dynamic range (Figure 1). However it appears that the colour reaction is too intense at

around 10 mg nitrate-N L^{-1} and a top standard of 5 mg N L^{-1} is therefore recommended. The

296 overall and single-day LOD for direct nitrate quantification was 0.016 mg N L^{-1} being close to

the LOD of 0.007 mg N L^{-1} reported by (Miranda et al., 2001). Blanks were about 0.38 ± 0.005

298 mg N L^{-1} (Table 1), the low standard deviation of the blanks allowing sensitive determination

of nitrate in soils (~0.16 µg N g^{-1} soil d.w.). In comparison anion chromatography was

300 superior in terms of LOD (0.001 mg N L^{-1}), linearity (R^2=0.999) and range (0.01-10 mg N L^{-1}

) for nitrate analysis. However, for most soil samples this extent of sensitivity is unnecessary,

302 and nitrate concentrations measured in the CaSO$_4$ extracts using anion chromatography or the

VCl$_3$/Griess method were highly comparable (Figure 4) with a slope of 0.9803 and a y-

304 intercept of -0.03 mg N L^{-1}. Since the 95% confidence limit of the slope included the value 1

306 this suggested the response was identical. The 95% confidence limit of the y-intercept also included the value zero which suggested no significant offset between both methods.

308 However, temperature and time affect the colour intensity of the reaction, demanding the addition of individual standard sets in each microtiter plate (Miranda et al., 2001). Colour intensity increased with time, effectively increasing the slope of calibration line and the

310 sensitivity of the assay (Figure 2). Moreover, the VCl_3/Griess method is sensitive to solution composition and salts present, e.g. 0.5 M K_2SO_4 decreased the slope of calibration line by 25-

312 38% compared to deionised water (Figure 2 and 3). The time kinetics of colour development followed an exponential rise to maximum model (Figure 3). Longer times of incubation (e.g.

314 2 hours) therefore yielded higher colour intensities of the dye and higher slopes of the regression line in the calibration procedure. Proper preparation of standards in the respective

316 extractant is therefore import to obtain unbiased results.

Though vanadium (III) does not exclusively reduce nitrate, interferences are only

318 known to derive from compound classes (e.g. reduced thiols, thiol-containing amino acids, nitro- and hydroxyl-L-arginine analogs and nitric oxide) that are uncommon to soils but may

320 be used in media and buffers and can be abundant in biological fluids such as urine or blood (Miranda et al., 2001). Interferences should therefore be negligible for soil analysis.

322 Soils are routinely extracted with 1 M KCl for inorganic NH_4^+ and NO_3^- analysis and therefore a comparison of nitrate concentration in KCl and $CaSO_4$ extracts using HPLC and

324 the VCl_3/Griess method was performed. Nitrate concentrations measured in either 1 M KCl (VCl_3/Griess method) or 10 mM $CaSO_4$ extracts (anion chromatography) of the same soil

326 batches were highly comparable ($R^2=0.994$, Figure 4). The 95% confidence limit of the slope (1.0007) included the value 1 and the 95% confidence limit of the intercept (0.1128) included

328 the value 0, suggesting no systemic difference in measurement. The modified VCl_3/Griess method therefore enables nitrate and ammonium to be measured in the same extract using an

330 extract volume of ca. 0.2 mL. The precision for a set of eight soil samples was 3.5 (CV; range 1.4-6.1) which is comparable or lower to other methods of soil N analysis e.g. ammonium

332 (2.1, Table 2) or HTCO ranging between 2.9 and 12.6 (Bronk et al., 2000). Overall the results demonstrate that VCl_3 reduction of nitrate to nitrite and subsequent reaction with the Griess

334 reagent followed by spectrophotometric analysis is a suitable method to measure NO_3^- concentration in $CaSO_4$, KCl and K_2SO_4 soil extracts. The VCl_3 method is simple and rapid to

336 perform, and does not require toxic chemicals and numerous samples can be run simultaneously. Given the low volumes required for analysis it is also possible to determine

338   nitrate N, ammonium N and TDN (see later) in soil water collected by soil suction tubes or

micro-tensionics.

340   It should be noted that it is also possible to measure nitrite in soil samples using the

microtiter plate technique. This is achieved simply by omitting VCl_3 from the reaction and

342   running appropriate standards and following appropriate soil extraction procedures for nitrite

(Stevens and Laughlin, 1995). The applied Griess reaction exhibits an LOD of 0.029 mg N L^{-1}

344   overall and on a single-day basis of 0.006 mg N L^{-1} (Table 1). Based on 0.5 M K_2SO_4 extracts

(2 g fresh weight, 15 mL extractant) nitrite concentrations in eight soils were low i.e. close to

346   the LOD and exhibited a precision (CV) of 5.3 (range 2.2-9.3, Table 2). Optimally, soils are

extracted using a blending procedure 1:1 with 2 M KCl adjusted to pH 8 for up to 40 min

348   (Stevens and Laughlin, 1995). Extracts are filtered through 0.7 µm glass fiber filters and

analysed within two days of extraction. This protocol would allow detecting nitrite at 0.02 µg

350   N g^{-1} soil d.w.

352   **Total soluble N and microbial biomass N analysis**

Concentrations of total soluble nitrogen and, based on this, of dissolved organic N or

354   microbial biomass N are commonly measured by HTCO or by persulphate digestion followed

by nitrate determination. After alkaline persulphate digestion nitrate is quantified by cadmium

356   reduction to nitrite and Griess reaction (flow injection analyzer), by UV absorbance of nitrate

or anion chromatography. We here propose to apply the VCl_3/Griess method for nitrate

358   determination after alkaline persulphate digestion.

