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Effect of Reduced Nitrogen Deposition on Microbial Activity, Abundance and Diversity in Forest Soils

Dissertation

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In loving memory of my mother,
Martha Tiku Enow

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Eidesstattliche Erklärung

Ich erkläre hiermit an Eides statt, dass ich die vorliegenden Dissertation selbständig angefertigt, nur die angegebenen Quellen und Hilfsmittel benutzt und inhaltlich oder wörtlich übernommene Stellen als solche gekennzeichnet habe. Ich habe noch keinen weiteren Promotionsversuch unternommen.

Stuttgart, 27. 06.2012 Enowashu Esther Eneckeh

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1 Summary

The deposition of nitrogen has increased many-fold due to anthropogenic activities. Since forest ecosystems are often limited by N availability, elevated N inputs from the atmosphere can have a fertilization effect but in the long-term, excess N can influence above- and below-ground production. One of the consequences of N deposition and increased N inputs is a shift in microbial community structure and function as ecosystems move towards N saturation. Soil microorganisms through the action of enzymes play an important role in N dynamics. Thus, the availability and turnover of N depends strongly on microbial abundance, diversity and activity which are in turn influenced by soil properties. Studies on the effects of high nitrogen inputs and the response of forest ecosystems to nitrogen saturation are many and well understood. However, the reversibility of N-induced shifts in forest ecosystem processes is largely unknown. This thesis was therefore designed to study the response of soil microorganisms to reduced N deposition. A biphasic approach was employed to look into (i) the general microbial functional status of the Solling forest site as well as (ii) the microbial community structure which may be a key regulator of two important processes of N transformation: denitrification and proteolysis.

The goal of the present thesis was addressed in three studies. Denitrification is considered sensitive to environmental changes and the response of nitrate-reducers and denitrifiers to reduced N deposition was determined in the first study. The goal of the second study was to investigate the overall microbial activity of the Solling forest profiles especially focussing on enzymes involved in the N cycle. This revealed a pronounced activity of peptidases whereby a set of novel *pepN* primers encoding alanine aminopeptidase enzyme was designed in the third study to determine the group of bacteria involved in proteolysis in forest as well as agricultural and grassland soils.

The Solling experimental station was established more than two decades ago and it gave the opportunity to study the N cycle in a natural forest ecosystem at different sampling dates and depths. A combination of classical biological methods and modern molecular techniques were used in the studies. Soil physico-chemical parameters (OC, N_t, NO_3^-, NH_4^+, pH, % Water content) were analysed to gain more information on mineralization and immobilization of N in the soil profiles. The analysis of microbial biomass, ergosterol content and the activity of several enzymes of the N, C and P cycles as well as enzyme activity of nitrate

reducers was determined in order to interpret microbial functions. The abundance of nitrate reducers and denitrifiers were determined by quantitative PCR of 16S rRNA, nitrate reductase (*narG* and *napA*) and denitrification (*nirK, nirS* and *nosZ*) genes. The diversity of peptide degrading bacteria was analysed by PCR, cloning and sequencing and the construction of *pepN* gene libraries.

The results of the first study indicated that time and space were the main drivers influencing the abundance and activity of the nitrate reducers and denitrifier communities in the forest soil profiles. Reduced N deposition had a of minimal effect. Interestingly, the ratios of *nosZ* to16S rRNA gene and *nosZ* to *nirK* increased with soil depth thereby tempting to conclude that the size of denitrifiers capable of reducing N_2O into N_2 might be bigger in the mineral horizons. In the second study, a stronger response of N cycling enzymes to reduced N deposition could be seen. However, these responses especially that of specific peptidases differed in magnitude which could be indicative of a modification of the reaction rates of the different N cycling enzymes. Correlation of nutrients (N, C, P) with microbial biomass and enzyme activities in the soil profiles revealed that substrate availability was the main factor influencing microbial activity. In the third study, analyses of gene libraries from extracted DNA from forest, agricultural and glacier soil samples revealed a high diversity of *pepN* sequences related to mainly α-Proteobacteria. A majority of the sequences showed similarity to published data revealing that the amplified region of *pepN* might be conserved. Linking diversity and enzymatic data, lowest diversity was observed in the agricultural soil where activity levels of alanine aminopeptidase were lowest indicating the importance of diversity studies for ecosystem functioning.

In conclusion, this thesis offers valuable contributions to understanding the impact of N deposition. The approach used was suitable to assess the response of the different microbial communities to reduced N deposition. The magnitude of the response depended strongly on space, time and substrate availability in soils as well as their interactions.

2 Zusammenfassung

Anthropogene Aktivitäten haben die Deposition von Stickstoff (N) um ein Vielfaches erhöht. Da Waldböden häufig N-limitiert sind, können erhöhte N-Einträge aus der Atmosphäre einen Düngungseffekt haben. Dies kann langfristig die oberirdische und unterirdische Pflanzenproduktion beeinflussen. Eine Folge von erhöhtem N-Eintrag ist die Veränderung der mikrobiellen Gemeinschaft sowie deren Funktion sobald das Ökosystem in Richtung N-Sättigung strebt. Aufgrund ihrer Enzymaktivitäten spielen Bodenmikroorganismen eine wichtige Rolle in N-Dynamiken. Die Verfügbarkeit und Umsetzung von N hängt daher stark von der mikrobiellen Abundanz, Diversität und Aktivität ab, die wiederum durch Bodeneigenschaften beeinflusst wird. Studien, die den Effekt von hohem N Eintrag und die Folgen von N-Sättigung in Waldökosystemen untersuchen, sind zahlreich und die Effekte und Folgen gut verstanden. Hingegen ist wenig bekannt inwieweit sich diese Veränderungen bei reduzierter N-Deposition umkehren. Daher wurden in dieser Doktorarbeit die Folgen einer reduzierten N-Deposition auf Bodenmikroorganismen untersucht. Ein biphasischer Ansatz wurde verwendet um 1) den generellen funktionellen mikrobiellen Status des Waldstandortes Solling und 2) die mikrobielle Gemeinschaftsstruktur, die ein wichtiger Regulator für zwei wichtige N-Transformationsprozesse, die Denitrifikation und die Proteolyse, sein könnte, zu untersuchen.

Zur Erreichung des Ziels dieser Arbeit wurden drei Studien durchgeführt. Denitrifikation reagiert sensitiv auf Veränderungen der Umweltbedingungen und die Reaktion von nitratreduzierenden und denitrifizierenden Mikroorganismen auf reduzierte N Bedingungen wurde in der ersten Studie untersucht. Das Ziel der zweiten Studie war es, mit dem Fokus auf die Enzyme, die in den N-Kreislauf involviert sind, die mikrobielle Aktivität der Solling Waldprofile zu untersuchen. Eine ausgeprägte Aktivität von Peptidasen wurde festgestellt woraufhin in der dritten Studie ein neuer Satz *pepN* primer entwickelt wurden, die Alanin Aminopeptidasen kodieren, um die Gruppen der Bakterien zu bestimmen, die an der Proteolyse in Waldböden sowie in landwirtschaftlich genutzten und Grünland Böden beteiligt sind.

Die Solling Forschungsstation wurde vor mehr als 20 Jahren aufgebaut und gab uns die Möglichkeit den N-Kreislauf in einem natürlichen Waldökosystem zu unterschiedlichen Zeitpunkten und Bodentiefen zu untersuchen. Eine Kombination klassischer und moderner molekularer Methoden wurde in den Studien verwendet. Wir analysierten

bodenphysikochemische Parameter (OC, N_t, NO_3^-, NH_4^+, pH, % Wassergehalt), um mehr Informationen über Mineralisation und Immobilisation von N in den Bodenprofilen zu erhalten. Die Bestimmung der mikrobiellen Biomasse, des Ergosterol Gehalts und die Aktivität von mehreren Enzymen des N, C und P-Kreislaufs wie auch Enzymaktivitäten nitratreduzierender Organismen wurde genutzt, um die mikrobiellen Funktionen interpretieren zu können. Die Abundanz von nitratreduzierenden und denitrifizierenden Bakterien wurde mit quantitativer PCR der 16S rRNA, Nitratreduktase (*narG* und *napA*) und denitrifizierenden (*nirK*, *nirS* und *nosZ*) Funktionsgenen bestimmt. Die Diversität der peptidabbauenden Bakterien wurde mittels PCR, Klonierung und Sequenzierung sowie des Aufbaus von *pepN* Genbibliotheken untersucht.

Die Ergebnisse der ersten Studie deuten darauf hin, dass Zeitpunkt und Bodentiefe die Haupteinflussfaktoren für die Abundanz und die Aktivität der nitratreduzierenden und denitrifizierenden Gemeinschaften im Waldprofil darstellen. Reduzierte N Deposition war von geringer Bedeutung. Interessanterweise erhöhten sich die Verhältnisse von *nosZ* zu 16S rRNA Genen und *nosZ* zu *nirK* in der Tiefe, was darauf schließen lässt, dass denitrifizierende Organismen, die fähig sind N_2O zu N_2 zu reduzieren, im Mineralbodenhorizont in größerer Zahl vorkommen könnten als in den oberen Horizonten. In der zweiten Studie wurde eine stärkere Reaktion der am N-Kreislauf beteiligten Enzyme bei reduzierter N Deposition festgestellt. Diese Reaktionen, insbesondere die der spezifischen Peptidasen, veränderten sich, was darauf hindeuten könnte, dass sich die Effizienz der unterschiedlichen am N-Kreislauf beteiligten Enzyme anpassen. Korrelationen von Nährstoffen (N, C, P) mit der mikrobiellen Biomasse and den Enzymaktivitäten im Bodenprofil verdeutlichen, dass die Substratverfügbarkeit der Hauptfaktor für die mikrobielle Aktivität war. In der dritten Studie, in der Gen-Bibliotheken von aus Wald-, landwirtschaftlichen und Gletscher Böden extrahierter DNA analysiert wurden, zeigte sich eine hohe Diversität der *pepN* Sequenzen, die hauptsächlich α-Proteobakterien zugeordnet werden konnten. Ein Großteil der Sequenz zeigte Ähnlichkeit mit bereits publizierten Daten, was darauf hindeuten könnte, dass die amplifizierte Region *pepN* konserviert ist. Bei der Betrachtung der Diversitäts- und Enzymdaten wurde die geringste Diversität in dem landwirtschaftlich genutzten Boden gefunden, der auch die geringsten Aktivitäten der Alanin-Aminopeptidase aufwies, was auf die Bedeutung von Diversitätsstudien für das Funktionieren von Ökosystemen hindeutet.

Zusammenfassend leistet diese Doktorarbeit wertvolle Beiträge, um den Einfluss von N Deposition zu verstehen. Der verwendete Ansatz war geeignet, um die Reaktion der

unterschiedlichen mikrobiellen Gemeinschaften auf reduzierte N Deposition einzuschätzen. Die Höhe der Reaktion hing stark von der Bodentiefe, des Zeitpunktes und der Substratverfügbarkeit sowie deren Interaktion ab.

3 General introduction

3.1 The N cycle

Nitrogen (N) is the very stuff of life (Galloway and Cowling, 2002) and makes up about 80% of the total mass of the atmosphere. This abundant amount of N is, however, in a form not available to plants, animals and most microorganisms. The global nitrogen cycle is strongly influenced by anthropogenic activities such as industrial combustion processes and fertilizer application (Vitousek et al., 1997; Gruber and Galloway, 2008). Galloway et al. (2003) estimated the global nitrogen at 165 Tg N yr^{-1}, which is tenfold more than in pre-industrial times. This nitrogen is mostly derived from biological nitrogen fixation (BNF) (≈ 100 – 140 Tg N yr^{-1}) with about 55% being emitted back to the atmosphere as NOx and NH_3. Most of the emitted N (≈ 70 – 80%) is returned as N deposition on land, with about one-third being deposited on forest ecosystems (Hudson et al., 1994). Other pathways or processes contributing to nitrogen losses are leaching of nitrates, run-off to surface water, nitrification and denitrification.

The N cycle in undisturbed forests ecosystem is relatively closed; most of the nitrogen being recycled within the soil-microbe-plant system (Nômmik, 1982). However, with increasing N deposition, soils rather than plants become an important long-term sink for the added N (Nadelhoffer et al., 1999; Providoli et al., 2006). Soil N availability largely regulates both biomass production and species composition in forest ecosystems. Total N stocks in forest soils and biomass could be as high as 500 g N m^{-2} (Galloway et al., 2003) of which over 80% is present in organic form (Schulten and Schnitzer, 1998). Organic N is primarily derived from plant materials and enters the soil as litter in addition to dead animals and microorganisms. The dynamics of organic N in forest soils as influenced by N deposition is governed by many factors and processes as well as their interaction, most of which are not well understood (Magill and Aber, 1998; Neff et al., 2002; Sinsabaugh et al., 2005). Microorganisms through the coordinated action of several enzymes are an important factor in N dynamics and as such, their activities can be used as indicator of nutrient cycling (Tan et al., 2008). They are involved in the decomposition of plant biomass and the mineralization and immobilization of organic N. Thus, organic N dynamics depends strongly on microbial abundance, diversity and activity which are in turn influenced by soil properties.

3.2 N deposition

The production of forest ecosystems and their responses to environmental factors are highly dependent on the sustainable functioning of soils on which they grow, especially the responses to organic matter decomposition and nutrient cycling. Over the past centuries, industrialisation and agricultural intensification have increased the flux of biologically available nitrogen into natural ecosystems (Vitousek et al., 2003; Sinsabough et al., 2004; Galloway et al., 2003; Gruber and Galloway, 2008). Though there is increased political awareness and concern, the rate of N emissions is still on the rise. For example, in regions of Central Europe, where intensive agriculture and forestry areas are interspersed N deposition into forest ecosystems has been reported to be in the range of 10–20 kg^{-1} N ha^{-1} $year^{-1}$ (Gauger et al., 2008). Even higher rates of atmospheric N deposition well above 20–30 kg^{-1} N ha^{-1} $year^{-1}$ have been reported in other regions, such as the Benelux countries, north Germany and parts of south Germany (Pilegaard et al., 2006; Simpson et al., 2006; Gauger et al., 2008). Following a long-term intensive monitoring of 89 forests stands in Germany, the mean annual nitrogen (NH_4^+ and NO_3^-) input to forest soils by through fall was 21.3 kg N $ha^{-1}y^{-1}$ and the critical load was by far (84%) exceeded in all cases (BFH, 2003). Projecting into the future, Denterer et al. (2006) reported increased rates of N deposition at a global scale by 2030. Since forest ecosystems are often limited by N availability, elevated N inputs from the atmosphere can influence above- and below-ground production (Knutte et al., 2000). On the one hand, increased N deposition has had a positive effect by increasing N cycling rates, above-ground biomass accumulation and soil mineralization rates (Chapin et al., 1995; Venterea et al., 2003; Magill et al., 2004; Kreutzer et al., 2009). The environmental impacts of N deposition are equally grave and include: N saturation (Aber et al., 1989), nitrate leaching to groundwater (Gundersen et al., 2006), emission of greenhouse gasses (Butterbach-Bahl et al., 2002), shifts in microbial and community composition and loss of biological diversity (Suding et al., 2005) and forest decline (Schulte, 1989).

3.3 Reduction of N deposition

The above mentioned adverse effects of N deposition triggered the signing of many clean rain protocols during the last decades to reduce the emissions of SO_2, and nitrogen (NO_x and NH_3) in Europe and North America (Van Egmond et al., 2002; EEA, 1999). Many studies have been carried out in Europe, for example, under the NITREX (NITRogen saturation EXperiment) and EXMAN (EXperimental MANipulation of forest ecosystem) programs and in the USA (Peterjohn et el. 1996; Fenn et al., 1998; Magill et al., 2000) in order to identify a solution to soil acidification and nitrogen fertilization. Policies to reduce atmospheric pollutants in Germany have resulted in a slight decrease in nitrogen depositions in the 1990s compared to the period between 1970-1980 (Matzner and Meiwes, 1994; Meesenburg et al., 1995).

Studies on the effects of high nitrogen loads and the response of forest ecosystems to nitrogen saturation are many and well understood (Aber et al., 1998). However, the reversibility of N-induced shifts in forest ecosystem processes is largely unknown (Clark et al., 2009). Some studies have also been done to determine the effects of reduced nitrogen on forest ecosystems. For example, in an experiment to determine the effect of reduced nitrogen ("clean rain") on the growth of fine roots and soil respiration, Lamersdorf et al. (2004) reduced acid and nitrogen input of spruce forest at the Solling experimental site by 50-80% by way of a roof construction below the canopy of the forest. They observed an increase in the growth and biomass of fine roots under reduced nitrogen inputs also accompanied by a 24% increase in soil respiration rate in the "clean rain" plots. In addition to the increase in fine roots growth, gross nitrogen mineralization of soil organic nitrogen increased in the "clean rain" plot (Corre and Lamersdorf, 2004) indicating that the enhanced internal nitrogen turnover could have compensated in part for the decreased nitrogen input.

3.4 Soil microbial diversity

Soil microorganisms are critical for the sustainable cycling of nutrients and for driving ecosystem functions (Øvreås, 2000). They take part in many fundamental processes including decomposition of organic matter and nutrient cycling (Frey et al., 2004). Soils contain 1–2 and 2–5 t ha^{-1} of bacterial and fungal biomass, respectively, that inhabit less than 5% of the available biological space. Microbial diversity in soil ecosystems exceeds, by far, that of eukaryotic organisms (Rosello-Mora and Amann, 2001). Based on DNA–DNA reassociation, Torsvik et al. (1990a, b) estimated that in 1 g of soil there are approximately 4000 different

bacterial genomic units and about 5000 bacterial species have been described (Pace, 1997, 1999). The ability of an ecosystem to withstand serious disturbances is dependent in part on the biological diversity of the system. Any adverse change in the environment impacting soil microbial communities can considerably affect ecosystem performance. While just a few number of species are necessary for certain functions, a large number of species, however, might be required to maintain ecosystem functions from major environmental changes like N deposition (Nannipirei et al., 2003). One anticipated consequence of N deposition and elevated N inputs is a change in microbial community structure and function as ecosystems move towards N saturation. For example, Nemergut et al. (2003) and Frey et al. (2004) observed a shift in the bacterial, fungal and archaea communities following N amendments in tundra and temperate forest ecosystems respectively. N deposition-induced shifts in soil microbial community have resulted in an increase in ammonium and nitrate both of which affect N loss to the environment (Paul and Clark, 1997). The application of molecular techniques (especially the application of functional gene makers) has greatly improved the study of microbial communities. For example, using molecular phylogenetic tools, changes in community structure of specific types of N cycling microorganisms as affected by increased N availability have been studied (He et al., 2007; Enwall et al., 2005; Tan et al., 2003).

3.5 Soil enzymes

In terrestrial ecosystems, the functioning of microorganisms depends primarily on the activities of extracellular enzymes. These enzymes are involved in the decomposition of plant litter, microbial cell walls and reduce complex macromolecules to soluble substrates for microbial assimilation (Burns and Dick, 2002). Extracellular enzymes therefore have a direct link to substrate quality and availability (Sinsabaugh et al., 2002), microbial status (Aon and Colaneri, 2001) as well as soil physical and chemical properties (Sinsabaugh, 1994). This makes soil enzymes excellent indicators of the soil microbial decomposition ability and functional diversity (Aon and Colaneri, 2001; Cadwell, 2005). Soil extracellular enzymes have been studied in relation to ecosystem responses to global change and other disturbances (Lipson et al., 2005; Sinsabaugh et al., 2005; Finzi et al., 2006). Anthropogenic nitrogen deposition and its effects on nutrient cycles and their enzyme catalyzed processes have sparked up a lot of research whose results are often contradictory. Studying different forest ecosystems, Grandy et al. (2008) observed that changes in enzyme activities which were related to changes in soil organic carbon chemistry were caused by elevated N deposition. According to resource allocation models, N enrichment should enhance the potential activity

of enzymes involved in P and C acquisition (e.g., cellulolysis) (Allison et al., 2007) but inhibit the degradation of lignin and its derivatives due to lowered oxidative enzyme activity (Sinsabaugh et al., 2005). Likewise N deposition decreased the activities of enzymes involved in the decomposition of organic N compounds (e.g., protein, chitin, peptidoglycan) (Allison et al., 2007).