Alkaline persulphate digestion followed by VCl_3-Griess reaction showed a highly

360   linear response between glycine concentration and absorbance (range 0.02 to 5 mg N L^{-1};

Figure 1). Standard deviations of absorbance increased with concentration for the whole data

362   set comprising five independent sets of measurements. While R^2 was 0.946 overall, on a

within-day basis the linearity was higher (R^2=0.990, Table 1), improving to 0.995 for the

364   range 0.02 to 2.5 mg N L^{-1}. Blank concentrations were ca. 0.800±0.045 mg N L^{-1}. Blank

concentrations linearly correlate with headspace-to-solution volume (Hagedorn and Schleppi,

366   2000) and therefore are lower when the volume of headspace above the digestion mix is

restricted. The blank concentrations here were higher than reported elsewhere, e.g. ranging

368   between 0.08 and 0.45 mg N L^{-1} (Doyle et al., 2004; Hagedorn and Schleppi, 2000).

However, blanks also derive from impurities in K_2SO_4, NaOH and persulphate that vary

370   between product batches and from digestion tubes and leaky septa. Re-crystallization,

changing the batches, decreasing headspace volume and work with muffled glass ware

372   therefore can greatly improve the sensitivity of the method, lowering blanks and LOD to

below 0.03 mg N L^{-1} (Doyle et al., 2004; Rogora et al., 2006). The overall and within-day

374   LOD for TSN determination was 0.13 and 0.08 mg N L^{-1}, allowing to detect TSN at a level of

about 0.8 to 1.4 µg N g^{-1} soil dw which is >5-fold below TSN concentrations encountered in

376   almost all agricultural soils assessed (see (Murphy et al., 2000) for review). Measurement of

nitrate in the same persulphate digests was compared between either a UV method (Hagedorn

378   and Schleppi, 2000) or the VCl$_3$-Griess method. This allowed direct comparison of two

methods for measuring nitrate in digested samples independent of digestion efficiency. Both

380   methods showed high comparability (R^2=0.914, Figure 5), and the slope (0.969) and its 95%

confidence limit included the value 1 suggesting the response was similar. The intercept

382   (0.338) and its 95% confidence limit included the value 0 (Figure 5). The absorbance of

nitrate by VCl$_3$/Griess reaction was not affected by alkaline persulphate digestion, comparing

384   nitrate standards with/without persulphate treatment (data not shown). The data therefore

suggest that the methods yield identical results and there were no systematic errors in

386   determination of nitrate concentration between UV or VCl$_3$/Griess reaction. The VCl$_3$-Griess

method represents a suitable and simple method for measuring nitrate in persulphate digests.

388   Moreover, compounds such as high Fe interfere with photometric nitrate detection and

thereby affect the UV absorbance of samples (Hagedorn and Schleppi, 2000), which may

390   explain the variance between both methods. Although the UV method simply requires a UV

spectrophotometer and the use of concentrated acids, it is also far more time consuming than

392   the VCl$_3$-Griess method. However, we advise not to use KCl but K$_2$SO$_4$ extracts for TDN

measurements in soils since chloride strongly interferes with the persulphate oxidation

394   reaction at high concentrations (data not shown; but see (Bronk et al., 2000). Additionally,

chloride can terminate free radical propagation (Peyton, 1993).

396   HTCO showed very high linearity (R^2=0.9995) across a wider range compared to the

persulphate oxidation/VCl$_3$-Griess method (Table 1). Moreover, the LOD of HTCO (0.047

398   mg N L^{-1}) was about half compared to the former method on a within-day basis. Total soluble

nitrogen in soil extracts was further investigated by HTCO or persulphate oxidation/VCl$_3$-

400   Griess method. The strength of relationship was high for extracts of fumigated and

unfumigated soils, i.e. R^2 was 0.937, comparable to 0.901-0.924 reported by (Doyle et al.,

402   2004). The slope of regression line was 1.11 (Figure 5) which was also in the range of 1.09

and 1.33 published by (Doyle et al., 2004). Considering that two very different, independent

404   methods for measurement of total soluble nitrogen were compared the results show that the

proposed persulphate oxidation/VCl$_3$-Griess method is as accurate and precise as the HTCO

406   method.

However, the data strongly indicate that persulphate digestion gives higher TSN

408   concentrations than HTCO. The deviation between both techniques was shown to derive from

differences in digestion efficiency (Rogora et al., 2006; Sharp et al., 2004). In this study we

410   evaluated TSN recovery of the persulphate oxidation – VCl$_3$/Griess method with a range of

amino acids standards, which showed linear and complete recovery following digestion.

412   Compared to external nitrate standards recoveries of nitrate-N from glycine, glutamate and

nitrate after persulphate oxidation were 99.0±2.7% (SD), 99.9±3.3% and 102.4±6.3% (three

414   independent measurements, based on comparison of slopes of regressions; data not shown). It

is further acknowledged that persulphate oxidation shows a greater recovery for reduced and

416   organic N compounds, recoveries for a wide range of labile and refractory standard

compounds tested being 93±13% while the HTCO method yielded recoveries of 87±14%

418   (Bronk et al., 2000). The persulphate digestion procedure also gives higher TSN

concentrations for a range of field samples (Sharp et al., 2004). Sharp et al. (2004)

420   undertaking a rigorous testing of Shimadzu TDN analyzers pointed to incomplete conversion

of some nitrogen compounds by the HTC instruments. Since HTCO was shown to give poor

422   recoveries for humic acids (60-86%) while persulphate oxidation quantitatively converts these

compounds (95-100%; (Bronk et al., 2000) we recommend the latter method particularly in

424   soil studies.

The precision of TDN measurements by persulphate digestion/VCl$_3$-Griess method for

426   a set of eight soil samples was 3.9 (range of CV 1.8-6.8) the precision being slightly higher

than found for HTCO ranging between 2.9 and 12.6 (Bronk et al., 2000) and for four HTCO

428   (Shimadzu) TN instruments i.e. 7.7 (Sharp et al., 2004). The precision of DON was lower as

for TSN alone, the mean CV being 12.5 (range of CV 5.3-24.6; Table 2). This is due to the

430   combined errors of three independent measurements that are propagated into the DON

estimates, a problem commonly encountered for calculation of DON concentration which

432   leads to an inflation of the CV (Sharp et al., 2004). For instance CVs of DON were reported to

increase 2-4 fold compared to CV of TDN (Bronk et al., 2000) and reached ±70% in deep

434   ocean samples (Sharp et al., 2004).

The CVs for soil microbial biomass N can be estimated by error propagation (square

436   root of two times SD^2 of TDN; e.g. Bronk et al. 2000) and, based on an assumed biomass N

identical to TDN level of unfertilized samples, ranged between 4.0-24.2 (mean 9.5; data not

438   shown). This level of precision is acceptable for most studies and persulphate oxidation/VCl$_3$-

Griess is therefore adequate for microbial biomass measurements. Moreover, multi-factorial

440 ANOVA of TDN analyzed by HTCO or persulphate oxidation/VCl_3-Griess showed no

significant difference between fumigated versus unfumigated soils. Doyle et al. (2004) also

442 concluded that persulphate oxidation is adequate for analysis of microbial biomass N, with

precisions ranging between 5 and 10%.

444   Since oxidation efficiency is not temperature sensitive as long as adequate time is

allowed for persulphate radical generation, autoclaving (120°C, 40 min) can be substituted by

446 overnight digestion at 80°C in a drying oven or by boiling in a water bath (Doyle et al., 2004).

This further simplifies the persulphate oxidation procedure and allows larger batches of

448 samples, blanks and standards to be processed simultaneously.

450 The simplified persulphate digestion procedure followed by detection of nitrate by the VCl_3-

Griess reaction with a microtiter plate reader greatly reduces sample preparation costs and

452 considerably shortens analysis times for microbial biomass N and TDN/DON. The simplicity,

rapidity and low cost of persulphate digestion followed by VCl_3-Griess detection of nitrate

454 render it a highly practical method for laboratory and field use.

456 **CONCLUSIONS**

The methods described based on the VCl_3-Griess reaction, have been shown to be

458 adequate to detect nitrate nitrogen in a range of soil preparations. The VCl_3-Griess method has

the advantage of low toxicity and has been shown to be rapid and accurate over a large range

460 of soil N concentrations. The methods described have the additional advantage of requiring

minimal quantities of solution for analysis, meaning that the sampling procedures can be

462 adjusted to small volumes and allows a larger number of samples to be processed

simultaneously. The techniques described are also complimentary to isotopic preparation

464 techniques leaving sufficient sample for sample preparation for isotopic analysis. Measuring

nitrogen concentrations in a range of soil pools is essential for managing this precious

466 resource. Often the capacity and scale of nitrogen cycling experiments are limited by the shear

practicalities and logistics of measuring large numbers of samples. The methods described

468 here therefore facilitate expansion of experimental scope.

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FIGURE CAPTIONS

548

Figure 1. Calibration of a suite of methods to quantify soluble soil N pools. Relationships

550   between standard concentration and absorbance or peak area are presented for ammonium
(Berthelot reaction), nitrite (Griess reaction), nitrate (VCl$_3$-Griess reaction; anion

552   chromatography-HPLC) and total soluble N (alkaline persulfate digestion with VCl$_3$-Griess
detection of nitrate; automated high temperature catalytic oxidation-HTCO). Range, linearity,

554   blank concentration, limit of detection and limit of quantification are given in Table 1.
Symbols represent means ± one standard error of three to six independent calibrations

556   performed on different days with each freshly prepared reagents and standards. Linear
regressions (Model I) were fitted to the data.

558

Figure 2. Sensitivity of nitrate assay by VCl$_3$-Griess reaction to time of incubation (30-135

560   min) and solution chemistry (deionized water; 0.5 M K$_2$SO$_4$). Relationships between nitrate
concentration and absorbance are given. Symbols represent means of duplicate measurements

562   of standards. Linear regressions (Model I) were fitted to the data.

564   Figure 3. Effect of time of incubation and solution chemistry on the slope of the calibration
line (see Figure 2). Data were fitted to an 'exponential rise to maximum' model (single, three

566   parameters).

568   Figure 4. Comparison of direct nitrate quantification by HPLC or photometry. (A)
Relationship between nitrate concentration in 10 mM CaSO$_4$ extracts of a range of different

570   soils measured by anion chromatography (HPLC) or VCl$_3$-Griess reaction, and (B) nitrate
concentration in CaSO$_4$ extracts measured by anion chromatography (HPLC) and nitrate

572   concentration in 1 M KCl measured by VCl$_3$-Griess assay in a range of different soils. Data
were tested by Model II linear regression models.