Extracellular enzyme activities have often been linked to microbial biomass since important organic matter transformations are carried out by microorganisms (Garcia and Rice, 1994; Zaman et al., 1999). Therefore microbial biomass represents a sensitive indicator of soil quality (Schloter et al., 2003) and ecological stability (Ajwal et al., 1999). Some research have shown that both soil extracellular enzyme activity and microbial biomass were influenced by N deposition (Carreiro et al., 2000; Michel and Matzner, 2003; DeForest et al., 2004; Allison et al., 2008; Wang et al., 2008; Guo et al., 2011). However, in addition to N deposition, other factors such as soil texture (Carreiro et al., 2000) and soil profile (Markonjic Fuka et al., 2008) may influence microbial biomass and enzyme activities.

3.6 Denitrification

Denitrification is one of the most sensitive soil processes as it is regulated by a complex web of biotic and abiotic factors (Tiedje, 1988). It is a major biological process in the nitrogen cycle in that it recycles between 50 and 110% of total N inputs, returning fixed nitrogen to the atmosphere and thus completing the N cycle. Denitrification is a microbial respiratory process whereby nitrate (NO_3^-) or nitrite (NO_2^-) is reduced to nitric oxide (NO), nitrous oxide (N_2O) or nitrogen gas. The nitrogenous oxides are used as alternative electron acceptors for the generation of energy when oxygen is limited (Phillipot, 2002). There exist arrays of enzymes (nitrate and nitrite reductases) that catalyze the four steps of the denitrification pathway. There are two enzymes, a membrane-bound (Nar) and a periplasmic (Nap) nitrate reductase, involved in the first step of the pathway, whereby NO_3^- is reduced to NO_2^-. The next step, termed dissimilatory denitrification, is the main step in the denitrification cascade. Here, a copper- and a cytochrome cd1-nitrite reductase catalyze reduction of nitrite to nitric oxide. The last step in the denitrification pathway, the reduction of nitrous oxide (N_2O) to nitrogen gas (N_2), is carried out by the multicopper homodimeric N_2O reductase (NosZ). All these catalytic processes are usually induced sequentially under anaerobic conditions mainly by the actions of bacteria and archaea which have the metabolic capability of converting organic nitrogen into inorganic nitrogen (Phillipot and Hallin, 2005). These microorganisms

are often isolated from natural environments such as soil, sediments, sewage and marine and fresh waters (Zumft, 1997). The ability to denitrify is performed by a polyphyletic diverse group of bacteria and not confined to a specific of taxonomic group. Consequently, to study and understand the diversity of the denitrifier communities, molecular techniques are based on the use of functional genes that encode key enzymes in the denitrification cascade or their transcript as molecular markers. *narG* and *napA* genes encoding the membrane-bound and the periplasmic nitrate reductase, respectively, as molecular markers of the nitrate reducer community (Bru et al., 2007). In addition, the *nirK*, *nirS* and *nosZ* genes encoding the copper and cytochrome cd1 nitrite reductases and the nitrous oxide reductase, respectively, were used as molecular markers of the denitrifier community (Philippot, 2005).

3.7 Peptide degradation

Proteins make up an integral part of soil organic nitrogen which makes them ecologically important (Lipson and Näsholm, 2001). In agricultural soils, peptides and proteins contribute 35–57% to soil organic N and up to 25% in forest soils (Paul and Williams, 2005; Kranabetter et al., 2007). The decomposition of proteins by microbial peptidases is fundamentally important since N is made available for plants and soil organisms (Bach and Munch, 2000) with a proportion being returned to the global N cycle. Through proteolysis, proteins are degraded into smaller membrane permeable substances (peptides and amino acids) that are easily assimilated and metabolized by soil organisms. The sources of soil peptidases have been directed mainly towards bacterial origin, although plant roots have been shown to exude peptidases (Adamczyk et al., 2009). Asmar (1992) indicated a high correlation between proteolytic activities and total bacteria counts in glucose-amended soils. By selective inhibition of fungal populations, Hayano and Watanabe (1990) revealed that peptidases are mainly of bacterial origin in paddy soils.

The indigenous bacterial community that harbour genes encoding extracellular peptidases are pivotal in the regulation of proteolysis and organic N mineralization in soils. Therefore, for ecosystem sustainability, the genetic diversity of proteolytic bacterial communities plays an important role. Several studies on proteolytic activity have shown differences in activity due to soil physicochemical properties, climatic conditions and management practices of agricultural systems (Schloter et al., 2003; Watanabe et al., 2003; Marx et al., 2005). Although a few studies have looked into proteolytic activity in other ecosystems (e.g., the Baltic sea; Nausch and Nausch, 2000), little is known about the activity and diversity of

peptide degrading bacterial communities and even less, the influence of environmental or anthropogenic factors.

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4 Objectives

N deposition being one of several environmental problems, different ecosystems would respond differently. More detailedly, different groups of microorganisms would differ in their response to N deposition. These responses might show high spatial and temporal variability due to differences in nutrient status of the different soil profiles as well as environmental and climatic changes that occur at the study sites. The aim of this thesis was to assess the effects of reduced N deposition on the activities, abundance and diversity of two bacterial communities. The specific objectives were to (i) localize and quantify the activities of enzymes involved in nutrient (N, C, P) decomposition, (ii) use functional genes coding for enzymes as biomarkers to characterize the bacterial communities involved in nitrate reduction and denitrification, (iii) evaluate the applicability of a new set of *pepN* primers to quantify genes involved in peptide degradation and (iv) improve understanding of the link between microbial diversity and function.

To realise these objectives, three studies were carried out. The first study aimed to evaluate the response of nitrate-reducing microorganisms towards environmental changes under reduced N deposition at the level of community abundance and enzyme activities. The hypothesis was that reduced N deposition in forest soils modifies the density, activity and function of microbial communities involved in denitrification in the different soil horizons. Using substrate-induced respiration (SIR), the active fraction of soil microbial biomass was characterized and potential nitrate reduction was used to quantify the activity of nitrate reducers. The abundance of total bacteria, nitrate reducers and denitrifiers in the different soil layers was analysed by quantitative PCR of 16S rRNA gene, nitrate reduction and denitrification genes.

The goal of the second study was to investigate the effects of reduced N deposition on several enzyme activities mediating soil organic matter decomposition with a special focus on the decay of different peptides. Given that the organic horizons of forest soils are nutrient-rich, the hypotheses were that (i) the reduction of N deposition might decrease the activity of enzymes involved in the decay of easily decomposable compounds within the soil profile and enhance the activity of enzymes involved in the decomposition of more recalcitrant compounds, (ii) enzymes involved in N cycling would show stronger response to N reduction than enzymes involved in C cycling and (iii) reduction of N deposition would have a more

pronounced effect in the upper soil layer than in lower soil horizons. Flourogenic substrates and standard colorimetric methods were selected to quantify the activities of these enzymes.

Proteins and peptides make up an integral fraction of dissolved organic N compounds. A focus on the decay of peptides in the second study revealed a significantly high activity of alanine aminopeptidase enzyme. Therefore the third study aimed to extend these findings to other ecosystems, namely, agricultural and grasslands affected by environmental/climate change. The main focus was to design a new set of primers targeting the functional gene, *pepN*, coding for alanine aminopeptidase and use it to investiggate in detail the function (enzyme activity) and diversity of those bacterial communities capable of peptide degradation. The diversity was analysed by sequencing and construction of clone libraries.

5 Response of total and nitrate-dissimilating bacteria to reduced N deposition in a spruce forest soil profile

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Abstract

A field-scale manipulation experiment conducted for 16 years in a Norway spruce forest at Solling, Central Germany, was used to follow the long-term response of total soil bacteria, nitrate reducers and denitrifiers under conditions of reduced N deposition. N was experimentally removed from throughfall by a roof construction (‘clean rain plot’). We used substrate-induced respiration (SIR) to characterize the active fraction of soil microbial biomass and potential nitrate reduction to quantify the activity of nitrate reducers. The abundance of total bacteria, nitrate reducers and denitrifiers in different soil layers was analysed by quantitative PCR of 16S rRNA gene, nitrate reduction and denitrification genes. Reduced N deposition temporarily affected the active fraction of the total microbial community (SIR) as well as nitrate reductase activity. However, the size of the total, nitrate reducer and denitrifier communities did not respond to reduced N deposition. Soil depth and sampling date had a greater influence on the density and activity of soil microorganisms than reduced deposition. An increase in the *nosZ*/16S rRNA gene and *nosZ*/*nirK* ratios with soil depth suggests that the proportion of denitrifiers capable of reducing N_2O into N_2 is larger in the mineral soil layer than in the organic layer.

Introduction

Nitrogen emissions and atmospheric deposition are globally significant in their potential to alter the nutrient balance in soils, triggering changes in the composition of plants and soil organisms (Gidman et al., 2006; McLauchlan et al., 2007). The current hypothesis suggests that increased N deposition promotes the rate of soil organic matter accumulation by either increasing leaf/needle biomass and litter production or by reducing the decomposition of organic matter (de Vries et al., 2006). Based on data collected at the monitoring plots of different European forest sites, the contribution of N deposition to net sequestration of C in trees and soil in the period 1960–2000 is c. 5.1 Mton $year^{-1}$ in tree wood and 6.7 Mton $year^{-1}$ soil – about 10% of the total C sequestration during that period (de Vries et al., 2006). Several N-addition experiments (partly including ^{15}N) in temperate forests revealed that soils, rather than plants, are a main long-term sink for the added nitrate and ammonium (Nadelhoffer et al., 1999; Providoli et al., 2006). Specific groups of soil microorganisms differed in their response to N deposition or N addition (Gundersen, 1998; Butterbach-Bahl et al., 2002; Hungate et al., 2007). For example, the composition of the ammonia-oxidizing community in acidic forest soil was not affected by nitrogen deposition (Jordan et al., 2005; Schmidt et al., 2007). In contrast, growth of ectomycorrhizal fungi in a Norway spruce forest soil was reduced under N deposition (Nilsson et al., 2007). In addition, the functioning of microorganisms was affected by N fertilization with a stimulation of the initial decomposition of cellulose and solubles and a suppression of the decomposition of older humus fractions (Hagedorn et al., 2003).

Whereas the mean chronic annual nitrogen deposition in Europe is still 17 kg N ha^{-1} $year^{-1}$ (Stevens et al., 2004), a reduction in emissions of nitrogenous pollutants under the Gothenburg Protocol is presupposed (Power et al., 2006). As one of seven NITREX sites across Europe (see Tietema et al., 1998), the ‘Solling roof project’ was established in 1989 in a Norway spruce forest at Solling, Central Germany, to simulate preindustrial N deposition. Partial deionization of rainfall was established in this field-scale roof experiment in 1991 to investigate whether the effects of N saturation on ecosystem functioning are reversible by decreasing N input (Bredemeier et al., 1995a, b, 1998). Three years after the start of the experiment, microbial biomass (C_{mic}) within the soil profile was not affected (Raubuch et al., 1999); after 10 years, the mean annual soil respiration rate was 24% higher in the clean rain vs. control plot (Lamersdorf & Borken, 2004). The long-term reduction of nitrogen and

proton inputs did not affect nitrous oxide emission, which ranged from 0.25 to 0.41 kg N_2O-N ha^{-1} $year^{-1}$ in the spruce forest after 10 years of the experiment (Borken et al., 2002). There are reasons to expect that lengthier reduction of N deposition alters the community composition of soil microorganisms in the acid forest ecosystem.

Thus, changes in the amount and composition of needles and fine roots might indirectly affect the density and activity of soil microorganisms, whereas altered N availability and pH might directly affect soil microorganisms under reduced atmospheric nitrogen loads and reduced proton input.

The present study evaluates the possible responses of the total microbial and the nitrate reducer communities within profiles of a spruce forest soil to reduction of chronic N deposition after an experimental duration of 16 years. Nitrate-reducing prokaryotes constitute a wide taxonomic group sharing the ability to produce energy from dissimilatory reduction of nitrate into nitrite, the first step of two different processes: denitrification and dissimilatory reduction of nitrate to ammonium. We used substrate-induced respiration (SIR) to characterize the active fraction of soil microbial biomass and potential nitrate reduction to quantify the activity of nitrate reducers. The densities of total bacteria, nitrate reducers and denitrifiers in different soil layers were analysed by quantitative PCR (qPCR) of the 16S rRNA genes, the nitrate reduction genes, and denitrification genes (Henry et al., 2004, 2006; López-Gutiérrez et al., 2004; Kandeler et al., 2006; Bru et al., 2007). We used the *narG* and *napA* genes encoding the membrane-bound and the periplasmic nitrate reductase, respectively, as molecular markers of the nitrate reducer community (Bru et al., 2007). In addition, the *nirK*, *nirS* and *nosZ* genes encoding the copper and cytochrome cd1 nitrite reductase and the nitrous oxide reductase, respectively, were used as molecular markers of the denitrifier community (Philippot, 2005). We hypothesized that reduced N deposition into different horizons of the acid forest soil would modify the densities and activities of the total soil microbial community as well as those of functional microbial communities involved in N cycling.

Materials and methods

Experimental site and soil sampling

The Solling roof project was established in 1989 in a 57-yearold Norway spruce plantation growing on strongly acidic and weakly podzolized loam-silt at the Solling plateau in Central

Germany (51°13'N, 9°34'E, elevation c. 500 m above sea level). The climate is dominated by Atlantic streams with evenly distributed precipitation (mean annual precipitation = 1090 mm) and a moderate variation in temperature (mean annual temperature = 6.4°C) throughout the year (Lamersdorf & Borken, 2004). The control plot is covered by a translucent roof (300 m^2). Throughfall water is permanently collected and immediately re-sprinkled without any chemical treatment in the control plot. The clean rain plot is also covered with an identical roof. There, throughfall water is partly deionized and re-sprinkled immediately on the plot since the start of the experiment. Controlling the efficiency of the deionization, Corre & Lamersdorf (2004) reported a reduction of the major elements in the clean rain plot compared with the control plot of 78% for protons, 53% for sulphate, 86% for ammonium and 49% for nitrate. The pH of the throughfall solution at the clean rain plot (pH = 5.0) was also higher than that at the control plot (pH = 4.4). The Ca and Mg input in the clean rain plot was twice as much as in the control plot during the first 4 years of the roof experiment (1992–1995), but thereafter, the levels were similar between plots (Corre & Lamersdorf, 2004). Soils of the roofed plots are separated from the surrounding area by a vertical plastic foil (Xu et al., 1998).

Four replicate soil samples ('subplots') were collected from the clean rain plot as well as from the control plot in late April and late October 2006. Because of the lack of roof replications, the results are based on pseudo-replications. Up to five soil cores (8 cm in diameter) per subplot were taken and mixed according to the horizons Oe, Oa, Ah and Bw. Samples were sieved through a 2-mm sieve (Oe: 5 mm) and stored at -20°C before analysis.

The C_{org} and N_t contents of soil were characterized for soil sampled in April, nitrate, ammonium and water contents were analysed for April and October samples (Tables 5.1 and 5.2). In spring, the moisture content of soils was much higher (organic layers: +22%, mineral layers: +12%) than for soils sampled in October (Table 5.2), mainly due to wet periods in March and April 2006 as well as due to a drought period in September and low precipitation in October 2006 (data not shown). The water contents of the clean rain and control plot did not differ significantly in April and October.

Table 5.1: Chemical soil properties of the acid forest site at Solling, Central Germany, in April 2006 in the control and clean rain plots at different soil depths.

	OC (g 100 g^{-1})		NT (g 100 g^{-1})		C/N		pH ($CaCl_2$)	
Soil depth	Control	Clean rain	Control	Clean rain	Control	Clean rain	Control	Clean rain
Oe	43.5	43.6	1.68	1.61	25.9	27.4	2.97	3.19
0-2 cm	(± 1.1)	(± 1.2)	(± 0.02)	(± 0.07)	(± 0.7)	(± 2.0)	(± 0.06)	(± 0.12)
Oa	35.0	32.7	1.39	1.36	25.3	24.0	2.63	2.77
2-5 cm	(± 3.2)	(± 1.2)	(± 0.12)	(± 0.06)	(± 0.7)	(± 0.6)	(± 0.07)	(± 0.03)
Ah	4.6	4.0	0.21	0.20	21.7	20.1	2.94	3.19
5-12 cm	(± 0.4)	(± 0.3)	(± 0.02)	(± 0.02)	(± 0.5)	(± 0.6)	(± 0.06)	(± 0.04)
Bw	1.7	1.6	0.09	0.09	19.1	17.5	3.80	4.05
12- cm	(± 0.1)	(± 0.2)	(± 0.01)	(± 0.01)	(± 0.4)	(± 0.9)	(± 0.02)	(± 0.05)

Results are given as means ± SE. OC, organic carbon; NT, total nitrogen.

Soil chemical analyses, microbial biomass and nitrate reductase activity

Organic carbon (OC) and total nitrogen (NT) were measured with a CNS analyzer (Vario MAX, Elementar GmbH, Hanau, Germany) using 250 mg of the organic soils and 800 mg of the mineral soils. Soil pH was measured in a 0.01 M $CaCl_2$ solution (soil to solution ratio 1: 10 for the organic layers and 1: 2.5 for the mineral soils). After extraction of inorganic N with 1 M KCl (soil to solution ratio of 1: 10 for the organic layers and 1: 5 for the mineral soils), nitrate and ammonium were measured at the SPINMAS [automated sample preparation unit for inorganic nitrogen (SPIN) species coupled to a quadruple Mass Spectrometer (MAS)] according to Stange et al. (2007).

For the SIR measurement (determination in duplicate), substrate saturation and the maximum initial respiration response were obtained at an amendment rate of 8.0 mg glucose g^{-1}. CO_2 evolved was trapped in 50 mM NaOH for a 4-h incubation at 25°C and measured by titration (Anderson & Domsch, 1978). The release of CO_2 was linear over a period of 4 h and the SD of the analytical replicates was < 15%.

The potential activity of the nitrate reductase was determined by anaerobic incubation of soil following a modified protocol of Kandeler (1996). The method was based on the determination of the NO_2^--N production after adding nitrate as a substrate and 2,4-dinitrophenol as an uncoupler of oxidative phosphorylation that interfered with electron transfer, but allowed nitrate reduction to continue. Substrate as well as inhibitor concentrations were optimized in pre-experiments. In detail, 0.2 g soil was weighed in five replicates into 2.0-mL reaction tubes. Two hundred micrograms of 2,4-dinitrophenol per gram soil (fresh weight) was added to inhibit the nitrite reductases. After a 24-h incubation in 1 mM KNO3 in a total volume of 1 mL at 25°C in the dark, the soil mixture was extracted

with 4 M KCl and centrifuged for 1 min at 1400 g. The accumulated nitrite in the supernatant was determined by a colorimetric reaction. All analytical results were calculated on the basis of the oven-dry (105^{o}C) weight of soil.

Table 5.2: NH_4+, NO_3^- and percentage water content of the acid forest site at Solling, Central Germany, in April and October 2006 in the control and clean rain plots at different soil depths

	NH_4^+ (µg N g^{-1})				NO_3^- (µg N g^{-1})				% water content			
	April		October		April		October		April		October	
Soil depth	Control	Clean rain	Control	Clean rain	Control	Clean rain	Control	Clean rain	Control	Clean rain	Control	Clean rain
Oe 0-2 cm	213.99 *(± 30.46)*	220.10 *(± 29.93)*	169.06 *(±1.81)*	263.59 *(± 35.74)*	9.20 *(± 5.25)*	5.83 *(± 0.74)*	46.35 *(± 0.79)*	8.25 *(± 0.13)*	68.2 *(± 0.9)*	68.7 *(± 1.2)*	41.50 *(± 1.0)*	48.93 *(±3.3)*
Oa 2-5 cm	85.30 *(± 14.95)*	131.93 *(± 26.93)*	163.73 *(± 15.52)*	202.64 *(± 37.51)*	9.03 *(± 3.61)*	2.78 *(± 0.38)*	21.54 *(± 5.36)*	8.05 *(± 1.56)*	65.0 *(± 1.4)*	66.5 *(± 0.7)*	43.95 *(± 2.0)*	47.32 *(± 4.9)*
Ah 5-12 cm	6.95 *(± 0.30)*	9.25 *(± 1.38)*	16.29 *(± 1.15)*	27.73 *(± 7.42)*	0.75 *(± 0.34)*	0.54 *(± 0.19)*	6.21 *(± 1.87)*	1.55 *(± 0.55)*	30.9 *(± 0.9)*	34.4 *(± 1.3)*	17.02 *(± 0.8)*	18.05 *(± 1.2)*
Bv 12- cm	3.46 *(± 0.23)*	4.04 *(± 0.13)*	9.22 *(± 0.68)*	10.68 *(± 1.27)*	0.70 *(± 0.20)*	0.64 *(± 0.19)*	8.38 *(± 2.49)*	4.68 *(± 2.51)*	25.9 *(± 0.2)*	26.5 *(± 0.6)*	15.76 *(± 1.2)*	17.54 *(± 0.8)*

Results are given as mean ± SE.

Quantification of 16S rRNA gene, nitrate reductase genes (narG and napA) and denitrification genes (nirK, nirS and nosZ)

DNA was extracted from 0.3 g of soil using the FastDNA Spin Kit for soil (BIO 101, Qbiogene, France), according to the protocol of the manufacturer. Because of the large amounts of PCR-inhibiting substances such as humid acids, an additional purification step with polyvinylpolypyrrolidone-loaded columns (Sigma Aldrich) was performed according to Martin-Laurent et al. (2001). DNA quantity was checked using a BioPhotometer (Eppendorf) at 260 nm. qPCR products were amplified with an ABI Prism 7900 (Applied Biosystems) using SYBR green as the detection system in a 25-µL reaction mixture containing 0.5 µM (each) primer, 12.5 µL of SYBR green PCR master mix (QuantiTect SYBR green PCR Kit; Qiagen, France), 1.25 µL of DNA-diluted template corresponding to 12.5 ng of total DNA and 500 ng of T4gp32 (Qbiogene). The thermal cycling conditions for the 16S rRNA gene and the *nirS, nirK* and *nosZ* genes were as described previously (Henry et al., 2004, 2006; López-Gutiérrez et al., 2004; Kandeler et al., 2006). The *narG* and *napA* qPCR was performed as described in Bru et al. (2007). Thermal cycling, fluorescent data collection and data analysis were carried out using the ABI Prism 7900 sequence detection system according to the manufacturer's instructions.

Standard curves were obtained with serial plasmid dilutions of a known amount of plasmid DNA containing a fragment of the 16S rRNA gene or the *narG*, *napA*, *nirK*, *nirS* or *nosZ* gene. Sequences of the primers and the thermal conditions used for the real-time PCR are given in Supporting Information. Purified soil DNAs were tested for inhibitory effects of coextracted substances by diluting soil DNA extracts and by quantifying by qPCR of a known amount of plasmid DNA mixed to soil DNA extracts. In all cases, no inhibition was detected.

Statistical analysis

If necessary, data were Box-Cox, log or sin transformed before analysis. The influence of reduced N deposition on the chemical and physical soil properties and on SIR for each soil depth was determined using the paired *t*-test. Two- and three-way univariate ANOVA was applied to test differences of the means of respiration, nitrate reductase activity and gene copy numbers of 16S rRNA gene, *narG*, *napA*, *nirK* and *nosZ* between depths, treatments and sampling dates. Homogeneity of variances was proved by a Levene test.

Multiple regression analysis was applied to evaluate the relationship between soil

environmental factors (water content, OC, NT, NH_4^+, NO_3^- and pH) and respiration, nitrate reductase activity, the density of total bacteria and the nitrate-reducing/denitrifying community. Significance was accepted at the $P < 0.05$ level of probability.

Results

Soil chemical properties

The results clearly showed that 16 years of reduced N deposition did not affect the organic C and N pools (Tables 5.1 and 5.3). Organic layers (Oe and Oa) were characterized by much higher OC and NT contents than both of the deeper layers (Ah and Bw). Because of high spatial heterogeneity, NO_3^- contents were not significantly different between the treatments. Long-term reduced N deposition showed a tendency towards higher NH_4^+ contents in all layers of the clean rain plots compared with the control plots (Table 5.2). As expected, the contents of inorganic N (NH_4^+, NO_3^- depended on the soil depth (Table 5.3). Although the roof construction removed the nitrogen and proton input into the ecosystem, the higher pH values in the clean rain treatment were statistically significant only for the Oa horizon. The pH values varied within the soil profile, showing the highest values in the Bw horizon. The soil water content was not affected by N deposition at either sampling date (April and October, Table 5.2).

Table 5.3: Influence of reduction of N deposition on chemical soil properties

	Univariate ANOVA *P*						
				†NH_4^+		†NO_3^-	
	* OC	* NT	pH	April	October	April	October
Nitrogen	0.202	0.626	**0.003**	**0.000**	**0.001**	0.919	0.700
Depth	**0. 000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
Nitrogen X Depth	0.820	0.974	0.902	0.418	0.058	0.520	0.260

* Data log transformed. †Data Box-Cox transformed. Effects of treatment and depths were estimated by univariate ANOVA. *P*-values ≤ 0.05 are indicated in bold.

Activity of microorganisms and nitrate reducers

Measurement of SIR yielded estimates of the CO_2 released by the soil microbial community under optimal substrate availability. At both sampling dates, reduced N deposition did not significantly affect SIR in the organic layers. In the mineral layers, a significant interaction between treatment and sampling date was observed in the Ah horizon, but not in the Bw horizon (Fig. 5.1). Sampling date had a significant influence on SIR in the Oe and Bw horizons. The active microbial community strongly decreased with depth (Fig. 5.1).

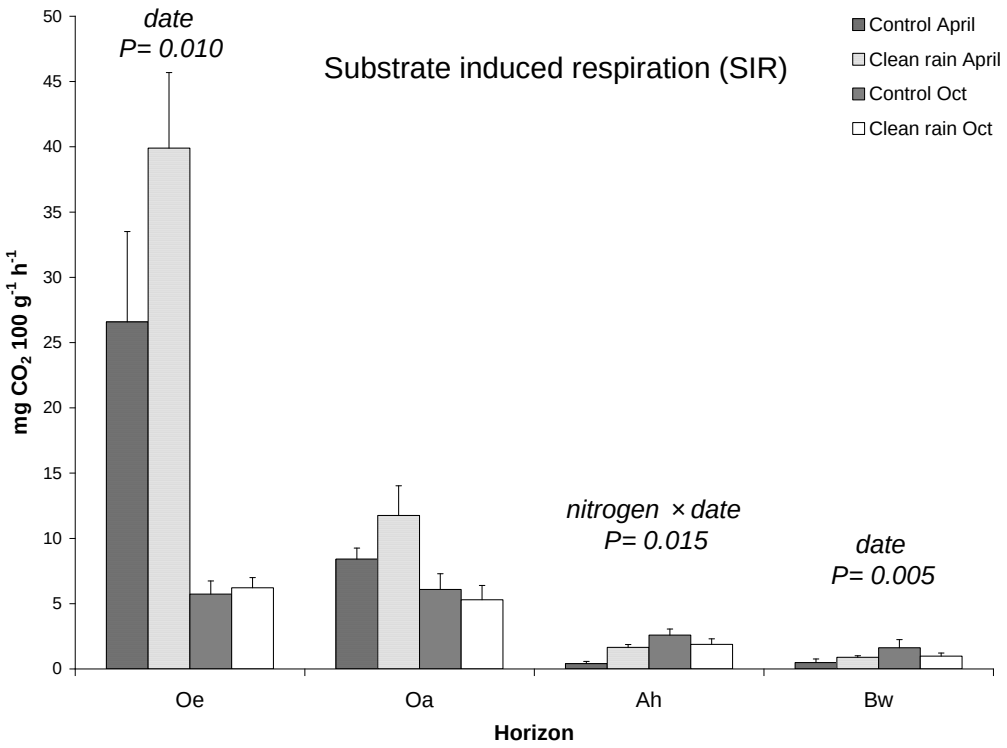

Fig. 5.1: Response of SIR to reduced N deposition within the soil profile (means ±SE of four replicates). *P*-values (< 0.05) of two-way ANOVA including N deposition and sampling date are given.

Nitrate reductase activity provided an insight into the potential activity of the nitrate reducers. Activity ranged from 0.1 to 1.2 µg N g^{-1} day^{-1} and was modified by N deposition, soil depth and sampling time. Whereas reduced N deposition did not influence nitrate reductase in April, significant treatment effects – lower activity of the clean rain treatment – were detected in October (Fig. 5.2). Generally, organic layers were characterized by a higher activity than the mineral soil layers.

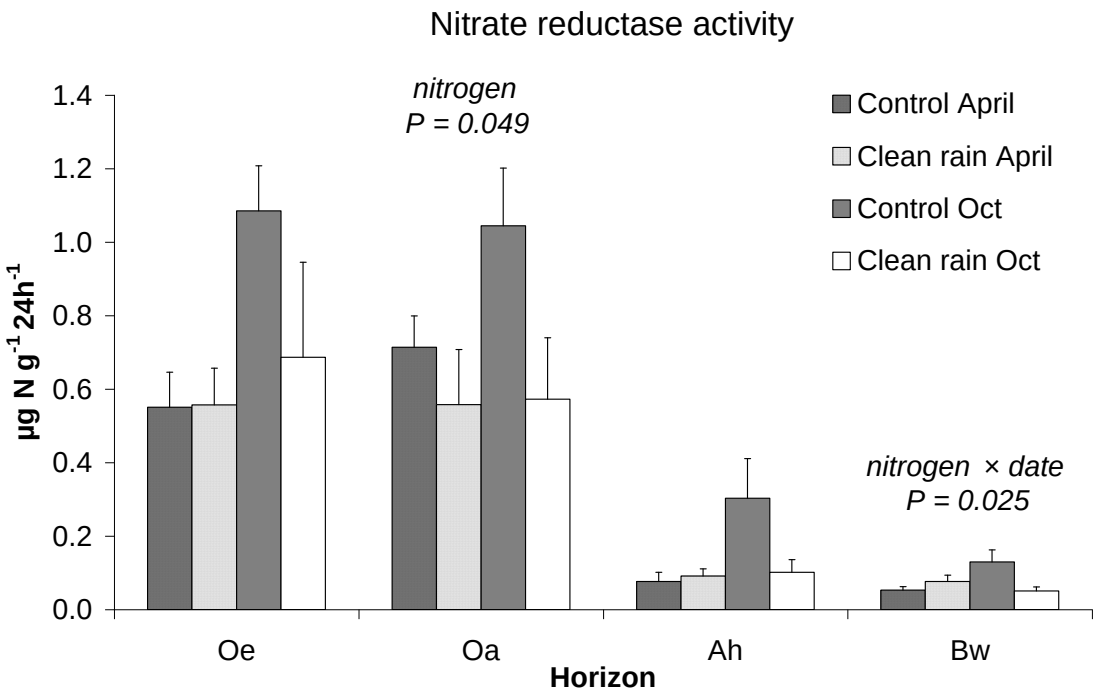

Fig. 5.2: Response of nitrate activity to reduced N deposition within the soil profile (means ±SE of four replicates). *P*-values (< 0.05) of two-way ANOVA including N deposition and sampling date are given.

Densities of total bacteria, nitrate reducers and denitrifiers

Samples from the forest site collected at four different soil depths and at two different dates contained amounts of 16S rRNA gene target molecules ranging from 3.8 $x10^4$ to 1.9 $x10^5$ copies ng^{-1} DNA (Fig. 5.3). The *narG, napA, nirK* and *nosZ* gene copy numbers were two to three logs lower than the 16S rRNA gene. The gene copy number of *narG* was higher than the other functional genes, with densities ranging from 79 to 1.4 $x10^3$ copies ng^{-1} DNA, while *napA* ranged from 23 to 3.3 $x10^2$, *nirK* from 25 to 9.0 $x10^2$ and *nosZ* from 18 to 1.9 $x10^2$ copies ng^{-1} DNA (Figs 5.4 and 5.5). The copy numbers of nirS gene fragments were below the detection limit of the *nirS* qPCR assay (10^2 copies ng^{-1} DNA).

Reduction of N deposition did not affect the abundance of 16S rRNA gene copy numbers in the soil profile at any time (Fig. 5.3). The densities of 16S rRNA genes decreased within the soil profiles, but this effect was less obvious than that for overall soil microbial activity (Figs 5.1 and 5.3).

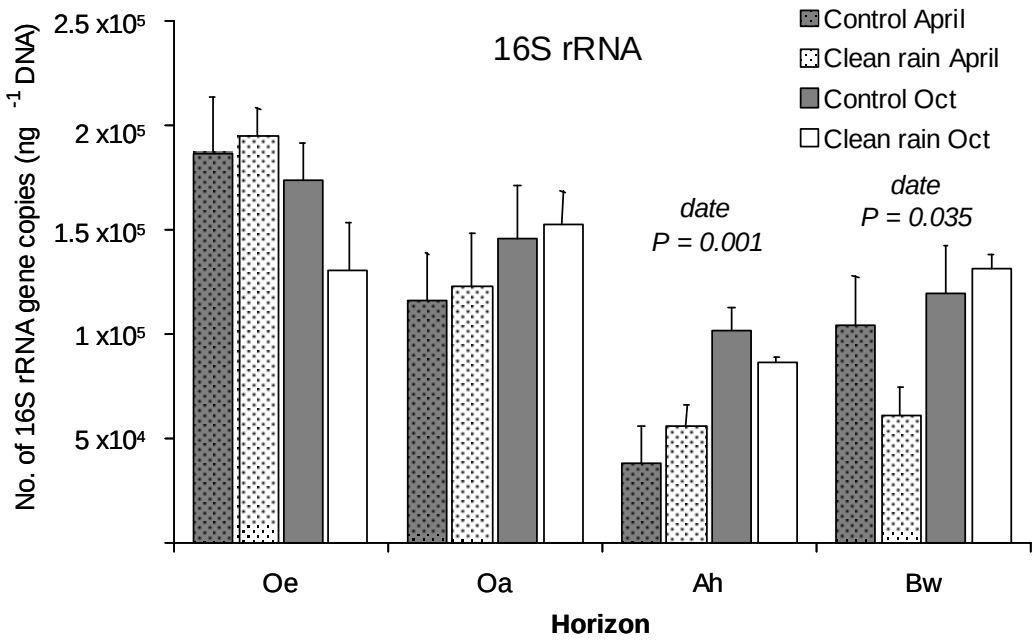

Fig. 5.3: Size of the total bacterial community estimated by quantifying the 16S rRNA gene copy numbers in relation to reduced N deposition and to soil depth (means ± SE of four replicates). Effect of reduced nitrogen deposition is significant neither for soil samples of April nor for October. *P*-values (< 0.05) of two-way ANOVA including N deposition and sampling date are given.

Similar to the 16S rRNA gene, the densities of the nitrate reduction genes (*narG* and *napA*) or of the *nirK* denitrification gene were not affected by reduced N deposition (Figs 5.4 and 5.5, Table 5.4). In contrast, a slight effect was observed for the *nosZ* gene (Table 5.4). All functional genes showed a significant depth effect (Table 5.4). Thus, the *narG*, *napA* and *nirK* gene copy numbers decreased within the soil profile, whereas *nosZ* showed the highest abundance in the Bw horizon (Figs 5.4 and 5.5). A significant sampling date effect was also observed for all genes, except for *nosZ* (Table 5.4).

The ratios of the functional genes to 16S rRNA gene from total eubacteria revealed maximum proportions of 0.91% for *narG*, 0.28% for *napA*, 0.42% for *nirK* and 0.05% for *nosZ* (data not shown). The relative abundance of *napA* and *nirK* genes was constant within the soil profile, whereas *narG* was mainly enriched in the upper horizons and *nosZ* in deeper soil layers. Therefore, the ratio of *nosZ*/16S rRNA gene and *nosZ*/*nirK* copy numbers increased from about 0.02 and 0.15 in the organic horizons to about 0.16 and 0.51 in the mineral soils, respectively.

Multiple regression analysis including microbiological data as independent factors (Table 5.5a) revealed no significant correlation between SIR and 16S rRNA gene but between nitrate

reductase activity and the copy number of *narG* ($P < 0.05$).

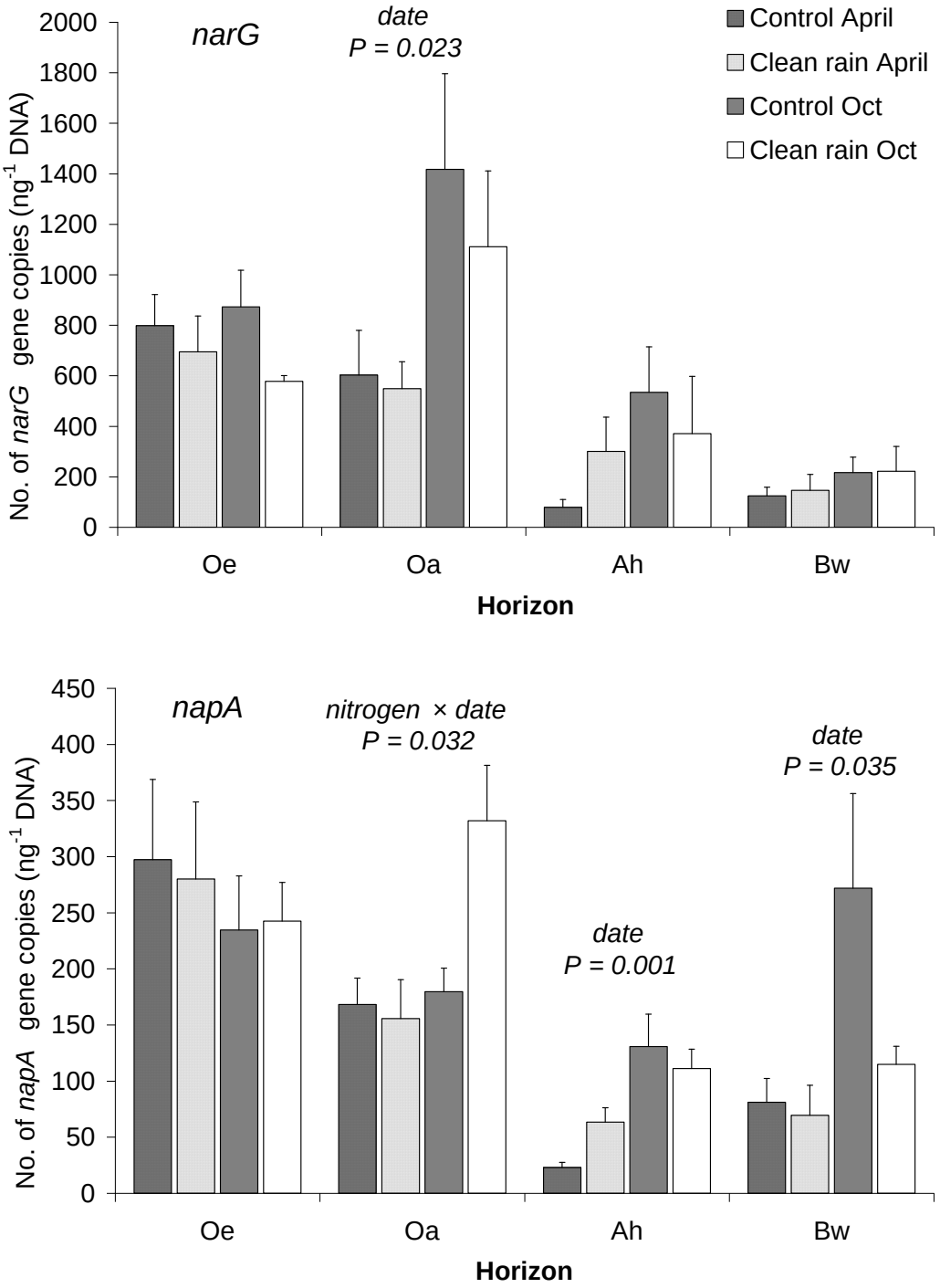

Fig. 5.4: Size of the nitrate reducer community estimated based on *narG* and *napA* gene copy numbers in relation to reduced N deposition and to soil depth (means ±SE of four replicates). *P*-values (< 0.05) of two-way ANOVA including N deposition and sampling date are given.