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Figure 5. Comparison of total soluble N measurements by (A) VCl$_3$-Griess reaction or UV

576   absorbance of nitrate after alkaline persulphate (AP) digestion, and (B) by high temperature
catalytic oxidation (HTCO) and alkaline persulphate digestion with subsequent nitrate

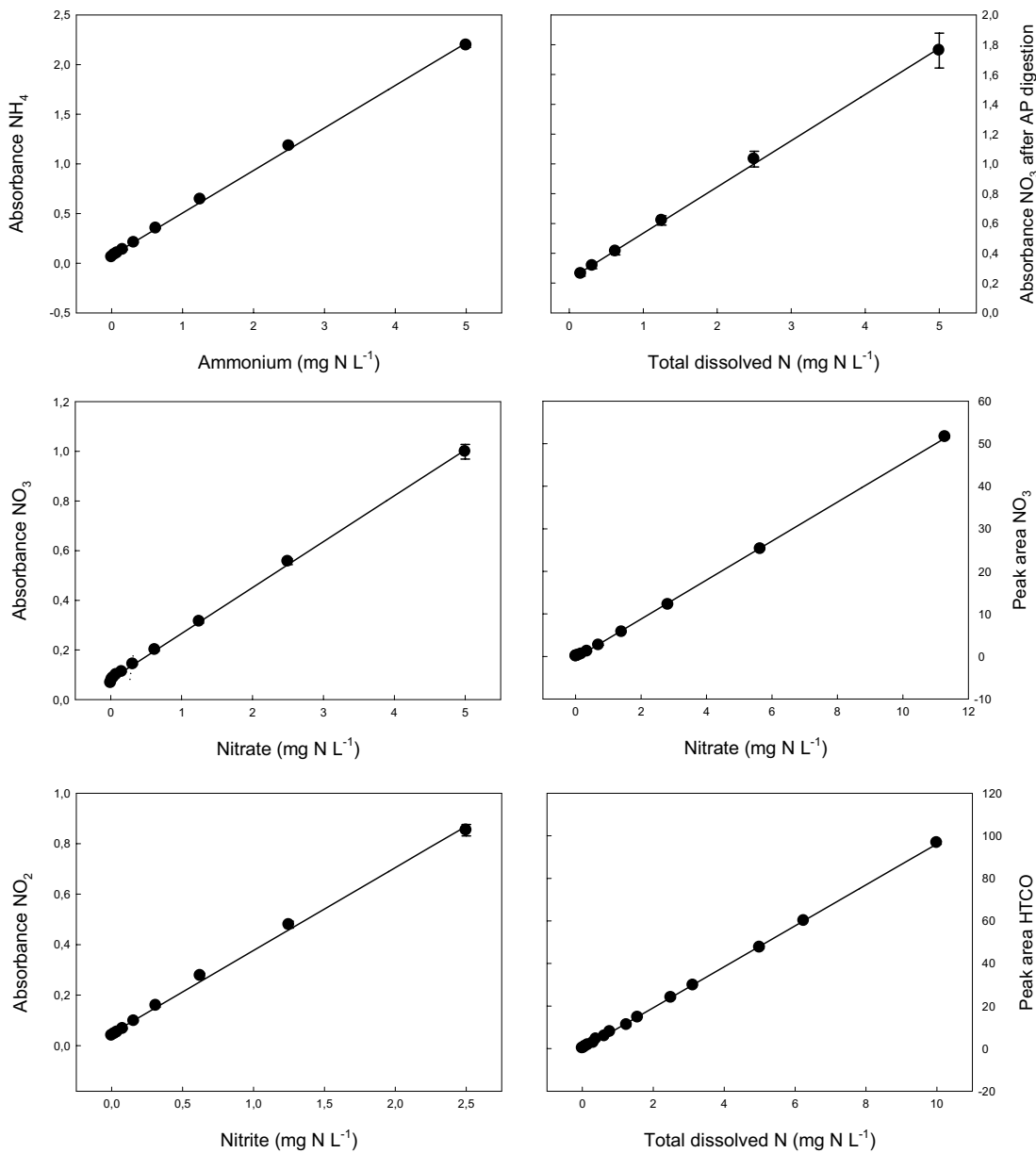578   quantification by VCl$_3$-Griess reaction. Data were tested by Model II linear regression
models.

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584 Table 1. Validation of a suite of methods to determine soluble soil N pools (ammonium-NH_4^+, nitrate-NO_3^-, nitrite-NO_2^-, total soluble N-TSN). Ranges, calibration lines, linearity, blanks (mean and SD of concentration), limits of detection (LOD) and limits of quantification (LOQ) were derived from data in Figure 1 and are presented (i) overall i.e. including data of all independent calibrations or (ii) as mean and standard deviation of three to six independent calibrations. Blank absorptions were measured directly in five replicates.

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N form Method	Range mg N L⁻¹	Averaging	Calibration line slope	intercept	Linearity R²	Blank mg N L⁻¹	Blank SD	LOD mg N L⁻¹	LOD µg N g⁻¹ dw	LOQ mg N L⁻¹	LOQ µg N g⁻¹ dw
NH₄ Berthelot	0.02-5	overall	0.429	0.073	0.9985	0.142	0.0054	0.0162	0.162	0.054	0.540
		mean	0.425	0.076	0.9990	0.142	0.0007	0.0020	0.020	0.007	0.0657
		SD	0.011	0.006	0.001	0.008	0.00008	0.0002	0.002	0.0018	0.0079
NO₂ Griess	0.02-2.5	overall	0.328	0.049	0.9903	0.122	0.0097	0.0291	0.291	0.0969	0.969
		mean	0.327	0.050	0.9972	0.123	0.0019	0.0058	0.058	0.0195	0.1948
		SD	0.021	0.005	0.0013	0.007	0.0001	0.0004	0.004	0.0013	0.0132
NO₃ VCl₃–Griess	0.02-5	overall	0.185	0.080	0.9923	0.378	0.0053	0.0158	0.158	0.0526	0.526
		mean	0.180	0.081	0.9992	0.371	0.0055	0.0165	0.165	0.0550	0.54999
		SD	0.007	0.006	0.0004	0.013	0.0037	0.0112	0.112	0.0373	0.37245
NO₃ HPLC	0.01-11.3	overall	4.575	-0.357	0.9995		0.00037	0.0011	0.011	0.0037	0.037
		mean	4.575	-0.357	0.9997		0.00091	0.0027	0.027	0.0091	0.09062
		SD	0.059	0.022	0.0001		0.00001	0.00004	0.0004	0.0001	0.00119
TSN HTCO	0.08-10	overall	9.673	-0.112	0.9995	0.013	0.016	0.047	0.465	0.155	1.551
		mean	9.673	-0.248	0.9995	0.013	0.028	0.085	0.852	0.284	2.838
		SD	0.156	0.371	0.0005	0.010	0.034	0.101	1.005	0.335	3.351
TSN PO/VCl₃- Griess		overall	0.310	0.224	0.9457	0.795	0.045	0.1363	1.363	0.454	4.544
		mean	0.310	0.224	0.9901	0.810	0.028	0.0825	0.825	0.275	2.7498
		SD	0.059	0.024	0.0040	0.118	0.006	0.0170	0.170	0.057	0.5678