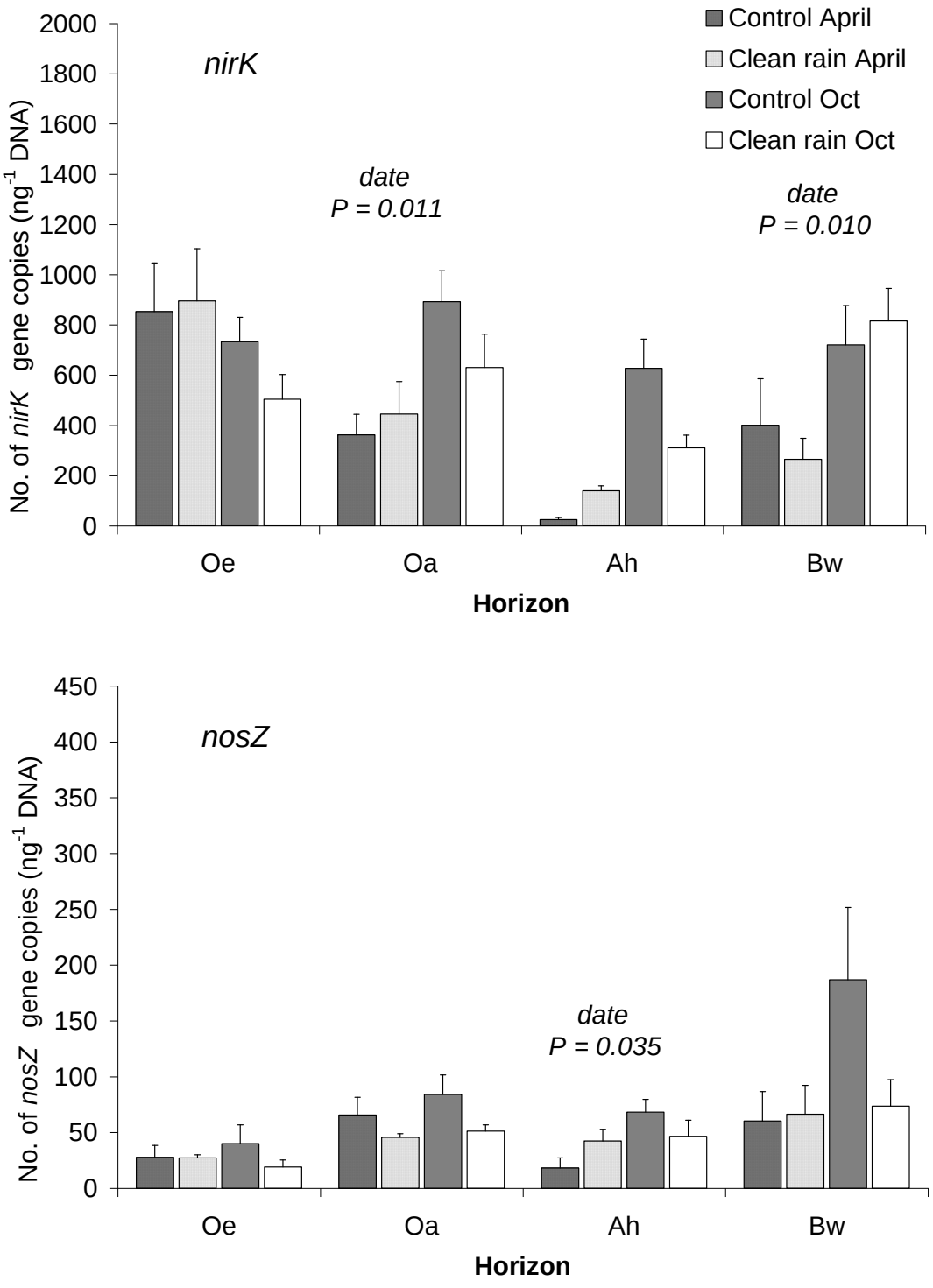

Fig. 5.5: Size of the denitrifier community estimated based on *nirK* and *nosZ* gene copy numbers in relation to reduced N deposition and to soil depth (means ± SE of four replicates). *P*-values (< 0.05) of two-way ANOVA including N deposition and sampling date are given

Table 5.4: Influence of reduced N deposit , soil depth and sampling date on nitrate reductase activity (NRA), SIR and on the density of total bacterial community, nitrate reducers and denitrifiers

	Univariate ANOVA *P*						
	16S rRNA	*narG*	*napA*	*nirK*	*nosZ*	NRA	SIR
Nitrogen	0.500	0.604	0.998	0.236	**0.049**	**0.005**	0.613
Depth	**0.000**	**0.000**	**0.000**	**0.000**	**0.025**	**0.000**	**0.000**
Date	**0.036**	**0.002**	**0.000**	**0.001**	0.601	**0.007**	0.062
Nitrogen × Depth	0.734	0.742	0.087	0.966	0.751	0.732	0.836
Nitrogen × Date	0.694	0.132	0.779	0.112	0.076	**0.003**	**0.035**
Depth × Date	**0.007**	0.176	**0.016**	**0.001**	0.574	0.623	**0.000**
Nitrogen × Depth × Date	0.215	0.392	0.112	0.258	0.629	0.835	0.644
Levene test	0.241	0.543	0.084	0.139	0.844	0.097	0.059

*Data Box-Cox transformed. †Data sin transformed. ‡Data log transformed. *P*-values for the density and activity of microorganisms analysed by ANOVA are shown. *P*-values ≤ 0.05 are indicated in bold.

Table 5.5a: Multiple regression analysis of size of total and nitrate-dissimilating bacteria using the microbial parameters SIR and nitrate reductase activity (NRA) as independent factors

Parameter	Standardized coefficient (β)					Model *P*-value
	16S rRNA copies	*narG* copies	*napA* copies	*nirK* copies	*nosZ* copies	
SIR	0.241	-0.030	**0.262**	0.259	**-0.367**	0.000
NRA	0.167	**0.428**	0.144	0.004	-0.170	0.000

Significant correlations with *P*-values ≤ 0.05 are indicated in bold.

Linking soil microbiological to environmental properties

Multiple regression analysis, including soil water content, OC, NT, NH_4^+, NO_3^- and pH as independent factors and copy numbers per nanogram DNA, respiration rates and activity rates as dependent factors, was used to relate changes in enzyme activities and gene densities to soil properties in April (Table 5.5b): SIR was positively related to OC content and negatively related to NT. Nitrate reductase activity depended on the soil water content. None of the soil properties analysed could explain the abundance of 16S rRNA gene, *narG* or *nosZ* genes, while the abundance of the *napA* gene was significantly influenced by the OC content. Multiple regressions also revealed that NT, NH_4^+ and pH could explain the copy numbers of *nirK* (Table 5.5b).

Table5.5b: Multiple regression analysis of the size and activity of total* and nitrate-dissimilating bacteria using soil water content, NT, OC, NH_4^+ ,NO_3^- pH as independent factors

	Standardized coefficient (β)						Model *P* value
Parameter	Soil water content	NT	OC	NH_4^+	NO_3^-	pH	
16S rRNA level	0.423	-0.799	0.758	0.521	0.048	0.235	0.000
narG copies	0.740	-1.917	1.831	0.076	0.116	0.009	0.000
napA copies	-0.865	-0.901	**2.457**	0.209	-0.205	0.025	0.000
nirK copies	0.654	**-1.729**	1.080	**0.879**	0.084	**0.347**	0.000
nosZ copies	1.727	0.433	-1.890	-0.229	0.130	0.461	0.520
SIR	0.015	**-2.280**	**3.243**	0.043	-0.179	0.283	0.000
NRA	**1.598**	-1.294	0.801	-0.164	-0.100	0.088	0.000

*Activity of all microorganisms. Data from the April sampling date were used for the analysis. Significant correlations with *P*-values ≤ 0.05 are indicated in bold. NRA, nitrate reductase activity.

Discussion

N deposition and soil acidification affect N cycling in many forest ecosystems (Lamersdorf & Borken, 2004). The Solling roof experiment, one of the rare long-term field experiments, was designed to provide an insight into future developments of acidified and nitrogen-saturated forest ecosystems in an envisaged environment of reduced N emissions. Our study, performed 16 years after the start of the experiment, investigated the effects of reduced N deposition on both the total microbial community and on the functional microbial guilds involved in N-cycling.

The total microbial community response to reduced N deposition and soil depth

The impact of reduced deposition was investigated by quantifying the activity and the size of the total microbial community using SIR and qPCR of 16S rRNA gene target N deposition and proton input can also be explained by molecules. Whereas community size did not respond, SIR temporarily increased in the Ah horizon of the clean rain plot. Because the Ah horizon was characterized by increased growth of fine roots (Lamersdorf & Borken, 2004), we suggest that rhizodeposition stimulated the heterotrophic soil microbial community here. The weak effect of reduced N deposition and proton input can also be explained by enhanced internal nitrogen turnover of soil microorganisms: because long-term reduction of N had a minor effect on the inorganic N contents (NH_4^+, NO_3^-) and on the active fraction of the soil

microbial community, enhanced internal nitrogen turnover might have partly compensated the lower N input by throughfall in the clean rain plot during recent years. This hypothesis is also supported by higher gross mineralization of soil organic nitrogen in that plot (Corre & Lamersdorf, 2004).

SIR showed values up to 40 mg CO_2 100 g^{-1} h^{-1} in the Oe horizon, in the range of previous results for spruce forests (Kandeler et al., 1999), while the values were 2–4 mg CO_2 100 g^{-1} h^{-1} in the Bw horizon in the mineral layer. 16S rRNA gene target molecules of the forest site ranged from 3.8 $x10^4$ to 1.9 $x10^5$ copies ng^{-1} DNA and are in agreement with previous reports (López-Gutiérrez et al., 2004; Kandeler et al., 2006). A significant decrease with soil depth was also observed for the 16S rRNA gene copy numbers (Table 5.4). However, comparison of SIR and 16S rRNA gene depth profiles revealed a stronger depth effect for the former than for soil microorganism density, suggesting that soil microorganisms were less active in deeper soil layers. Alternatively, this could be due to a higher fungal biomass in Oe because SIR covers the active fraction of both bacterial and fungal biomass while only bacteria are targeted using the 16S rRNA gene.

Multiple regression analyses were used to test whether the results of SIR and 16S rRNA genes depended on specific chemical and physical soil properties (Table 5.5b). Whereas the active fraction of the soil microbial community (SIR) was significantly related to OC, there was no relationship between 16S rRNA gene copy numbers and substrate pools (NT, OC and N_{min}), pH or water content. This suggests that other factors such as temperature, redox potential or distribution of roots might be important.

Activity and size of the nitrate-reducing community within the soil profile

Quantification of potential nitrate reductase activity as well as genes encoding enzymes involved in nitrate reduction was used to assess the response of this specific functional community involved in nitrogen cycling to reduced N deposition. Nitrate reductase in the Solling spruce forest did not exceed 1.5 µg NO_2^--N g^{-1} day^{-1}. These values were about two to 20 times lower than the activities of alpine grasslands (Deiglmayr et al., 2004) and even more than one thousand times lower than the activities of agricultural soils (Philippot et al., 2006).

Lower N deposition significantly affected nitrate reductase activity in October. Thus, lower rates were observed in the clean rain vs. control plot at all depths, but this difference was only significant for the Oa layer (Fig. 5.2). Because nitrate reductase is an enzyme that is generally

induced by nitrate and oxygen limitation, we expected that nitrate in the soil solution as well as moisture content would be important regulating factors. Accordingly, multiple regression analysis revealed that soil water content was related to nitrate reductase activity (Table 5.5b). However, the soil water content can only explain differences in nitrate reductase activity within the soil profile and not between the two treatments. Reduced N deposition induced a trend towards a lower nitrate concentration that was not significant between the treatments. Therefore, reduced nitrate reductase activity in the clean rain treatment cannot be attributed solely to differences in the nitrate contents of the soil solution, but also to other environmental factors.

The reduction of nitrate to nitrite is catalysed by two different types: a membrane-bound reductase (Nar) encoded by the *narG* gene and a periplasmic nitrate reductase (Nap) encoded by the *napA* gene. Nitrate reducers can carry either one or both nitrate reductase enzymes (Philippot & Hojberg, 1999). Whereas Nar has been purified from a large variety of microorganisms including Archaea, the periplasmic enzyme Nap is present only in Gram-negative bacteria. The *narG* and *napA* gene copy numbers estimated by qPCR were similar to those described for other ecosystems (López Gutiérrez et al., 2004; Kandeler et al., 2006; Bru et al., 2007). Reduced N deposition did not modify the size of the nitrate reducer community, whatever the gene targeted (Figs 5.4 and 5.5). Therefore, the densities of both nitrate reducers and total bacteria seemed to be buffered against environmental changes resulting from the lower N deposition. This stability of the soil microbial community is also evident in the percentage of nitrate reducers to total bacteria, which was affected neither by N deposition nor by soil depth (data not shown). An alternative explanation is that the between-treatment variability in the chemical compositions of the soil solution was too high to yield differences in the relative abundance or size of the nitrate reducer community. Surprisingly, important regulating factors for the nitrate reducer community – such as organic and inorganic N pools, pH and soil water content – did not correlate with the density of nitrate reducers (Table 5.5b). On the other hand, univariate ANOVA clearly showed that the densities of nitrate reducers were controlled by spatial and seasonal variation. Our plot-scale study did not account for small-scale heterogeneity of nitrate reducers and its physico-chemical controls. Further studies should clarify whether the size of the nitrate reducer community is driven by the micro-topography of the acid forest soil as described by Mohn et al. (2000) and Hafner & Groffman (2005) for the spatial variation of denitrifying activity and N transformation, respectively.

Quantification of the genes encoding the nitrate reductase and of the nitrate reductase activity in relation to N deposition, soil depth and season allows us to study the possible linkage between the abundance and the activity of the nitrate reducer community in the acid forest soil. We hypothesized that the potential nitrate reductase activity is controlled by the abundance of nitrate reducers under suboptimal environmental conditions. Indeed, Pearson correlation analysis indicated that the density of the *narG*-carrying community – the more abundant group of nitrate reducers in this forest soil – is positively correlated to nitrate reductase activity (r^2 = 0.487, P < 0.001). Linkages between the size and the activity of functional communities involved in the N cycle have been reported previously for nitrifiers or denitrifiers (Patra et al., 2005).

Size of the denitrifier community within the soil profile

Quantification of *nirK*, *nirS* and *nosZ* genes should yield information on the effect of N deposition on the density of denitrifiers capable of reducing the nitrite produced by nitrate reducers into gaseous nitrogen in the acid forest soil. Nitrite is reduced to nitric oxide by microorganisms having either a Cu-containing nitrite reductase enzyme encoded by the *nirK* gene or a cd1 nitrite reductase encoded by the *nirS* gene (Zumft, 1997). Nitrous oxide is reduced to N_2 by the nitrous oxide reductase encoded by *nosZ*. We were unable to detect any *nirS* gene encoding the cytochrome cd1 nitrite reductase, due to the lower sensitivity of the *nirS* assay compared with the assays of other denitrification genes. Lower N deposition did not affect the copy numbers of *nirK* and only weakly impacted the copy numbers of *nosZ* (Fig. 5.5, Table 5.4). This is probably due to the relatively constant conditions of soil solution chemistry since more than a decade (e.g. almost no detectable nitrate in the above-ground soil solution since 1995; see Lamersdorf & Borken, 2004). Similarly, no significant intertreatment differences were recorded in the relative abundance of denitrifiers to total bacteria. Mergel et al. (2001b) reported that nitrogen fertilization in an acid forest soil also had no impact on the relative abundance of denitrifiers.

The densities of *nirK* and *nosZ* genes were differently influenced by soil depth: whereas copy numbers of the *nirK* gene decreased within the soil profile, values of *nosZ* in the Bw layer were higher than those in the organic layers (Fig. 5.5). Applying the most probable number (MPN) method and colony hybridization using denitrification genes as probes, Mergel et al. (2001a) also showed a depth effect on denitrifier density with decreasing bacterial and denitrifier numbers with soil depth. Although the amount of organic substances is the most

important factor determining the size of the denitrifier community in other ecosystems (Tiedje, 1988; Kandeler et al., 2006), we detected no correlation between OC and the number of *nirK* and *nosZ* functional genes (Table 5.5b). The higher *nosZ*/16S rRNA gene and *nosZ*/*nirK* ratios in the Ah and Bw horizons suggest that the proportion of denitrifiers capable of reducing the greenhouse gas N_2O into N_2 is higher in the mineral soil layer than that in the organic layer.

In conclusion, our study showed that reducing the N deposition had a smaller effect on the abundance and function of soil microorganisms, nitrate reducers and denitrifiers than soil depth and sampling date. The negligible effects of lower N deposition on nitrate reducers and denitrifiers in this acid forest ecosystem are likely due to the low level of nitrogen oxides respiration and the dominance of microbial NH_4^+ turnover in the internal N cycling. Variations of the *nosZ*/16S rRNA gene and *nosZ*/*nirK* ratios in the different soil horizons suggest that the relative abundance of microorganisms capable of performing complete denitrification is unequally distributed within the acid soil profile. This study provides first evidence that denitrifiers that can reduce nitrous oxide might be enriched in deeper soil layers. Studies on expression of denitrification genes and on N_2O fluxes in relation to soil depth are necessary to confirm whether the risk of N_2O release from mineral soil layers can be neglected.

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6 Microbial biomass and enzyme activities under reduced nitrogen deposition in a spruce forest soil

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Abstract

High N availability resulting from anthropogenic emissions can alter the activities of enzymes involved in the breakdown of organic compounds in ecosystems. In a spruce forest stand in Solling, central Germany, the nitrogen contained in throughfall water was adjusted to pre-industrial concentrations and resprinkled as ‘‘clean rain’’ onto the forest floor. Soil was sampled from the clean rain and control plot at three different dates and four horizons. We investigated the response of active microbial biomass (SIR) and fungal biomass (ergosterol content) and 15 enzymes to the reduction of N deposition. Some N-cycling enzymes (urease, arginine deaminase, alanyl aminopeptidase, lysyl-alanyl aminopeptidase) showed increased activities whereas others (N-acetyl-glucosaminidase, protease, leucyl aminopeptidase, alanyl-alanyl-phenyl aminopeptidase) decreased under reduced N treatment indicating a modification of the reaction rates of different enzymes involved in N cycling. For the C-and P-cycling enzymes, α-and β-glucosidase as well as phosphatase activities increased in the clean rain treatment in spring 2006 in Oe and Oa horizons, respectively, but did not affect the pattern of substrate decomposition. Spatial variability of microbial biomass and enzyme activities within the soil profile indicated that the decrease in microbial activity with depth was driven by resource allocation.

Introduction

In recent decades nitrogen emissions into the atmosphere have promoted increased mobilization and deposition of reactive forms of N into ecosystems (Galloway et al., 2003) resulting in increased acidification and N saturation of soils (Aber et al., 1989; Matson et al., 2000; Puhe and Ulrich, 2001). In this way natural ecosystems have received atmospheric N inputs in an order greater than those presumed for pre-industrial conditions (Carreiro et al., 2000). Since nitrogen is generally a limiting nutrient for plant growth, Spieker et al. (1996) suggested that high nitrogen depositions have substantially increased tree growth in European forests. However, forest ecosystems have been severely affected by high N inputs. For example, increased N deposition can have dramatic impacts on ecosystem processes and microbial communities. N deposition increases N leaching and trace gas losses, alters soil carbon (C) stocks, and changes plant community composition (Mack et al., 2004; Waldrop et al., 2004a, b; Suding et al., 2005). When N availability exceeds biological demand in forested ecosystems, N saturation may occur, leading to changes in soil pH, forest decline, and massive N losses (Allison et al., 2007).

Tree needle litter is the main raw material for humus formation in the organic horizons of spruce forest ecosystems and may represent a major source of nutrients and substrates for microbial decomposition. Decomposition processes that control nutrient (carbon and nitrogen) fluxes in soil are liable to changes due to anthropogenic nitrogen enrichment of ecosystems (Sinsabaugh et al., 2005) and enzymes play a pivotal role in the catalysis of these soil nutrient transformations (Burns and Dick, 2002). High N availability has been observed to alter the activities of enzymes involved in the breakdown of organic compounds. Studies on the interrelationship between organic carbon and nitrogen in soils utilizing manipulation experiments showed that in nitrogen saturated soils, a decreased decomposition of recalcitrant organic matter occurs due to inhibitory effects of high nitrogen levels on extracellular enzyme activities (Sinsabaugh et al., 2002). Additionally, Sinsabaugh et al. (2002) reported that high nitrogen concentrations reduced the activity of microorganisms involved in the degradation of nitrogenous organic matter. Measurement of soil enzyme activities has, therefore, been recommended as a suitable method for measuring changes in soil quality (Dick, 1992), soil recovery from disturbance or stress (Decker et al., 1999), and as the most appropriate indicator of microbial function (Cadwell, 2005).

Most of the studies about N deposition have concentrated on the effects of high nitrogen

loads and the response of forest ecosystems to nitrogen saturation, but little is known on the response of microbial function and enzyme activities to the reduction of N deposition on forest soils. However, at Solling, Central Germany, as one of the former EXMAN NITRIX networks of European ecosystem manipulation studies long-term reduction of atmospheric N input is still applied. Here throughfall is permanently collected by a roof construction below the canopy, partially deionised and resprinkled on the experimental plots since 1991. Substantial information from previous studies carried out in the Solling roof project is available on soil water chemistry (Bredemeier et al., 1995a,b), hydrochemical input–output budgets (Xu et al., 1998), root growth and aboveground-stand response (Bredemeier et al., 1998), soil microbial parameters (microbial biomass and respiration) (Raubuch et al., 1999), gross microbial N cycling rates (Corre and Lamersdorf, 2004), and fine root growth and soil respiration (Lamersdorf and Borken, 2004).

After 16 years of manipulations to reduce N deposition to preindustrial levels, the goal of this study was to investigate the effects of reduced N deposition on several enzyme activities mediating soil organic matter turnover of cellulose, hemicellulose, organic phosphorus compounds, proteins and chitin in the Solling spruce forest soil profile. In addition, we focused on decay of different peptides, an important fraction of dissolved organic N compounds. We hypothesize that (1) the reduction of N deposition might decrease the activity of enzymes involved in the decay of easily decomposable compounds within the soil profile and enhance the activity of enzymes involved in the decomposition of more recalcitrant compounds, (2) the enzymes involved in N cycling would show stronger response to N manipulation than enzymes involved in C cycling and (3) the reduction of N deposition would have a more pronounced effect in the upper soil layer than in lower soil horizons.

Material and methods

Study site and soil sampling

The study site was a 75-year-old Norway spruce (Picea abies) plantation at the Solling plateau in Central Germany (51°31'N, 9°34'E, elevation ≈500 m above sea level). The soil is classified as strongly acidic Dystric Cambisol (FAO, 1987) with a base saturation of about 5–10% in the whole profile down to 1 m depth (Raubuch et al., 1999). Considerable amount of nutrients and organic matter are accumulated in the humus layer (moder humus form). The bedrock material is sandstone, overlaid by a loess layer. More detailed information on chemical and physical soil properties of the individual plots and soil profiles are given by

Bredemeier et al. (1995a, b), Raubuch et al. (1999), Theuerl et al. (2009) and Kandeler et al. (2009). The climate is dominated by Atlantic streams with evenly distributed precipitation (MAP = 1090 mm) and moderate variation in temperature (MAT = 6.4^{o}C) throughout the year (Lamersdorf and Borken, 2004).

The Solling roof project was established in 1989 with translucent roofs (each 300 m^2), constructed below canopy in 1991. Two roofed plots were used in this study. One plot acts as the so called ‘‘clean rain’’ plot, where throughfall water is permanently collected, partly deionised to adjust to pre-industrial ion concentrations, and resprinkled immediately to the plot. The second plot is used as the roof control plot, i.e. throughfall water is also collected but immediately resprinkled without any prior chemical treatment. Compared to the control plot the clean rain plot receives about 65% less N (2.1 kg NH_4^+-N ha^{-1} $year^{-1}$; 7.6 kg NO_3^--N ha^{-1} $year^{-1}$; 1.8 kg DON ha^{-1} $year^{-1}$) in the throughfall water (Lamersdorf and Borken, 2004). The content of soil inorganic N species (NH_4^+, NO_3^-) was not significantly affected by treatment within the soil profile. Reduction of N deposition did not change organic carbon (OC) and total nitrogen (NT) contents of soil profiles. OC and NT in the different horizons (Oe–Bw) were 43.5, 33.9, 4.6, 1.7 g 100 g^{-1} soil and 1.7, 1.4, 0.2, 0.09 g 100 g^{-1} soil, respectively. These values were similar for both the clean rain and the control plots and at both sampling dates in 2006. Although the roof construction removed nitrogen and proton input into the ecosystem, the pH ($CaCl_2$) values in the clean rain treatment were still low, ranging from 2.6 in the Oa horizon to 4.1 in the Bw horizon and were not different from the control.

Soil samples were collected in April and October 2006 and April 2007 by driving a 10 cm diameter core borer into the ground from the soil surface to 40 cm soil depth. Because of the lack of roof replications, the plots were separated into four subplots. Four soil cores were sampled per subplot and the different horizons (Oe, Oa, Ah and Bw) were separated in order to investigate the response to reduced N deposition in both the upper and lower soil layers since nutrient fluxes are dynamic. After sieving with a 2 mm sieve, the soil samples were homogenised and stored at -20^{o}C until analysis.

Microbial biomass

Microbial biomass was measured by the substrate-induced respiration (SIR) method (in duplicate). Substrate saturation and maximum initial respiratory response were obtained with glucose amendment (10 mg glucose g^{-1} soil). CO_2 evolved was trapped in 50 ml 0.1 M NaOH

during 4 h incubation at 25°C and measured by titration with 0.1 M HCl (Anderson and Domsch, 1978).

Fungal biomass

Soil fungal biomass was determined by extraction and quantification of ergosterol content using the method of Djajakirana et al. (1996) with slight modifications. One gram of soil was suspended in 50 ml ethanol (HPLC-grade) in 100 ml wide-mouth brown bottles and extracted in a shaker for 30 min at 250 rev. min^{-1} followed by centrifugation in 50 ml tube at 4560 rev. min^{-1} for 30 min. An aliquot of 20 ml was transferred into a test tube and evaporated in a rotary evaporator at 50°C under vacuum. The dry extract was then dissolved in 2 ml methanol and percolated through a syringe filter (cellulose– acetate, 0.45 mm pore size) into brown glass HPLC-vial. Extracts were measured by injection of 20 ml into a HPLC autosampler (Beckmann Coulter, System Gold 125 Solvent Module). Extracts were passed through a column (250 mm x 4.6 mm, 5 mm diameter = solid phase, Spherisorb octadecyl silan, ODS II). Pure methanol was used as mobile phase at a flow rate of 1 ml min^{-1}. The column was conditioned with methanol eluent at a flow rate of 1 ml for 30 min before measurement started. The detection was carried out with an UV-detector (Beckmann Coulter, System Gold 166) at a wavelength of 282 nm. Identification of ergosterol was performed by retention time and quantification by peak area.

Enzymatic activity using fluorogenic substrates

The activities of the following 10 enzymes involved in pathways of N-, C-, and P-cycling were determined using fluorogenic substrates according to Marx et al. (2001); leucyl aminopeptidase, alanyl aminopeptidase, lysyl-alanyl aminopeptidase, alanyl-alanyl-phenyl aminopeptidase, α-glucosidase, β-glucosidase, Nacetyl-glucosaminidase, β-xylosidase, cellobiohydrolase and phosphatase. All substrates, standards and buffers were obtained from Sigma–Aldrich (St. Louis, USA). The substrates contained the fluorescent compounds 4-methylumbelliferone (4-MUF) and 7-amido-4-methylcoumarin (7-AMC). Buffers and substrates were prepared according to Poll et al. (2006).

Soil suspensions were prepared by adding 0.5 g fresh soil into 50 ml of autoclaved water and dispersed by ultrasonication for 2 min with 50 J s^{-1} sonication energy. The suspensions were continuously stirred using a magnetic stir plate while 50 ml aliquots (with four analytical replicates per sample per assay) were dispensed into 96-well microplate (PPF black 96 well; Greiner Bioone GmbH, Frickenhausen, Germany), followed by 50 ml of the appropriate

autoclaved buffer (MES or TRIZMA buffer) and 100 ml of substrate solution. Standard wells received 50 ml of soil suspension, standard solution (MUF or AMC) and appropriate amount of buffer. Controls were buffer and MUF-or AMC-substrates. All microplate wells had a final volume of 200 ml as reaction medium. The plates were incubated at 30^{o}C. Fluorescence was measured at 360/460 nm wavelength in a microplate fluorescence reader (Bio-Tek Instruments Inc., FLX 800, Germany) after 0, 30, 60, 120 and 180 min. The enzyme activity corresponded to an increase in fluorescence and was calculated in nmol g^{-1} soil h^{-1}.

Enzymatic activity by standard colorimetric method

The following enzymes involved in C and N cycling were measured by colorimetric methods: invertase, xylanase, protease, urease and arginine deaminase according to Kandeler (1996) with some modifications. All measurements were done with four analytical replicates.

For the determination of invertase activity, 0.3 g of fresh soil was incubated with 5 ml of 5 mM sucrose substrate solution and 5 ml of 2 M acetate buffer (pH 5.5) in 50 ml test tubes while shaking for 3 h at 50^{o}C. The controls received acetate buffer only. At the end of incubation reducing sugars released during incubation were measured photometrically (UV-1601, Shimadzu) at 690 nm by the ferric-ferrocyanide reaction (Schinner et al., 1996).

Due to insufficient amount of sample material, xylanase activity was not determined for the October 2006 sampling date. Xylanase activity was determined by incubating 0.3 g of soil with5ml substrate solution (1.2% (w/v) xylan from oat speltss suspended in 2 M acetate buffer, pH 5.5) and 5 ml of 2 M acetate buffer (pH 5.5) for 24 h at 50^{o}C. The reducing sugars released during the incubation period were determined by the ferric-ferrocyanide reaction (Schinner et al., 1996).

Protease activity was measured as follows: 0.3 g of fresh soil was incubated with 5 ml of 2% sodium caseinate as substrate and 0.05 M Tris buffer (pH 8.1) at 50^{o}C for 2 h. The aromatic amino acids released were extracted and the remaining substrate precipitated with 0.92 M tricholoroacetic acid (TCA). The amino acids reacted with 5 ml of Folin-Ciocalteu's phenol reagent and were measured colorimetrically. Protease activity was expressed as tyrosine equivalents per gram dry soil per 2 h.

Urease activity was determined by incubating 0.3 g of fresh soil with 1.5 ml of 0.08 M substrate (urea) solution at 37^{o}C for 2 h. Released ammonium was extracted with 12 ml 1 M potassium chloride/0.01 M hydrochloric solution and determined by a modified Berthelot

reaction. Urease activity was expressed as N equivalents per gram dry soil per 2 h.

The determination of arginine deaminase activity was performed with 5 g of fresh soil, 2 ml of aqueous arginine–substrate solution and incubated at 37^{o}C for 3 h. Two molar KCl solution was added after incubation to extract the ammonium released and was determined colorimetrically by the indophenol reaction.

Statistical analyses

After data were separated into the organic and mineral horizons, independent *t*-tests were used to test the effect of reduced N deposition at different sampling dates on microbial parameters (SIR and ergosterol content) and soil enzyme activities. Three-way univariate analysis of variance (ANOVA) was applied to test for differences of the means of substrate-induced respiration, ergosterol content and enzyme activities among treatments, depths and sampling dates. Levene test was used to test for homogeneity of variances. Where necessary, data sets were log (SIR, urease, alanyl aminopeptidase, leucyl aminopeptidase, lysylalanyl aminopeptidase, β-xylosidase, phosphatase) or sin (xylanase, cellobiohydrolase) transformed. The data for some of the enzymes (even after application of several transformations) could not be normalized to give a satisfactory homogeneity test. Multiple regression analysis was applied to evaluate the relationship between microbial biomass, enzyme activity and nutrient pools and between microbial biomass and enzyme activities. Significant differences were accepted at the $P < 0.05$ level of probability.

Results

Microbial and fungal biomass

SIR was used to determine the active component of soil microbial community while the content of ergosterol in soil was used as an estimate of fungal biomass. There was a significant increase of both SIR and ergosterol content in the reduced N treatment in spring 2006 (Table 6.1); however, this treatment effect was restricted to the Oa horizon. Sampling time and treatment significantly ($P = 0.035$) interacted to have an influence on SIR (Table 6.2). SIR and ergosterol content declined substantially with depth, from Oe to Bw horizons (Fig. 6.1).

Table 6.1: *t*-Test results testing the effect of reduced N deposition on microbial biomass (SIR), ergosterol (Ergl) content, and enzyme activities at different soil depths and sampling dates. Significance (in bold) is considered at $P < 0.05$.

Soil depth	Sampling date	SIR	Ergl	Urease	Argn	Prot	N-ac	Ala	Leu	LA-aP	Ala-Ala	α-Glu	β-Glu	β-Xyl	CBH	Invt	Xylan	Phos
Oe	April 06	0.588	0.052	0.143	0.597	0.596	**0.043**	0.741	0.334	0.671	0.334	0.110	**0.048**	0.162	0.640	0.826	0.470	0.212
	Oct. 06	0.565	0.681	0.180	0.493	0.882	0.538	0.676	0.366	0.914	0.897	0.560	0.512	0.950	0.235	0.119	ND	0.270
	April 07	0.371	0.529	0.180	**0.016**	0.399	0.890	0.985	0.524	0.607	0.540	0.711	0.712	0.630	0.922	0.918	0.278	0.114
Oa	April 06	**0.013**	**0.014**	**0.018**	**0.015**	0.346	0.082	0.251	0.733	0.356	0.193	**0.036**	**0.010**	0.184	0.485	0.259	0.601	**0.027**
	Oct. 06	0.698	0.325	0.600	0.159	0.728	0.913	0.585	0.682	0.881	0.820	0.396	0.527	0.860	0.753	0.869	ND	0.857
	April 07	0.068	0.175	**0.035**	0.530	0.077	0.406	0.204	0.906	0.879	0.660	0.933	0.967	0.482	0.971	0.577	0.424	0.809
Ah	April 06	0.154	0.215	0.071	0.491	0.592	0.244	0.167	0.781	0.083	0.419	0.727	0.237	0.171	0.463	0.216	0.615	0.759
	Oct. 06	0.144	0.241	**0.017**	0.389	0.829	0.220	0.701	0.744	0.550	0.255	0.664	0.136	0.081	0.353	0.291	ND	0.251
	April 07	0.918	0.784	0.083	0.974	0.113	0.720	0.758	0.514	0.710	0.968	0.825	0.300	0.293	0.153	0.316	0.464	0.959
Bw	April 06	0.606	0.252	0.366	0.918	0.840	0.542	0.482	0.356	0.066	0.239	0.336	0.648	0.083	0.086	0.681	0.128	0.542
	Oct. 06	0.577	0.221	**0.027**	0.250	0.544	0.167	0.668	0.442	**0.043**	0.197	0.067	0.081	0.111	0.617	0.733	ND	0.192
	April 07	0.499	0.222	0.224	0.875	**0.010**	0.650	0.926	0.852	0.735	0.111	**0.020**	0.877	0.487	0.537	0.310	0.475	0.479

Argn, arginine deaminase; Prot, protease; N-ac, N-acetyl-glucosaminidase; Ala, alanyl aminopeptidase; Leu, leucyl aminopeptidase; LA-aP, lysyl-alanyl aminopeptidase; Ala-Ala, alanyl-alanyl-phenyl aminopeptidase; α-Glu, α-glucosidase; β-Glu, β-glucosidase; β-Xyl, β-xylosidase; CBH, cellobiohydrolase; Invt, invertase; Xylan, xylanase; Phos, phosphatase.

Table 6.2: Three-way ANOVA results testing the response of microbial biomass (SIR) and some enzyme activities investigated to reduced N deposition, soil depth and sampling date. Significance (in bold) is considered at $P < 0.05$.

	Univariate ANOVA								
	[a]SIR	[a]Urease	[a]Ala	[a]Leu	[a]LA-aP	[a]β -Xyl	[b]CBH	[b]Xylan	[a]Phos
Nitrogen	0.354	**0.000**	0.062	0.831	**0.022**	0.260	0.115	0.353	**0.268**
Depth	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.020**	**0.000**	**0.000**
Date	**0.005**	**0.008**	**0.000**	**0.000**	**0.000**	0.067	0.694	0.072	**0.000**
Nitrogen × Depth	0.850	0.747	0.993	0.865	0.276	0.500	0.298	0.066	**0.664**
Nitrogen × Date	**0.034**	0.271	0.782	0.623	0.137	0.985	0.756	0.082	**0.681**
Depth × Date	**0.000**	**0.000**	**0.000**	**0.000**	**0.027**	**0.011**	0.451	**0.010**	**0.012**
Nitrogen × Depth × Date	0.973	0.802	0.836	0.803	0.943	0.349	0.148	0.334	0.121

Ala, alanyl aminopeptidase; Leu, leucyl aminopeptidase; LA-aP, Lysyl-alanyl aminopeptidase; β-Xyl, β-xylosidase; CBH, cellobiohydrolase; Xylan, Xylanase; Phos, phosphatase. [a] Data log transformed. [b] Data sin transformed.

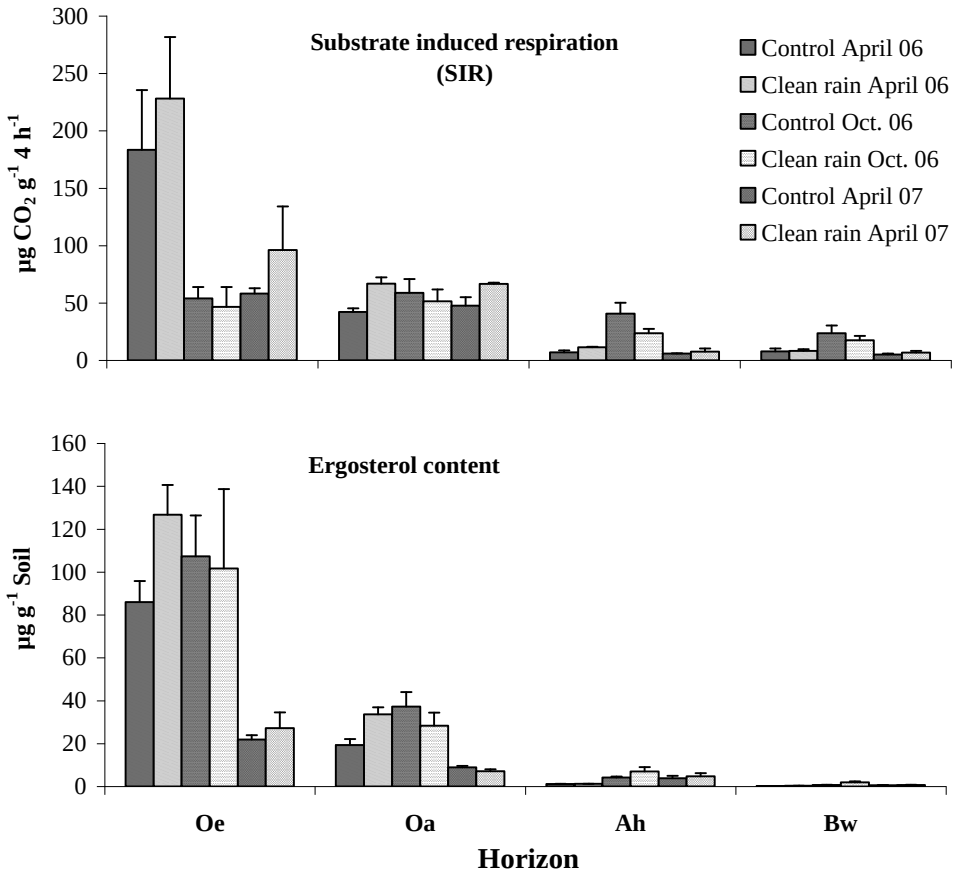

Fig. 6.1: Response of substrate-induced respiration and ergosterol content to reduced N deposition at four horizons. Error bars indicate standard error (SE).

Enzyme activities involved in N cycling

Key processes in N cycling responded differently to the reduction of N deposition. In general, some enzymes involved in N cycling (urease, arginine deaminase and lysyl-alanyl-amino-peptidase) showed a tendency towards higher activity under the clean rain treatment, whereas others showed little or no treatment effects (Figs. 6.2 and 6.3).

Reduction of N deposition increased urease activity in the soil profile (Fig. 6.2). However, increase in urease activity were significant only in the Oa horizon (P = 0.018 and 0.035) at the spring sampling dates of 2006 and 2007, respectively, and in the Ah (P = 0.017) and Bw (P = 0.027) horizons at the autumn 2006 sampling date (Table 6.1). There were significant treatments, sampling date and depth effects, shown by three-way ANOVA (Table 6.2). Arginine deaminase also showed a tendency towards higher activity at the reduced N plot compared to the control at all horizons and sampling dates (Fig. 6.2). Treatment effect was significant only for Oe horizon (Table 6.1).