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Table 2. Mean concentrations and coefficients of variance (CV; SD/mean x 100) of ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-), total soluble N (TSN), and dissolved organic N (DON) in 0.5 M K_2SO_4 extracts of four agricultural soils collected from the vicinity of Vienna, Austria: T…Tulln, N…Niederschleinz, P…Purkersdorf, and R…Riederberg. For further details on soil characteristics see Inselsbacher et al. (2009). One set of samples was directly extracted with 0.5 M K_2SO_4, the second set of samples (+) was spiked with a solution of NH_4NO_3 (0.2 mg N g^{-1} soil fresh weight), incubated at room temperature for 24 hours and thereafter extracted. Data represent means of five replicates. Extracts and digests were diluted if concentrations were outside of the linear calibration range.

Soil	NH_4^+ mean mg N L^{-1}	CV %	NO_2^- mean mg N L^{-1}	CV %	NO_3^- mean mg N L^{-1}	CV %	TSN mean mg N L^{-1}	CV %	DON mean mg N L^{-1}	CV %
P	0.21	2.5	0.021	9.3	5.82	3.3	8.66	5.0	2.63	17.9
N	0.11	2.5	0.018	7.3	5.26	3.4	9.12	3.4	3.76	9.4
R	0.17	2.4	0.029	4.1	11.23	3.4	20.32	6.8	8.92	16.1
T	0.07	2.7	0.017	4.5	3.60	2.1	6.46	2.8	2.79	7.1
P+	6.54	2.7	0.014	7.0	9.73	6.1	22.80	6.5	6.54	24.6
N+	6.56	1.1	0.087	5.5	9.74	4.1	22.06	1.8	5.77	9.7
R+	4.16	1.4	0.078	2.2	15.06	1.4	32.71	2.1	13.50	5.3
T+	4.36	1.6	0.072	2.4	9.27	4.0	21.91	3.2	8.28	9.6
Mean CV		2.1		5.3		3.5		3.9		12.5

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Figure 1.

Figure 2.

Figure 3.

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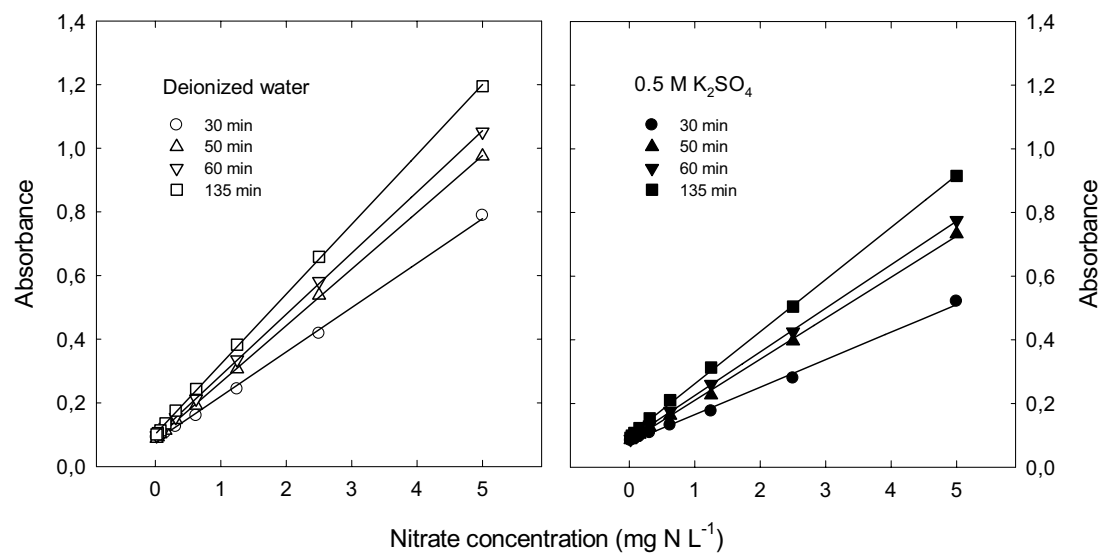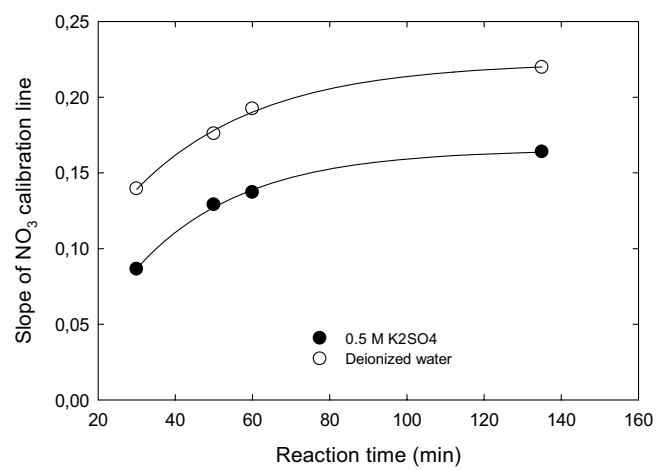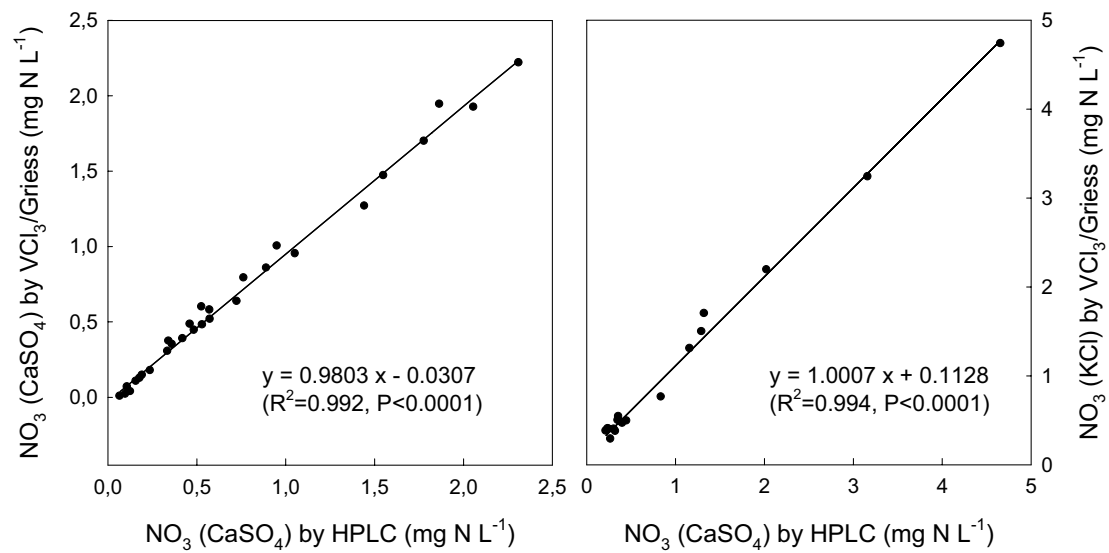

Figure 3.
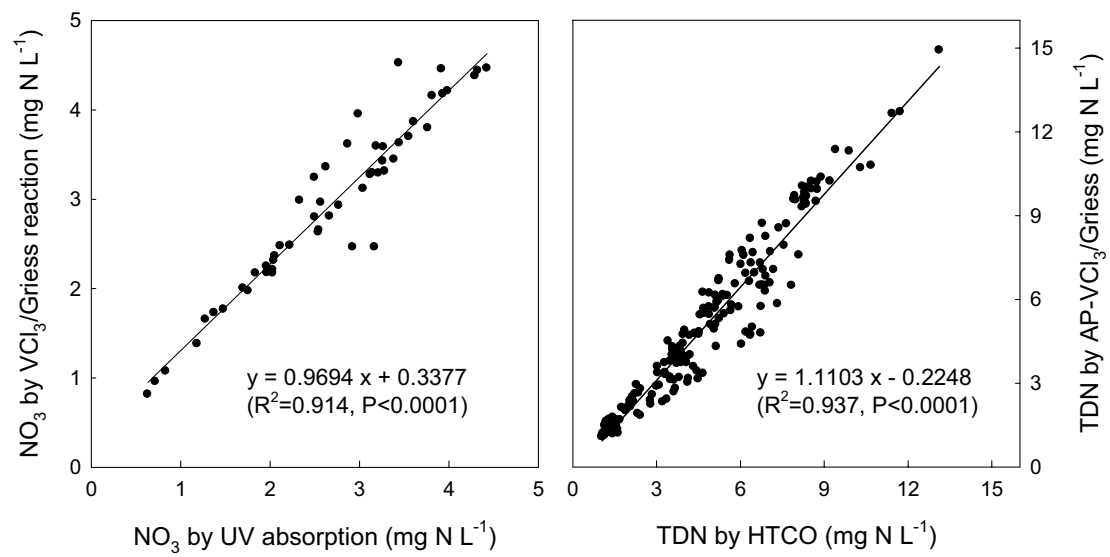
610   Figure 4.

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614   Figure 5.

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Appendix B

Conference contributions

"NITROGENOM" – A standardized microcosm system to study nitrogen pathways and soil-plant-microbe relationships in agricultural soils
Inselsbacher, E; Hackl, E; Klaubauf, S; Ripka, K; Sessitsch, A; Strauss, J; Von Wirén, N; Wanek, W; Zechmeister-Boltenstern, S
Poster presented at the 4th International Nitrogen Conference 2007, Costa do Sauípe, Brazil

The NITRO-GENOME Project
Increasing Nitrogen Fertilizer Use Efficiency by Understanding Microbial Nitrogen Cycling
Strauss, J, Inselsbacher, E, Von Wirén, N; Wanek, W; Zechmeister-Boltenstern, S,
Poster presented 4th International Nitrogen Conference 2007, Costa do Sauípe, Brazil

"NITROGENOM" – Studying nitrogen pathways and soil-plant-microbe relationships in agricultural soils within a standardized microcosm system
Inselsbacher E, Hackl E, Hood R, Klaubauf S, Ripka K, Sessitsch A, Strauss J, Von Wirén N , Zechmeister-Boltenstern S, Wanek W
Oral presentation at the Eurosoil 2008 Conference, Vienna, Austria

Publications in preparation:

Working title:
Process-specific analysis of N dynamics in agricultural soil-plant microcosms by using a Monte Carlo sampling technique
Erich Inselsbacher[1,2], Wolfgang Wanek[1], Joseph Strauss[3], Sophie Zechmeister-Boltenstern[2], Christoph Müller[4]

[1]Department of Chemical Ecology and Ecosystem Research, Faculty Centre for Ecology, Faculty of Life Sciences, University of Vienna, Austria

[2]Department of Forest Ecology and Soils, Unit of Soil Biology, Federal Research and Training Centre for Forests, Natural Hazards and Landscape (BFW), Vienna, Austria

[3]Department for Applied Genetics and Cell Biology, Fungal Genomics Unit, Austrian Institute of Technology and BOKU University Vienna, Vienna, Austria

[4] UCD Agriculture and Food Science Centre, University College Dublin, Ireland

"NITROGENOM" – A standardized microcosm systems to study nitrogen pathways and soil-plant-microbe relationships in agricultural soils

Inselsbacher E[1,2], Wanek W[1], Zechmeister-Boltenstern S[2], Hackl E[3], Hood R[1], Klaubauf S[4], Ripka K[3], Sessitsch A[3], Strauss J[4]

[1]Department of Chemical Ecology and Ecosystem Research, Faculty Center for Ecology, Faculty of Life Sciences, University of Vienna, A-1090 Vienna, AUSTRIA
e.mail: erich.inselsbacher@bfw.gv.at
[2]Federal Research and Training Centre for Forests, Natural Hazards and Landscape (BFW), Department of Forest Ecology and Soils, Unit of Soil Biology, A-1131 Vienna, AUSTRIA
[3]ARC Seibersdorf research GmbH, Department of Bioresources, A-2444 Seibersdorf, AUSTRIA
[4]Institute for Applied Genetics and Cell Biology, University of Natural Resources and Applied Life Sciences Vienna, A-1190 Vienna, AUSTRIA

Presentation: Poster

We developed a new, easily available and cheap laboratory format microcosm system for high throughput analysis of soil N pools, N transformation rates, N losses, as well as microbial populations and their activities in soil-plant systems. The aim of this investigation is to improve N fertilizer use by plants by targeted manipulation of bacterial and fungal communities. The system consists of polypropylene centrifuge tubes complemented with two sieves for soil and plant containment, aerated cones for soil water sampling, a retrofit kit for elongation and butyl rubber seals for gas sampling. The advantages of this microcosm design are the possibility to work in a highly replicated and standardized way, and the fact that only small sample amounts are necessary.

In order to evaluate the system we compared five soils selected in Lower Austria to represent contrasting textures, pH and C-contents. After being sieved and homogenized, soil aliquots were weighed and centrifuged into the test tubes and kept under controlled conditions in a climate chamber for four weeks at a water content of 70%. Soil samples were taken every week and analyzed for N pools, gross N mineralization, enzyme activities, microbial biomass, fluxes of N_2O, CO_2 and CH_4, and microbial and fungal population dynamics. Overall we found highly significant effects of soil type on N pools and gas emissions but not on gross N turnover rates. Nitrate reducing and NH_4 oxidizing bacterial communities varied between different soils and did not change during the test period of four weeks.

The results show that this system is practical, reliable and suitable for our objectives and that conditions remained stable for the test period. The adjustment and maintenance of homogenous soil water contents occurred to be a major challenge of the system, which may be overcome by introducing a porous medium for improved drainage. We hope that this new approach to study soil-plant-microbe relationships for understanding the biotic "black-box" may help to improve nitrogen efficiency in the future.

"NITROGENOM" – Increasing nitrogen efficiency in agricultural systems by understanding and manipulating the biotic *"Black-Box"*

Strauss J[1], <u>Inselsbacher E[2,3]</u>, Hackl E[4], Hood R[2], Klaubauf S[1], Luschnig C[1], Ripka K[4], Sessitsch A[4], , Von Wirén NRW[5], Wanek W[2], Zechmeister-Boltenstern S[3]

[1]Institute for Applied Genetics and Cell Biology, University of Natural Resources and Applied Life Sciences Vienna, A-1190 Vienna, AUSTRIA
e.mail: joseph.strauss@boku.ac.at
[2]Department of Chemical Ecology and Ecosystem Research, Faculty Center for Ecology, Faculty of Life Sciences, University of Vienna, A-1090 Vienna, AUSTRIA
[3]Federal Research and Training Centre for Forests, Natural Hazards and Landscape (BFW), Department of Forest Ecology and Soils, Unit of Soil Biology, A-1131 Vienna, AUSTRIA
[4]ARC Seibersdorf research GmbH, Department of Bioresources, A-2444 Seibersdorf, AUSTRIA
[5]Molecular Plant Nutrition, Faculty of Agricultural Sciences, University of Hohenheim, D-70593 Stuttgart, GERMANY

Presentation: Poster

The present project addresses the impact of microbial and fungal communities on the N fertilizer use by plants. Using a unique combination of molecular, genetic and biochemical approaches allows us to open the microbial "black-box" (plant root-fungi-bacteria interaction) and to reveal the mechanisms by which fungi and bacteria influence the nutrient pathways to different compartments after N-fertilizer application. For this purpose we developed a microcosm system which allows to study plant-soil-microbe interactions in a highly reproducible, standardized laboratory format design.
By using ^{15}N tracers, it will be possible to elucidate the exact fate of applied fertilizer N within the test system. The amount of N taken up by the model plant *barley* is set in relation to N losses from the system, by leaching and volatilization, as well as to the amount of N immobilized by fungi and microorganisms in the soil. Variation of the ratio of nitrate fertilizer to ammonium fertilizer will permit the study of the impact of different N forms on the N uptake by plants, fungi and microorganisms. This project further aims at revealing new perspectives of microbial activities and population dynamics, and of transcriptional responses of bacteria, fungi and plants to different fertilizer applications.
Overall, investigating and understanding N cycling dynamics within the presented laboratory format microcosm system will provide novel insights into the mechanisms regulating the distribution and compartmentalization of fertilizer-N to plant, soil, fungal, bacterial and environmental pools. The gained knowledge will help to set up further extensive studies in the field and to provide concepts for targeted strategies to improve N fertilizer use efficiency, by specific agricultural management practices, fungal "nitrate-traps" and growth promoting bacterial strain additions to the soils.