In contrast, protease and N-acetyl-aminoglucosaminidase activities decreased in the reduced N treatment plots. Protease activity decreased in the first two sampling dates and increased at the last sampling date in the Oe, Oa and Bw horizons (Fig. 6.2). Distinct (P = 0.010) reduced N treatment effect was detected in the Bw horizon in April 2007 (Table 6.1). N-acetyl-aminoglucosaminidase activity was lower in the reduced N plot than the control at all sampling dates in the organic layers, except for spring 2006 in the Oa horizon (Fig. 6.2). There was a significant (P = 0.043) treatment effect observed in Oe in spring 2006 (Table 6.1). The activity in the deeper layers was more or less constant irrespective of treatment or sampling date (Fig. 6.2).

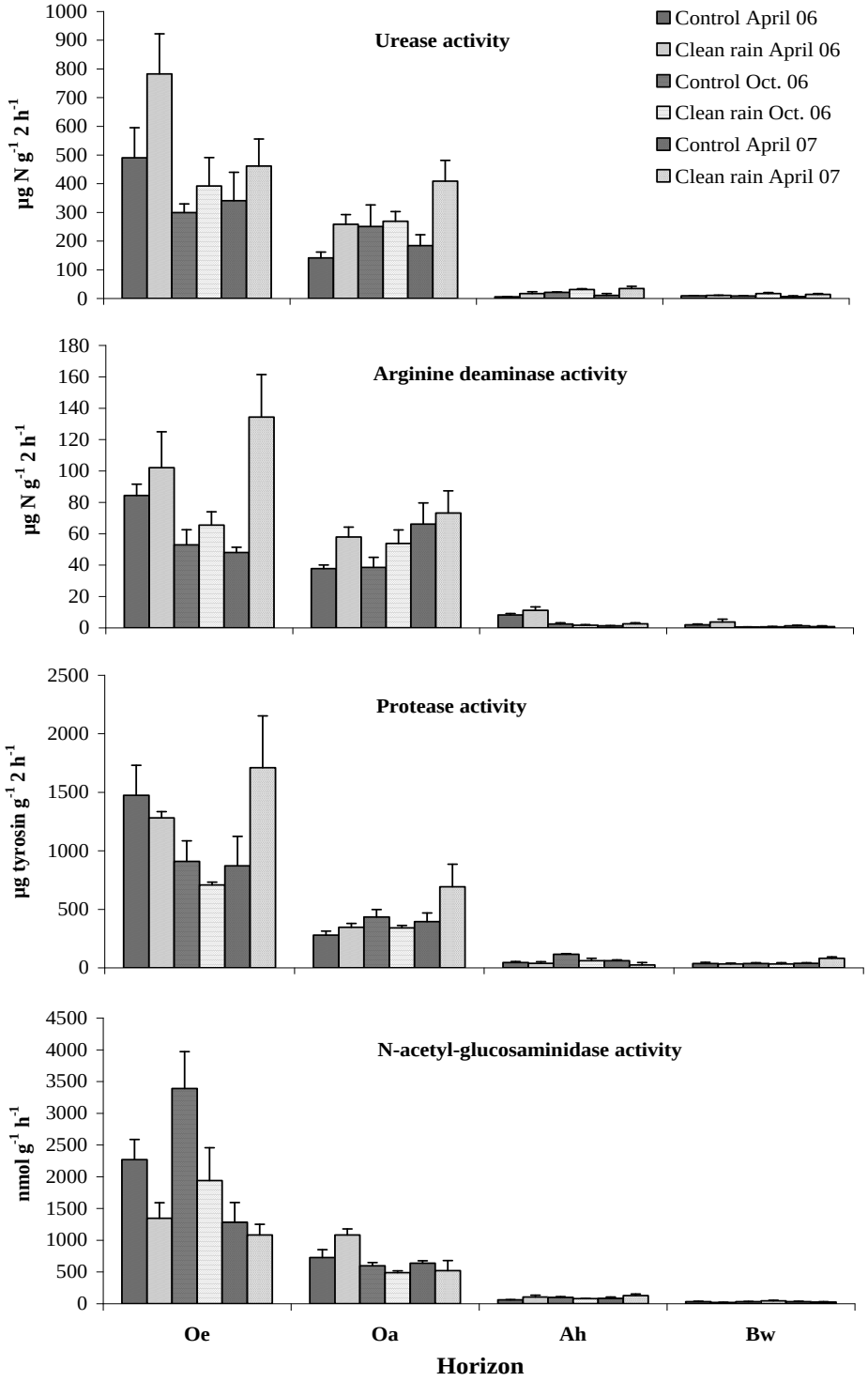

Fig. 6.2: Response of urease, arginine deaminase, protease and N-acetyl-glucosaminidase activities to reduced N deposition at four horizons. Error bars indicate standard error (SE).

In general, alanyl aminopeptidase and lysyl-alanyl aminopeptidase showed a similar pattern to urease activity, whereas leucyl aminopeptidase and alanyl-alanyl-phenyl aminopeptidase showed a similar pattern to protease activity (Fig. 6.3). We found one exception for the Bw horizon where lysyl-alanyl aminopeptidase showed a significantly higher activity (P = 0.043) in autumn 2006 (Table 6.1).

In all treatments, enzymes involved in N cycling showed higher activities in the organic (Oe and Oa) than in the mineral layers (Ah and Bw) (Figs. 6.2 and 6.3).

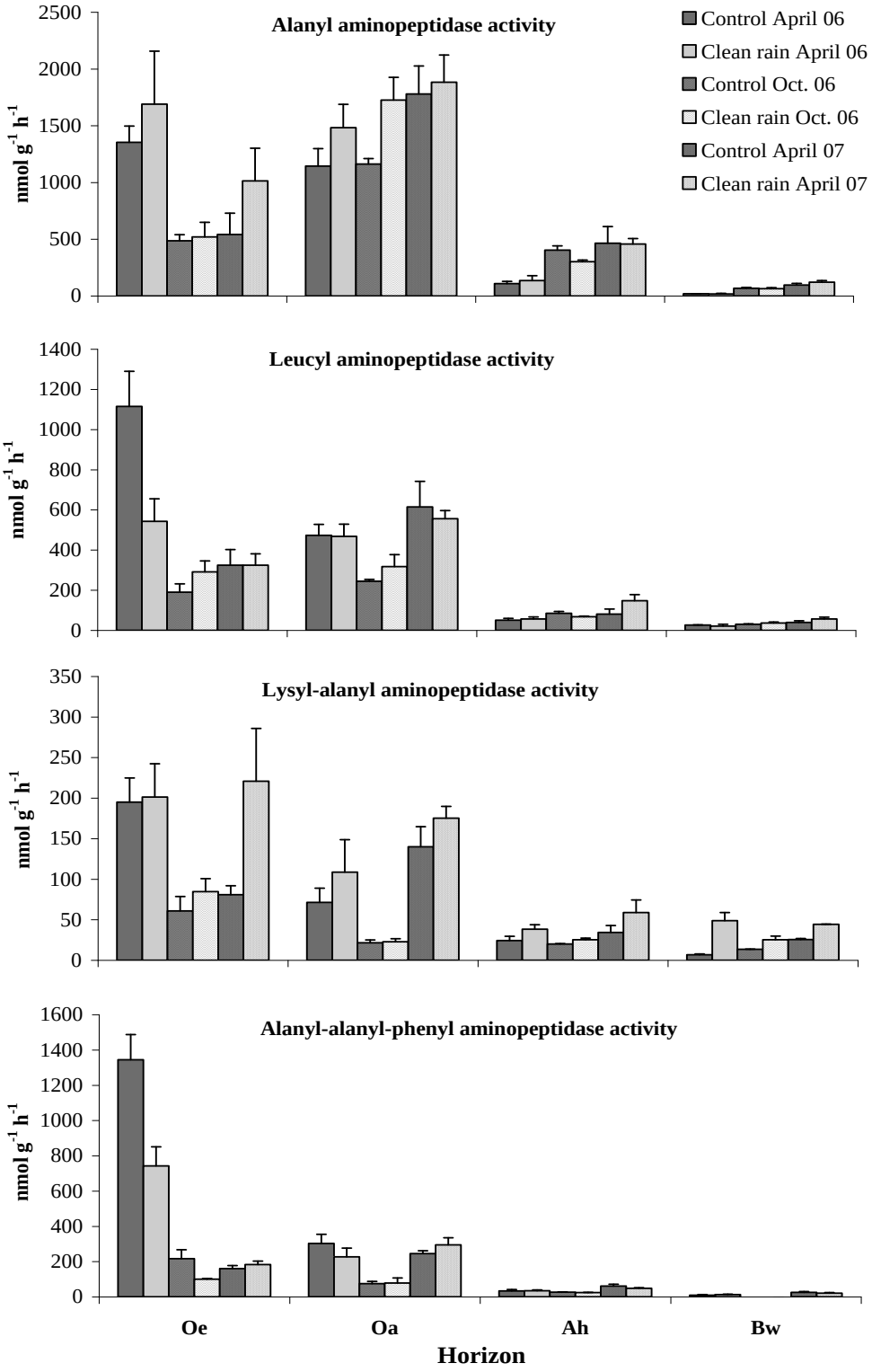

Fig. 6.3: Response of alanyl aminopeptidase, leucyl aminopeptidase, lysyl-alanyl aminopeptidase and alanyl-alanyl-phenyl aminopeptidase activities to reduced N deposition at four horizons. Error bars indicate standard error (SE).

Enzymes activities involved in C cycling

Univariate statistical analyses revealed that reduction of N deposition did not affect different key processes in C cycling (β-xylosidase, cellobiohydrolase and xylanase) (Table 6.2). Nevertheless, pair-wise comparison of each horizon at each sampling time showed some exceptions for α-glucosidase and β-glucosidase where activities increased significantly in the reduced N treatment (Table 6.1 and Fig. 6.4). In all treatments, enzymes involved in C cycling showed higher activities in the organic (Oe and Oa) than in the mineral layers (Ah and Bw) (Figs. 6.4 and 6.5).

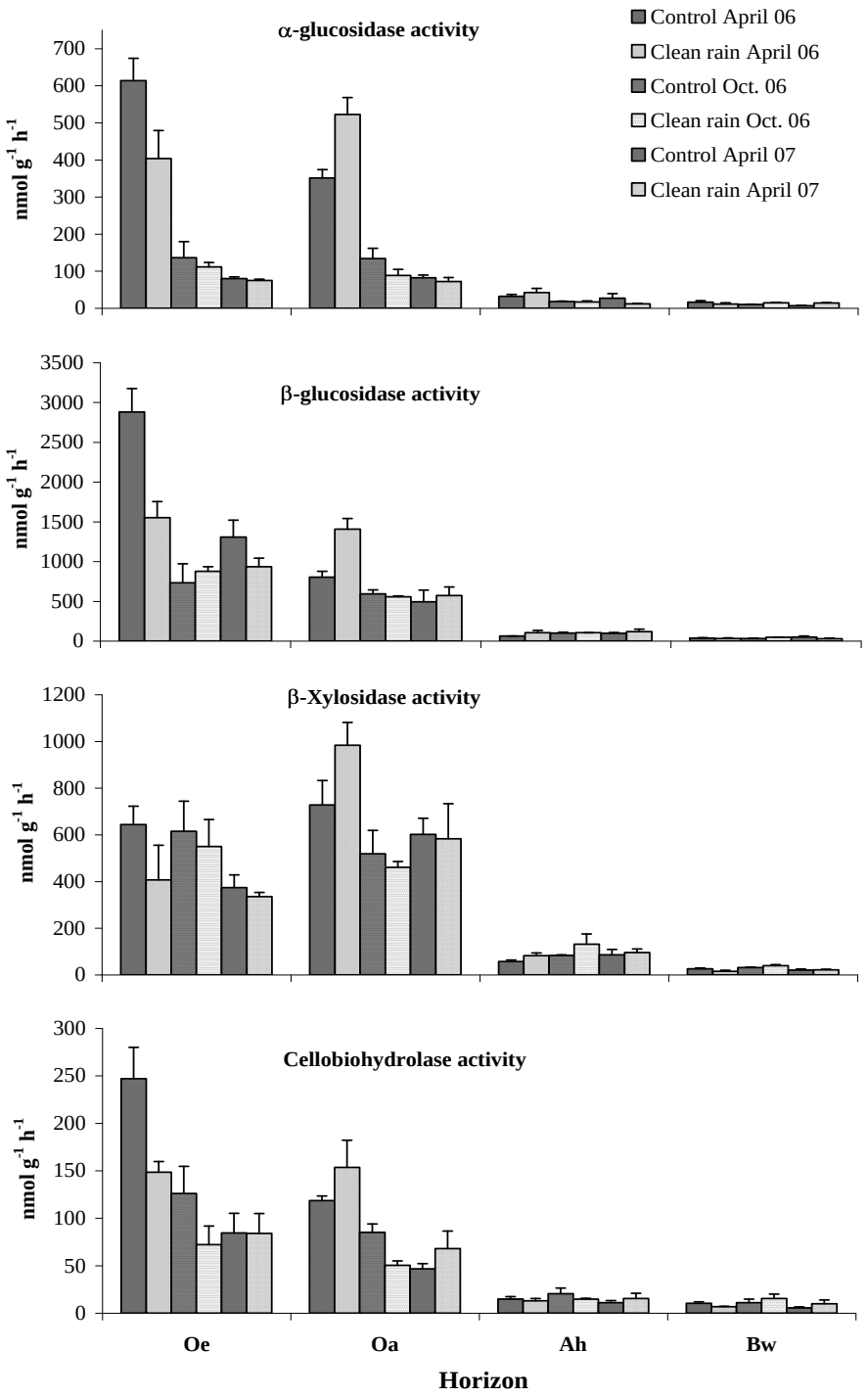

Fig. 6.4: Response of α-glucosidase, β-glucosidase, β-xylosidase and cellobiohydrolase activities to reduced N deposition at four horizons. Error bars indicate standard error (SE).

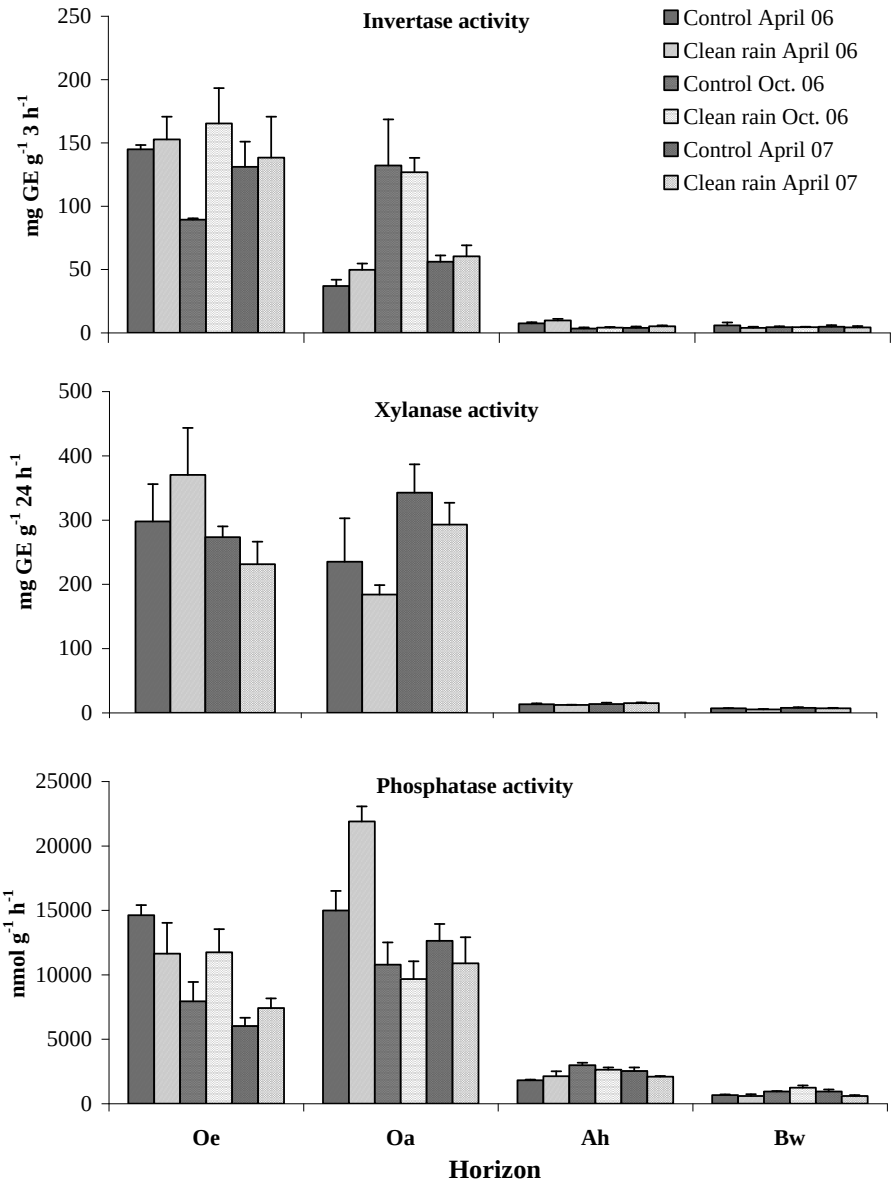

Fig. 6.5: Response of invertase, xylanase and phosphatase activities to reduced N deposition at four horizons. Error bars indicate standard error (SE).

Enzyme activity involved in P cycling

Depending on the sampling time, we detected either an increase or decrease of phosphatase activity of specific soil layers due to reduced N deposition (Fig. 6.5). For example, phosphatase activity showed a significant (P = 0.027) increase under reduced treatment at the Oa horizon in spring 2006 (Table 6.1), but showed a decrease, although not significant, in autumn 2006 and spring 2007. The three-way ANOVA analysis of the data showed only effects of depth and sampling date.

Relation between soil properties, microbial biomass and enzyme activity

Multiple regression analysis was used to examine relationships between soil microbial properties (SIR, ergosterol content and enzyme activities) and soil chemical properties (OC, NT, NH_4^+ and NO_3^-) (Table 6.3). OC, NT and NH_4^+ were closely related to the abundance and function of soil microorganisms in the forest site, whereas nitrate content showed significant *P*-values only for arginine deaminase and alanyl-alanyl-phenyl aminopeptidase. SIR, ergosterol content as well as enzymes involved in N cycling (urease, arginine and leucyl aminopeptidase) were positively correlated to OC and negatively to NT. Ammonium was closely linked to five out of seven enzymes involved in N cycling (protease, alanyl aminopeptidase, leucyl aminopeptidase, lysyl-alanyl aminopeptidase and alanyl-alanyl-phenyl aminopeptidase). We could not detect a general pattern for enzymes involved in C cycling. There was a positive linear relationship for three enzymes involved in N cycling (protease, urease and alanyl-alanyl-phenyl aminopeptidase) with SIR, while ergosterol content was positively correlated with α-glucosidase, alanyl aminopeptidase, alanyl-alanyl-phenyl aminopeptidase, invertase and negatively with β-xylosidase (Table 6.4).

Table 6.3: Multiple regression analysis of microbial biomass (SIR), ergosterol content and enzyme activities using soil OC, NT, NH_4^+ and NO_3^- as independent factors. Significant correlations with *P*-values equal ≤ 0.05 are indicated in bold.

Parameter	Standardized coefficient (β)																Model *P* value
	SIR	**Ergosterol content**	**Urease**	**Argn**	**Prot**	**N-ac**	**Ala**	**Leu**	**LA-aP**	**Ala-Ala**	**α-Glu**	**β-Glu**	**β-Xyl**	**CBH**	**Invt**	**Xylan**	**Phos**	
OC	**3.575**	**3.298**	**4.873**	**3.127**	1.512	-1.714	-1.325	**2.458**	1.354	0.731	**-3.600**	-1.104	**-4.475**	**-3.388**	**3.198**	1.022	**-4.688**	0.000
NT	**-2.731**	**-2.897**	**-4.939**	**-2.996**	0.235	**3.253**	-1.561	**-3.347**	-1.915	0.748	**3.928**	**2.138**	**2.956**	**3.610**	**-2.186**	**-2.532**	**3.348**	0.000
NH_4^+	0.649	**4.739**	1.765	1.614	**2.696**	1.954	**2.272**	**2.342**	**3.027**	**1.979**	**2.149**	**2.088**	-0.028	**4.730**	**-2.730**	**2.960**	-0.510	0.000
NO_3^-	-1.222	0.748	-0.838	**-2.147**	0.958	0.624	0.952	1.196	1.189	**2.648**	-0.810	0.817	0.208	0.987	0.748	0.089	-0.976	0.000

OC, organic carbon; NT, total nitrogen; Argn, arginine deaminase; Prot, protease; N-ac, N-acetyl-glucosaminidase; Ala, alanyl aminopeptidase; Leu, leucyl aminopeptidase; LA-aP, lysyl-alanyl aminopeptidase; Ala-Ala, alanyl-alanyl-phenyl aminopeptidase; α-Glu,α-glucosidase; β-Glu, β-glucosidase; β-Xyl, β-xylosidase; CBH, cellobiohydrolase; Invt, invertase; Xylan, xylanase; Phos, phosphatase.