"NITROGENOM" – Studying nitrogen pathways and soil-plant-microbe relationships in agricultural soils within a standardized microcosm system

Inselsbacher E[1,2], Hackl E[3], Hood R[1], Klaubauf S[4], Ripka K[3], Sessitsch A[3], Strauss J[4], Von Wirén N[5] , Zechmeister-Boltenstern S[2], Wanek W[1]

[1]Department of Chemical Ecology and Ecosystem Research, Faculty Center for Ecology, Faculty of Life Sciences, University of Vienna, A-1090 Vienna, AUSTRIA
e.mail: erich.inselsbacher@bfw.gv.at
[2]Federal Research and Training Centre for Forests, Natural Hazards and Landscape (BFW), Department of Forest Ecology and Soils, Unit of Soil Biology, A-1131 Vienna, AUSTRIA
[3]Austrian Research Centers GmbH - ARC, Department of Bioresources, A-2444 Seibersdorf
[4]Institute for Applied Genetics and Cell Biology, University of Natural Resources and Applied Life Sciences Vienna, A-1190 Vienna, AUSTRIA
[5]Molecular Plant Nutrition, Faculty of Agricultural Sciences, University of Hohenheim, D-70593 Stuttgart, GERMANY

Presentation: Oral

Within the present project we developed a new, easily available and cheap laboratory format microcosm system for high throughput analysis of soil N pools, N transformation rates, N losses, as well as microbial populations and their activities in soil-plant systems. The system consists of polypropylene centrifuge tubes complemented with two sieves for soil and plant containment, aerated cones for soil water sampling, a retrofit kit for elongation and butyl rubber seals for gas sampling.

Two agricultural soils from the vicinity of Vienna were used for growing *barley* plants in the microcosms. Within three experimental setups the ratio of nitrate to ammonium fertilizer was varied to study the impact of different N forms on the N uptake by plants, fungi and microorganisms. By using ^{15}N tracers, given either as $^{15}NH_4{}^{15}NO_3$, $^{15}NH_4Cl$ or $K^{15}NO_3$, it was possible to follow the exact fate of applied fertilizer N within the test system. The amount of N taken up by the plants was set in relation to N taken up by microorganisms and fungi, losses to the environment, and to N transformation rates. Investigating and quantifying the fluxes of the trace gases N_2O, CO_2 and CH_4 from the soils provided additional information on the release of climate relevant gases.

Overall, investigation, understanding and future modelling of N cycling dynamics within the presented laboratory format microcosm system will provide novel insights into the mechanisms regulating the distribution and compartmentalization of fertilizer-N to plant, soil, fungal, bacterial and environmental pools. The gained knowledge will help to set up further extensive studies in the field and to provide concepts for targeted strategies to improve N fertilizer use efficiency, by specific agricultural management practices, fungal "nitrate-traps" and growth promoting bacterial strain additions to the soils.

CURRICULUM VITAE

Mag. Erich Inselsbacher

Date of birth   18.07.1978 Steyr, Austria
Nationality   Austria
Address   Bräuhausgasse 52/18
A-1050 Vienna, Austria
E-mail   erich.inselsbacher@bfw.gv.at
e.insel@gmx.at

Education and Employment

1996   Graduation from High school
1998-2006   Studies in biology/ecology at the University of Vienna
2004-2006   Master thesis, University of Vienna
 "Microbial activities and foliar uptake of nitrogen in the epiphytic bromeliad *Vriesea Gigantea*."
carried out at the Department of Chemical Ecology and Ecosystem Research
Supervisors: Prof. Dr. Andreas Richter and Ass.Prof. Dr. Wolfgang Wanek
2006-2009   PhD thesis, University of Vienna
WWTF (Vienna Science and Technology Fund) project: NITRO-GENOM: Increasing nitrogen efficiency in agricultural systems by understanding and manipulating the biotic "Black-Box"
carried out at the Department of Chemical Ecology and Ecosystem Research, University of Vienna, and at the Department of Forest Ecology and Soils, Federal Research and Training Centre for Forests, Natural Hazards and Landscape, Vienna.
Supervisors: Ass.Prof. Dr. Wolfgang Wanek and Univ.-Doz. Dr. Sophie Zechmeister-Boltenstern
2006-2008 Research associate at the University of Natural
Resources and Applied Life Sciences, Vienna.
FWF-project: Decomposition, mineralization and nutrient release from soils in pure or mixed forests of Beech (Fagus sylvatica) and Spruce (Picea Abies) trees

Publications

Inselsbacher E., Cambui C.A., Richter A., Stange C.F., Mercier H. and Wanek W. (2007) Microbial activities and foliar uptake of nitrogen in the epiphytic bromeliad *Vriesea Gigantea*. *New Phytologist* **175***,311-320*

Inselsbacher E., Ripka K., Klaubauf S., Hackl E., Gorfer M., Hood-Novotny R., Von Wirén N., Sessitsch A., Zechmeister-Boltenstern S., Wanek W. and Strauss J. (2008) A cost-effective high-throughput microcosm system for studying nitrogen dynamics at the plant-microbe-soil interface. Plant and Soil (2009) 317:293-307