Table 6.4: Multiple regression analysis of the activities of C-, N-, and P-enzymes using SIR and ergosterol content as independent factors. Significant correlations with *P*-values equal ≤ 0.05 are indicated in bold.

Parameter	Standardized coefficient (β)															Model *P* value
	Urease	**Argn**	**Prot**	**N-ac**	**Ala**	**Leu**	**LYs-Ala**	**Ala-Ala**	**Invt**	**Xylan**	**α-Glu**	**β-Glu**	**β-Xyl**	**CBH**	**Phos**	
Ergosterol	0.247	0.004	-0.143	-0.160	**0.370**	-0.073	-0.035	**0.307**	**0.335**	-0.302	**0.349**	0.318	**-0.571**	0.128	0.253	0.000
SIR	**0.576**	-0.011	**0.474**	-0.354	-0.213	-0.321	-0.064	**0.342**	-0.104	0.288	0.057	0.448	-0.568	-0.257	0.591	0.000

SIR, substrate-induced respiration; Argn, arginine deaminase; Prot, protease; N-a-ac, N-acetyl-glucosaminidase; Ala, alanyl aminopeptidase; Leu, leucyl aminopeptidase; LA-aP, lysyl-alanyl aminopeptidase; Ala-Ala, alanyl-alanyl-phenyl aminopeptidase; α-Glu, α-glucosidase; β-Glu, β-glucosidase; β-Xyl, β-xylosidase; CBH, cellobiohydrolase; Inv, invertase; Xylan; xylanase; Phos, phosphatise.

Discussion

The deterioration of the soil environment with respect to high N depositions and acid stress has impacted not only the above but also below ground ecosystem processes. In the Solling N deposition manipulation experiment running since 1989, the reduced N plot receives about 65% less nitrogen than the control plots (Lamersdorf and Borken, 2004). Nitrogen loading on forest soils and the reversal of N saturation would produce different microbial functional responses. This study gives an insight into the utility of microbial biomass and microbial enzyme activities in monitoring directly the functional responses of microbial communities to the reversal of N deposition in this forest ecosystem.

Microbial and fungal biomass

The SIR method utilizes the respiration response of soil microorganisms to substrate enrichments to provide an estimate of soil microbial biomass (Anderson and Domsch, 1978), while ergosterol content has been recommended as a good index for fungal biomass (Eash et al., 1996). Microbial biomass parameters (SIR and ergosterol content) in the organic layer (Oa, spring 2006) were significantly higher in the reduced N plot. The high microbial biomass was probably due to enhanced microbial activity since there was significant microbial NH_4^+ immobilization rate and a faster turnover rate of NH_4^+ and microbial N pools in the organic layer of the clean rain plot (Corre and Lamersdorf, 2004). However, the overall response of microbial biomass to reduced N deposition was marginal (Tables 6.1 and 6.2). Since the long-term reduction of N had a minor effect on inorganic N contents (NH_4^+, NO_3^-) of soils (Kandeler et al., 2009), enhanced internal nitrogen turnover by soil microorganisms might have partly compensated the lower throughfall N input in the clean rain plot and might consequently have caused the negligible treatment effect (Corre and Lamersdorf, 2004). Our results of the fungus-specific compound ergosterol gave evidence that free-living as well as ectomycorrhizal fungi were not affected by the reduction of nitrogen. These results were also supported by more specific studies on the decomposition of recalcitrant litter compounds (e.g. phenolic compounds and lignin) and fungal abundance and diversity (e.g. basidiomycete genes and fungal derived lignolytic enzymes) at the same site (Theuerl et al., 2009).

Microbial biomass parameters decreased with depth in our soil profile and this is in accordance with Fierer et al. (2003) where decreased microbial biomass was observed through two soil depth profiles. A possible reason for the observed trend could be the decline in substrate availability through the soil profile which could be primarily driven by changes in

soil carbon content and reduced quality of soil organic matter with depth (Ajwa et al., 1998; Trumbore, 2000; Fierer et al., 2003). This finding is also supported by Agnelli et al. (2004) stating that the composition and structure of organic matter which represents the energy source for prevailing heterotrophic microorganisms, is liable to change throughout the profile of a forest soil (Taylor et al., 2002; Fierer et al., 2003).

Enzyme activities involved in N cycling

The N-cycling enzymes showed mixed responses to reduced N treatment. This is not unexpected since these enzymes attack substrates of different origins and classes. For example, the activity of urease depends on the release of nucleic acids from dead cells. In our experiment, urease activity increased significantly in the reduced N plot at some sampling dates and horizons (Fig. 6.2 and Table 6.1). This might indicate a higher turnover of microorganisms and roots. Given that annual soil respiration increased by 24% in the clean rain plot (Lamersdorf and Borken, 2004), these authors suggested that the reduced N treatment enhanced respiration of roots and proliferation of heterotrophic microorganisms within the rhizosphere. Another explanation could be attributed to high gross N mineralization rate in the reduced N treatment plot (Lamersdorf and Borken, 2004) and a high microbial N mineralization rate 14 years after suspension of N input to a boreal forest (Chen and Högberg, 2006). This hypothesis is supported by Saiya-Cork et al. (2002) reporting that increased gross N mineralization rates were accompanied by increased urease activity in a forest ecosystem. The stimulation of internal N cycling under reduced N deposition was also obvious from our results of alanyl aminopeptidase, lysyl-alanyl aminopeptidase and arginine deaminase showing higher activities under the clean rain treatment. Bach and Munch (2000) described also a stronger expression of several peptidases in a beech forest topsoil under N deficiency. Likewise Chróst (1991) found out that the activities of aminopeptidases were induced at limited N availability.

Some other enzymes involved in the degradation of chitin (N-acetyl-aminoglucosaminidase) and proteins and peptides (protease, leucyl aminopeptidase and alanyl-alanyl-phenyl aminopeptidase, see Figs. 6.2 and 6.3) showed either a trend for lower activity or no significant reaction under clean rain treatment. The activity of N-acetyl-aminoglucosaminidase was lower in the clean rain treatment. This enzyme is involved in the breakdown of chitin, which is a relevant source of N in soils. Sinsabaugh et al. (1993) assumed N-acetyl-aminoglucosaminidase activity to be induced by low N conditions whereas

at high N concentrations a non-competitive inhibition could occur. The present study did not support this assumption since N-acetyl-aminoglucosaminidase was reduced under the clean rain treatment in Oe and mineral soil layers. N-acetyl-aminoglucosaminidase was proposed as an adequate indicator of active fungal biomass (Miller et al., 1998). The missing correlation between ergosterol content and N-acetylaminoglucosaminidase revealed that abundance and function of active fungi are regulated by different factors in our study site (Table 6.4).

The activities of N-cycling enzymes showed higher activities in the upper soil horizons and varied with depth and sampling date and were probably dependent especially on substrate availability. The importance of substrate availability for the expression of different peptidases comes also from studies of Weintraub et al. (2007). For example, reducing rhizodeposition by girdling, Weintraub et al. (2007) observed a decrease in leucyl aminopeptidase activity in a subalpine forest ecosystem. Since data on the quality of root exudates and other rhizodeposition are not available for our experiment, we cannot decide whether the trend for lower values of some peptidases were caused by substrate limitation or by preferred decomposition of alternative substrates.

Enzyme activities involved in C cycling

Glucosidases are associated with degradation of cellulose and storage of carbohydrates (Saiya-Cork et al., 2002). α-and β-glucosidase activities showed significant treatment effects in the Oe and Oa horizons at some sampling dates (Table 6.1). Whereas activities decreased under reduced N deposition in the Oe horizon, these activities increased in the Oa horizon in spring 2006. This could possibly be associated with a different nutrient or substrate allocation pattern according to the N deposition. Moreover, the Oa horizon which accounts for >90% of forest floor material is primarily made of humus and fine roots supporting a high microbial biomass (Fisk and Fahey, 2001; Lamersdorf and Borken, 2004; Jansson et al., 1982). Similar patterns of α-and β-glucosidase, β-xylosidase and cellobiohydrolase activities as well as xylanase and peroxidase activities (Theuerl et al., 2009) did not support our hypothesis that reduction of N deposition decreases the decay of easily decomposable compounds and accelerates the decomposition of more recalcitrant compounds.

Enzyme activity involved in P cycling

Phosphatase plays a critical role in catalysing the hydrolysis of organic P compounds in soil. Since phosphatase activity under the clean rain treatment showed only temporally higher values than the control (Table 6.1 and Fig. 6.5), we suggest that phosphate limitation due to

increased microbial turnover in the organic layer (Oa horizon especially) induced phosphatase synthesis. As typical for forest soils (Turner et al., 2002), spatial distribution of phosphatase activity within the soil profile followed similar pattern like the other enzymes in this study, showing more than 50% higher values in the organic than in the mineral layers.

Relation between soil properties, microbial biomass and enzyme activity

Different microbial responses (stimulation, suppression and no effect) have been reported for different ecosystems following N deposition (Waldrop et al., 2004a, b). These responses might be in part a result of differences in microbial community composition (Agnelli et al., 2004). Nitrogen deposition could lead to ecosystem-specific alteration in enzyme activities which is related to the pattern of microbial biomass and soil carbon flow. After 16 years of experimental manipulation, reduced N deposition had little or no effect on the microbial biomass (SIR and ergosterol content) and activities of 15 different enzymes. The effects of depth and sampling date were more obvious, showing that in the Solling soil profile, spatial variability was more important than reduced N deposition. Microbial biomass and enzyme activities declined with soil depth and correlated with the nutrient pools (organic carbon and total nitrogen) which decreased with depth. These results gave a strong indication that decrease in microbial activity through the soil profile was a consequence of resource quantity, quality and allocation. The correlations between enzyme activities and microbial biomass (SIR and ergosterol content) on the other hand were not consistent for all the enzymes assayed. Ergosterol content correlated either positively or negatively with the C cycling enzymes (α-glucosidase, β-xylosidase and invertase) while microbial biomass (SIR) correlated positively with the N cycling enzymes (protease, urease and alanyl-alanyl-phenyl aminopeptidase). Enzyme activities might not always correlate with the biomass of microorganisms that produce them (Šnajdr et al., 2008). The reason is that soils might differ in their turnover time of microorganisms as well as extracellular enzymes (Bonmatti et al., 1991).

Conclusion

The Solling roof experiment gave us the opportunity to follow the response of microbial biomass and enzyme activities to long-term reduction of the N deposition within the soil profile of a spruce forest. Since cleaning throughfall water reduced external N input but did not reduce the total N pool within the ecosystem, we expected a change in internal element cycling within the ecosystem. Our study clarified that microbial processes involved in N

cycling are more affected than processes involved in C cycling. Specifically, we revealed that reduction of N deposition modified the reaction rates of different enzymes involved in N cycling. The stimulation or repression of specific peptidases under the clean rain treatment might be caused either by different turnover times of peptides or by different microbial acquisition of organic N compounds. Since reduction of N deposition did not change activities of enzymes hydrolysing low and high molecular weight C compounds, the current N status of the soil did not switch the preferred decomposition from easily available substrates to decomposition of recalcitrant compounds. Microbial biomass and enzyme activities were higher in the upper soil layer and decreased within the soil profile and correlated with the nutrient pools indicating that resource allocation and spatial availability of substrates are important factors regulating enzyme synthesis and activity.

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7 Development of a primer system to study abundance and diversity of the gene coding for alanine aminopeptidase *pepN* gene in Gram-negative soil bacteria

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Abstract

A new set of primers was developed allowing the specific detection of the *pepN* gene (coding for alanine aminopeptidase) from Gram-negative bacteria. The primers were designed *in silico* by sequence alignments based on available DNA sequence data. The PCR assay was validated using DNA from selected pure cultures. The analysis of gene libraries from extracted DNA from different soil samples revealed a high diversity of *pepN* related sequences mainly related to α-Proteobacteria. Most sequences obtained from clone libraries were closely related to already published sequences (<80 % homology on amino acid level), which may be related to the conserved character of the amplified region of *pepN*. By linking the diversity data obtained by the clone library studies to potential enzymatic activities of alanine aminopeptidase, lowest diversity of *pepN* was found in those soil samples which displayed lowest activity levels, which confirms the importance of diversity for ecosystem function mainly when transformation processes of complex molecules are studied.

Introduction

Up to 90 % of the total nitrogen (N) in soils is stored in the organic N fraction (Miltner and Zech, 1999). Compared to the turnover of inorganic N forms, like ammonia or nitrate, the cycling of organic N occurs often slowly over many years and thus it's steady mineralization which provides a sustainable source of N for above and below-ground processes that contribute to plant nutrition (Kirk et al., 2004) mainly in non-fertilized soils. The most abundant organic N-containing compounds in soils are proteins, peptides, amino acids and amino sugars (Stevenson, 1994; Schulten and Schnitzer, 1998). Whereas organisms of different trophic levels including several plant species, animals and microbes are able to uptake amino acids (Senwo and Tabatabai, 1998), which are either utilized as energy source or as matrix for biomass formation, proteins and peptidases are exclusively transformed by microbes (Kaye and Hart, 1997). Such microbes harbour the genetic potential to express of extracellular hydrolytic enzymes, including proteases and peptidases.

Only very few studies in the past investigated factors that drive the abundance and the diversity of microbes being able to catalyse protein and peptide degradation. These rare examples include several studies, where the influence of the soil type and the agricultural management on abundance, diversity and activity of microbes carrying the genes for neutral proteases and subtilisin was proven (Watanabe and Hayano, 1993a, b, 1994; Mrkonjic Fuka et al., 2008). However, data on drivers for peptide degrading microbes in nature is still missing.

In the present study, we describe a new set of primers to amplify the gene encoding alanine aminopeptidase (EC 3.4.11.12; *pepN*) from soils of different ecosystems to investigate abundance and diversity of the corresponding functional groups of microbes. *PepN* was first described as the major membrane bound aminopeptidase for *E.coli* in the 70s of the last century (Lazdunski et al., 1975a, b), but turned out to be well distributed among a wide range of Gram- positive and Gram-negative bacteria. Therefore not surprisingly enzymatic essays focussing on the detection of potential activities of *pepN* in soil revealed higher activities compared to other peptidases (e.g. leucine aminopeptidase) mainly in forest soils (Enowashu et al., 2009). The primers were constructed *in silico* and their suitability was tested using selected bacterial strains. Finally, gene libraries from three different soil samples were constructed and a diversity pattern for the alanine aminopeptidase was described to prove the suitability of the developed primer systems.

Materials and methods

Bacterial strains and culture conditions

Strains carrying the *pepN* gene covering different sub-groups of Proteobacteria (*Labrenza aggregate* (DMSZ 13394), *Pseudomonas putida* (DMSZ 3226), *Shinella zoogloeoides* (DMSZ 287) and *Rhizobium radiobacter* (DSMZ 5172)) as well as Gram-positive bacteria (*Corynebacterium glutamicum* (DSM 20300)) were purchased from the German collection of microorganisms and cell cultures (DSMZ, Germany). The strains were grown over night at 30^{o}C in peptone-glucose broth (per l distilled water: 10 g peptone, 5 g glucose, 10 g of NaCl, 5 g yeast extract; pH 7.5) and cells were harvested by centrifugation at 5000 x *g* for ten minutes.

Soils

We selected three different sites differing in their physico-chemical properties as well as in their vegetation cover for this study.

Two forest soil (FS) samples were taken in October 2007 from the "clean rain" (reduced N) experiment in Solling, central Germany (51^{o}31´N, 9^{o}34´E, elevation ~500 m above sea level (a.s.l.)). The soils were sampled from the Oa horizon (2 to 5 cm) with one sample being derived from the treatment "clean rain" (FS-RN) and the other from the control (FS-C) plot. The "clean rain" treatment is characterized by a 65% lower N input than the control plot due to prior deionization of throughfall water. The mean annual precipitation and variation in temperature were approximately 1090 mm and 6.4°C, respectively. The soil has been characterized as a strongly acidic dystric cambisol. (pH 2.6-2.8; organic carbon 32.8-35 g 100 g^{-1} soil; total N 1.4 g 100 g^{-1}soil). A detailed description of the experimental site is given by Enowashu et al. (2009).

Soil under agricultural use (AS) was obtained from the Hohenheim Climate Change experimental field located at the Heidfeldhof experimental field station (48°42´50´N, 9°11´26´E, 395 m a.s.l.) of the University of Hohenheim. Samples were collected in June 2009 from both the elevated temperature treatment (AS-ET) where the surface soil was heated (2.5°C) by means of heating cables to a depth of 4 cm, and the control treatment (AS-C) (ambient temperature) from the upper 20 cm (Ap horizont). In 2009, the mean annual temperature and precipitation was 9.9°C and 707.2 mm respectively. The soil has been

characterized as a loess derived stagnic Luvisol with silt-loam soil texture (pH 6.8, organic carbon content 15 g kg^{-1}).

Soils from a glacier forefield (GS) of three successional ages (1953 (GS-1953); 1900 (GS-1900) and 1850 (GS-1850)) were sampled in September 2006 from the glacial foreland of the Oedenwinkelkees (47°07′N, 12°38′E) in the Austrian Alps, at an altitude of 2068 – 2150 m above sea level. The soil (0 - 10 cm depth) was primarily leptic Regosols (pH 5.71-6.55; organic carbon 3.8-30.4 mg g^{-1}soil; inorganic nitrogen 1.98-12.10 mg kg^{-1}soil). For details of the site description and the soil sampling, see Philippot et al. (2011).

Soils were stored at –20°C immediately after sampling for molecular analysis or at 4°C for not longer than 2 weeks for enzymatic measurements.

Enzyme actvities

Potential activities of the alanine aminopeptidase enzyme (EC 3.4.11.2) were determined using flourogenic substrates according to Marx et al (2001). The substrate an L–alanine amino methyl coumarine (AMC) derivative, as well as standards and buffer were obtained from Sigma-Aldrich (Germany). Briefly, soil suspensions were prepared by adding 0.5 g soil into 50 ml of autoclaved water and dispersed by ultrasonication for 2 min with 50 J s^{-1} sonication energy. The suspensions were continuously stirred using a magnetic stir plate while 50 µl aliquots were dispensed into 96-well microplate (PP F black 96 well; Greiner Bio-one GmbH, Germany), followed by 50 µl of the buffer and 100 µl of the substrate solution. Standard wells received 50 µl of soil suspension, standard solution buffer. All microplate wells had a final volume of 200 µl as reaction medium. The plates were incubated at 30°C. Fluorescence was measured at 360/460 nm wavelength in a microplate fluorescence reader (Bio-Tek Instruments Inc., FLX 800, Germany) after 0, 30, 60, 120 and 180 minutes. The enzyme activity corresponded to an increase in fluorescence and was calculated in nmol AMC g^{-1} soil h^{-1}.

DNA extraction

Genomic DNA of pure cultures was obtained using the DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) as specified by the manufacturer. Total DNA of soils (300 mg) was extracted using the FastDNA® Spin Kit for soil (Qbiogene, Germany) as specified by the manufacturer. Isolated DNA was stored at -20°C.

In silico design of a PCR primer system for pepN

As the set of primers should be useful for qPCR as well as for diversity analysis of *pepN* an amplicon length of 400–450 bp has been considered as optimal, to obtain enough phylogenetic information for the diversity analysis, as well as to reach satisfactory efficiencies for qPCR. For the design of the primers, nucleotide sequences of known bacterial alanine aminopeptidases were extracted from NCBI data base and aligned using the clustalW based alignment tool implemented in biology workbench (http://workbench.sdsc.edu/). The specificity of the obtained forward and reversed primer was tested using the Genomatix software package (http://genomatix.gsf.de) against the Bacteria Data set (GenBank release 154) *in siilco*. The deduced sequences of the degenerated primers was as follows: *pepN* F 5'-CARTGYGARBCISARG-3' and *pepN* R 5'-CYYTTRTTYTCCATIGC-3'.

PCR reaction

PCR reactions were done in 25 µl volume and contained 5 ng (pure strains) respectively 10 ng (soils) of template DNA, 100 pmol of forward and reverse primer and 12.5 µl of a ready-to-use PCR mix (BioMix, Bioline, Germany). To improve efficiency of the PCR for soil DNA dimethylsulfoxide (DMSO) was used in a final concentration of 5%. The PCR started with a 5 min initial denaturation at 94°C, followed by 35 (pure cultures) respectively 37 cycles (soil) of 94°C for 50 s, 52°C for 30 s, 72°C for 60 s and a final elongation step for 10 min at 72°C. Purity of PCR products was checked using agarose gel electrophoresis and ethidiumbromide staining.

Cloning and sequence analysis of pepN amplicons

pepN PCR products were cloned into a pCR®2.1 vector (TA Cloning Kit; Invitrogen, Germany) according to the handbook of the manufacturer. Briefly 1.6 µl *pepN* PCR product; 1 µl 10X ligation buffer; 5.4 µl sterile water, 1 µl pCR®2.1 vector (25ng/µl) and 1 µl T4 DNA ligase were incubated at 14°C overnight. 2 µl of the ligation mixture was transformed into competent cells and grown on LB agar plates containing 50 µg ml^{-1} of kanamycin and 40 µl of 40 mg/ml stock of X gal solution (Qbiogene, Germany). Blue colonies were picked after 48 h of incubation at 37°C. Plasmids were isolated using the NucleoSpin Plasmid Mini Kit (Qiagen, Germany). Purified plasmids were tested for inserts by *Eco*RI digestion (MBI Fermentas, Heidelberg, Germany). Inserts were sequenced on a ABI PRISM® 3730 DNA Sequencer (Applied Biosystems, USA) using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

Sequence analyses were performed using the software package ARB (Ludwig et al., 2004; http://www.arb-home.de). Nucleotide sequences of amplicons and of reference genes retrieved from nucleotide collection of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were translated into amino acid sequences according to the correct reading frame and then aligned applying clustalW protein alignment implemented in ARB. Phylogenetic analyses on amino acid level of the sequences were performed using Maximum Likelihood (proML) algorithms. Collectors' curves analysis was performed using MOTHUR software (Schloss et al., 2009) based on a distance matrix calculated using ARB Neighbour Joining.

Results and discussion

Specificity of the pair of primers targeting pepN

The designed degenerate primer set was tested for specificity *in silico* by comparison with known GenBank DNA sequences using the Genomatix Matinspector program (http://genomatix.gsf.de/cgi-bin/matinspector/matinspector.pl), revealing that the detection system is specific for bacterial alanine aminopeptidases but discriminates fungal alanine aminopeptidase genes. Genomatix Modellinspector (http://genomatix.gsf.de/cgi-bin/gems/launch.pl) identified 137 perfect matches, mostly Gram-negative bacteria, if both primers were used together with a minimum length of the amplicons of 431 nt and a maximum length of 449 nt (supplemental material). The specificity of the *pepN* primer was tested empirically by PCR with genomic DNA of pure strains of Proteobacteria. Agarose gel of PCR products revealed one single band of the expected size for *Labrenza aggregate*, *Pseudomonas putida*, *Shinella zoogloeoides* and *Rhizobium radiobacter* respectively (Figure 7.1A). No PCR product was obtained when DNA from *Corynebacterium glutamicum* (high GC Gram-positive bacterium) was used as template.

According to the gene map published by Foglino et al. (1986), the amplicon is located close to the catalytic part of the enzyme with a rather conserved nucleic acid structure. This position of the amplicon is also confirmed on the basis of deletion mutants by Foglino and Lazdunski (1987). However as the gene sequence of this region differs in Gram-positive bacteria. e.g. *Streptococcus spp*. or *Lactobacillus spp*. (Rul et al., 1994; Varmanen et al., 1994), the constructed set of primers does not amplify DNA from those organisms despite the presence of *pepN* genes. Interestingly Chandu et al. (2003) found a close similarity of *pepN* related proteins in some Archaea and Eucaryotes. However, the *in silico* analysis using Genomatrix Modellinspector revealed no binding of the primer to any organisms other than

Gram-negative bacteria. As so far most complete genome sequences indicate only the presence of one *pepN* operon, primers are well suited to measure also the abundance of *pepN* harboring Gram-negative bacteria in different environments.

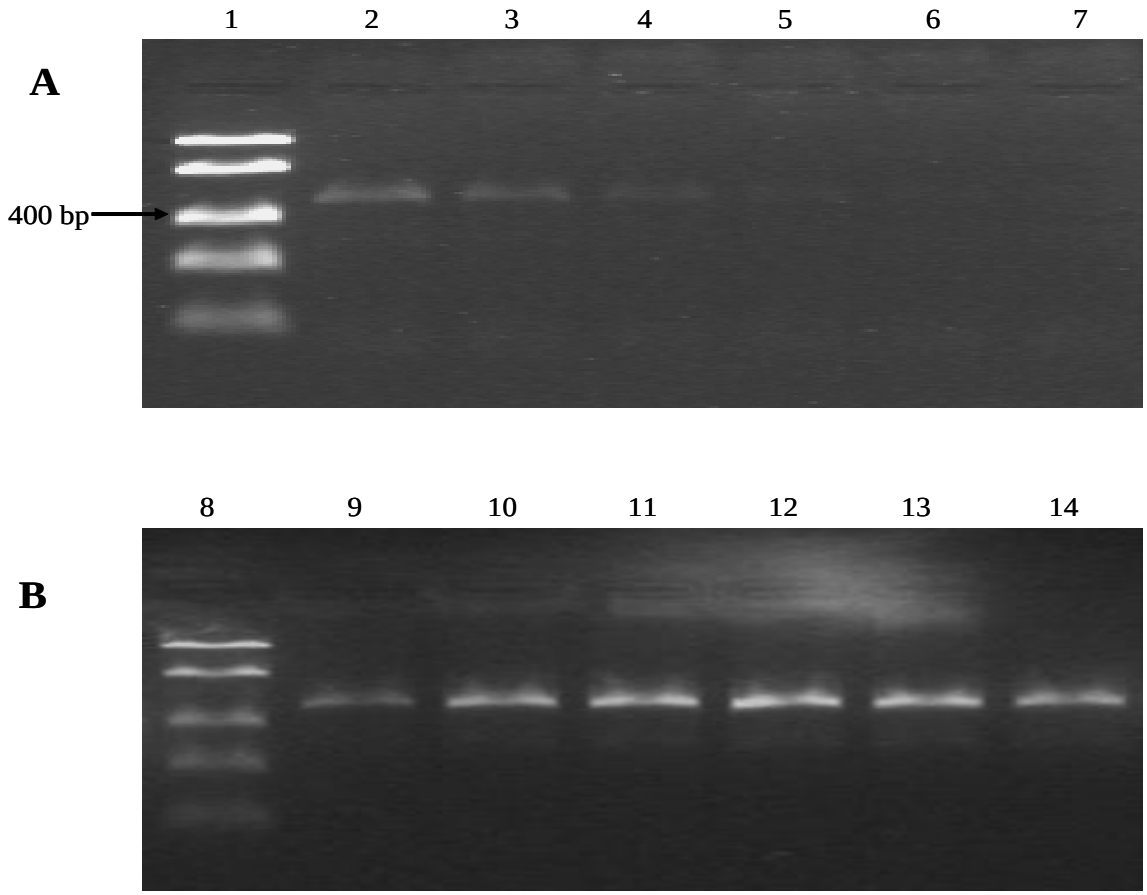

Figure 7.1: PCR amplicons of pure strain (A) and environmental soil DNA (B) using the newly developed primer set. A: Lanes 1+8: DNA ladder; lane 2: *Labrenza aggregate*, lane 3: *Pseudomonas putida*, lane 4: *Shinella Zoogloeoides*, lane 5: *Rhizobium radiobacter*, lane 6: *Corynebacterium glutamicum* (negative control), lane 7: no DNA. B: lane 9: *Pseudomonas putida* (positive control), lanes 10+11: arable soil, lanes 12+13: forest soil, lane 14: glacier soil.

Phylogenetic analysis

The designed primer set was further tested using community DNA extracts from three distinct environmental samples (forest, agricultural and glacier soils) to investigate the diversity and phylogenetic distribution of Gram-negative bacteria involved in peptide degradation carrying the *pepN* gene (Figure 7.3a,b,c). Electrophoretic separation of the PCR products revealed a single band which confirms the specificity of the primer set (Fig. 7.1B). After cloning of the PCR products and sequencing in total, 274 sequences were obtained from the different soil types of which 37 were most probably chimeres and were excluded

from subsequent analyses. The remaining 237 sequences (possibly encoding for *pepN*) were translated into amino acid sequences and subjected to phylogenetic analysis using the neighbour-joining and maximum-likelihood methods. Collector's curves were plotted at a 10% sequence difference level with the number of clones sampled against the number of OTUs observed. A plateau, as expected for full coverage of the libraries, was obtained only for extracted DNA from one soil sample obtained from grassland (AS-C) and two soil samples that were obtained from the glacier fore field (GS-1953 and GS-1850) after screening about 30 clones per sample (Fig. 7.2).

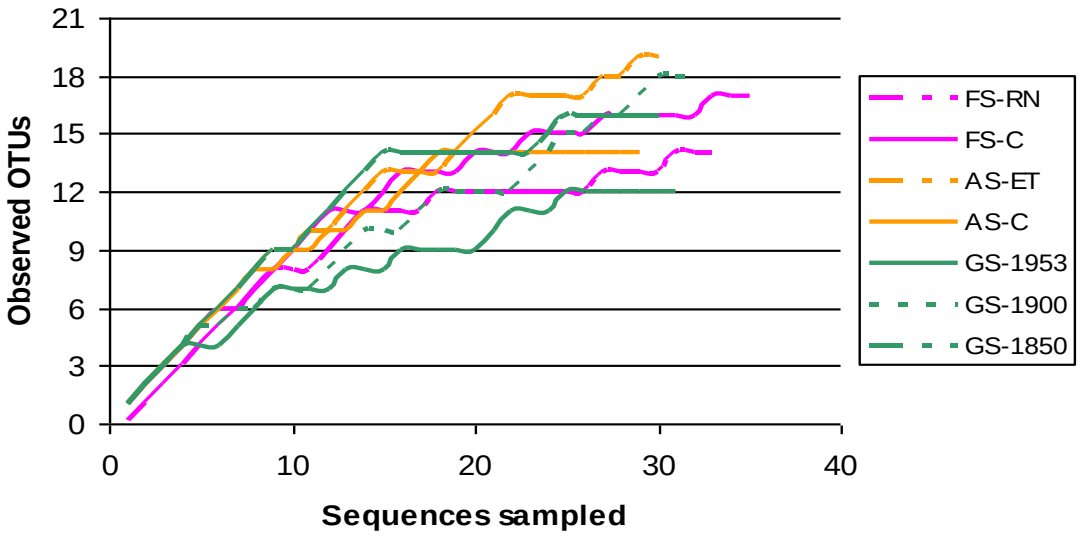

Figure 7.2: Collectors' curve constructed from observed OTUs at 10 % sequence difference for the three soil types.

A total of 63 *PepN* like sequences was obtained for the clone library constructed from the amplicons obtained from DNA of the two forest soil samples (FS; 35 for (FS-RN) and 28 (FS-C)). The majority of the translated amino acid sequences were closely related to sequences deposited in the NCBI database (80 – 95% identity to *PepN*). Only 5 of the obtained sequences showed lower homology levels on the amino acid level to so far described *PepN* sequences (70 – 79%). The abundance of clones affiliated to the different Gram-negative bacterial groups was the same for both treatments under investigation (reduced N and control). 96% of the clones were affiliated to *PepN* sequences of α-Proteobacteria. Here, two major clusters were observed; one closely related to *PepN* of *Acidiphilium cryptum* [148261095], which belongs to the class of metallopeptidases. The second cluster was closely related to *PepN* from *Methylobacterium extorquens* [254265931]. A third major cluster contained *PepN* sequences that were closely related to

Phenylobacterium zucineum [197106141]. The high contribution of sequences related to α-Proteobacteria might reflect the overall dominance of this group of bacteria in forest ecosystems which has been shown in previous studies by 16S rRNA gene barcoding, e.g. for pine forest soils in British Columbia (Axelrood et al. 2002; Chow et al. 2002) or Austria (Hackl et al., 2004), or soils from sub-tropical evergreen forests (Chan et al., 2006).

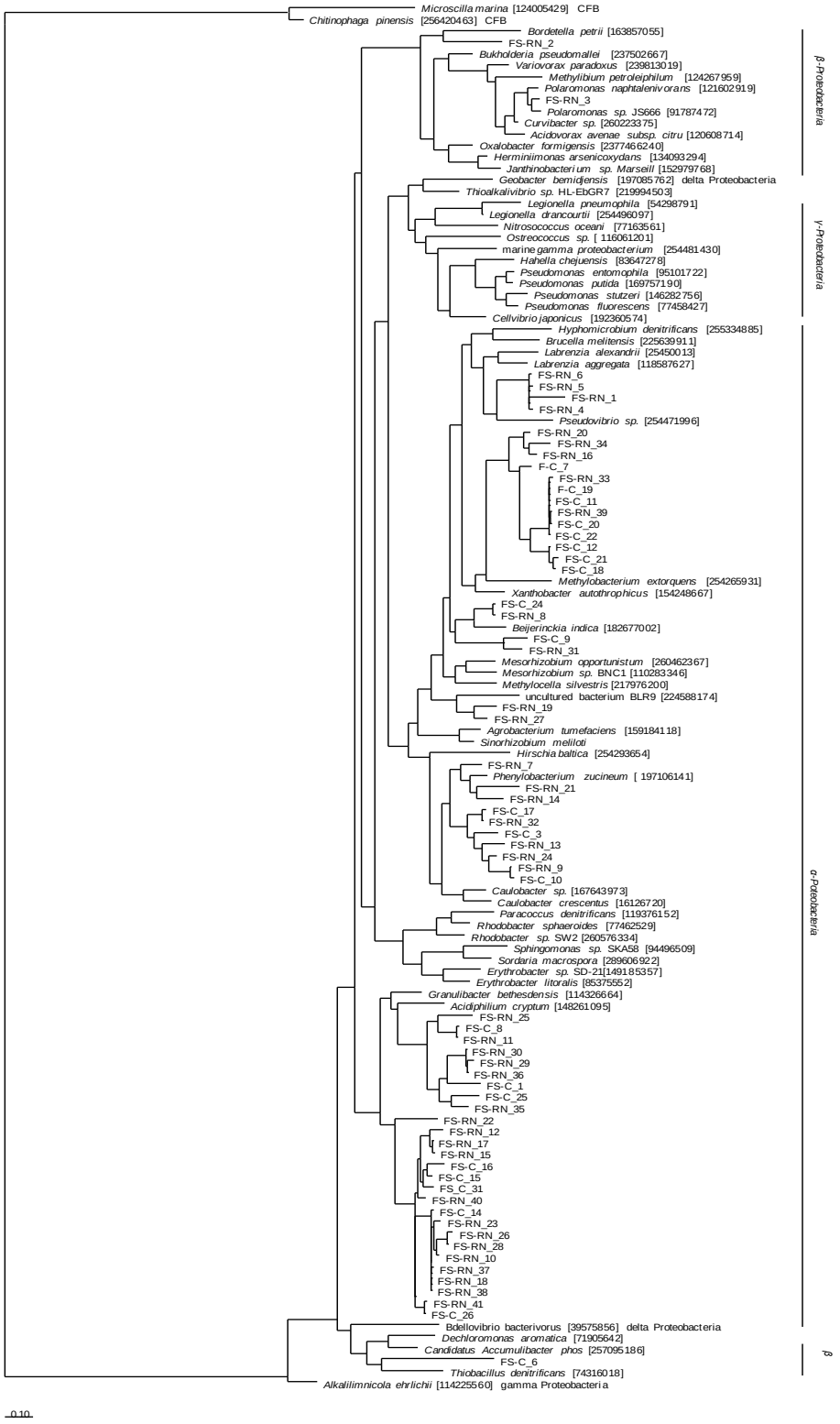

Figure 7.3a: Tree reconstructed using Maximum likelihood (ProML) based on ARB aligned amino acid sequences of *PepN* translated from nucleotide sequences derived from amplicon libraries of *pepN* from forest soil. Sequences of *Microscilla marina* and *Chitinophaga pinensi* were used as outgroups to root the tree. Bar indicates 10% estimated sequence divergence

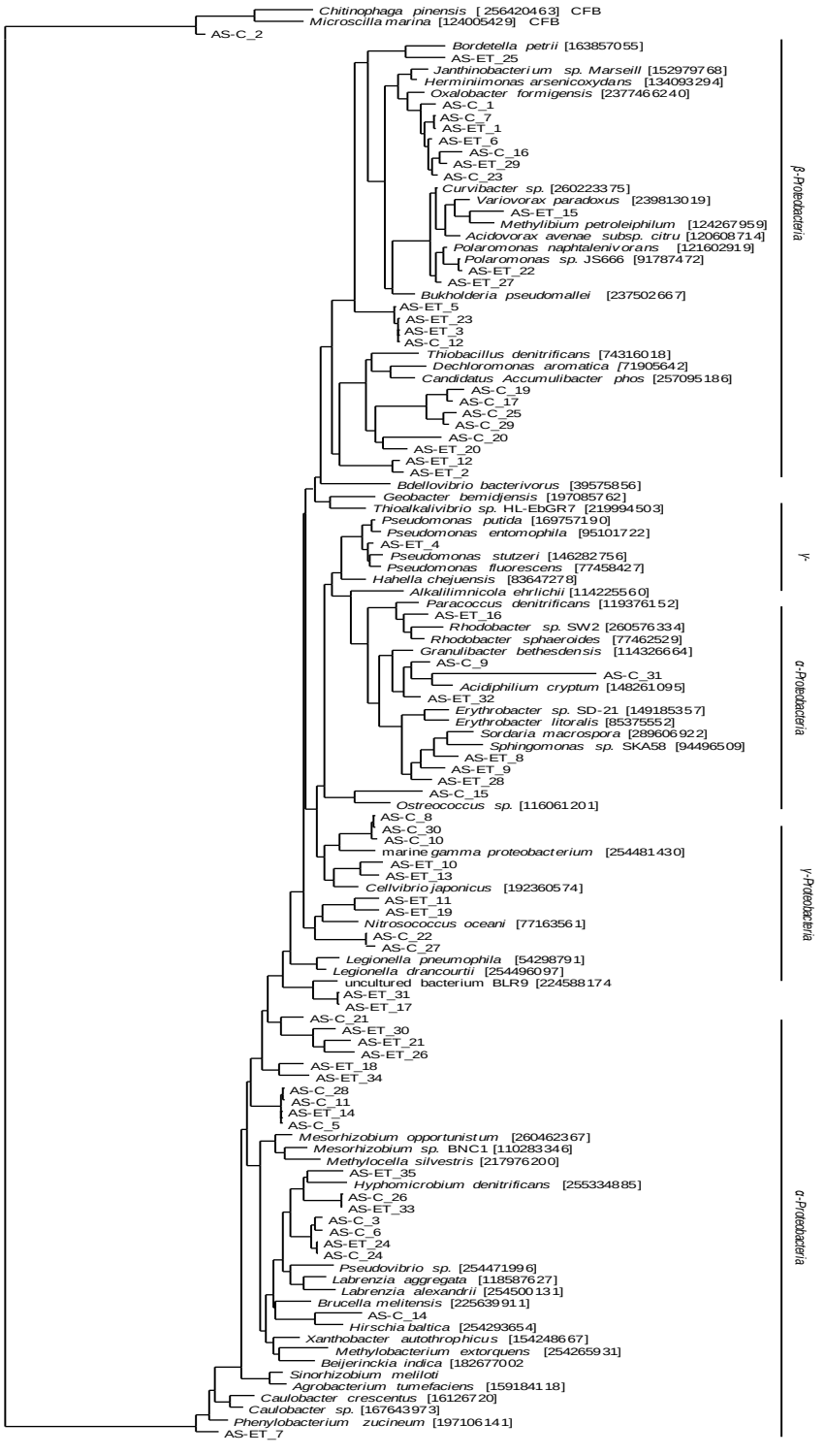

Figure 7.3b: Tree reconstructed using Maximum likelihood (ProML) based on ARB aligned amino acid sequences of *PepN* translated from nucleotide sequences derived from amplicon libraries of *pepN* from agricultural soils. Sequences of *Microscilla marina* and *Chitinophaga pinensi* were used as outgroups to root the tree. Bar indicates 10% estimated sequence divergence

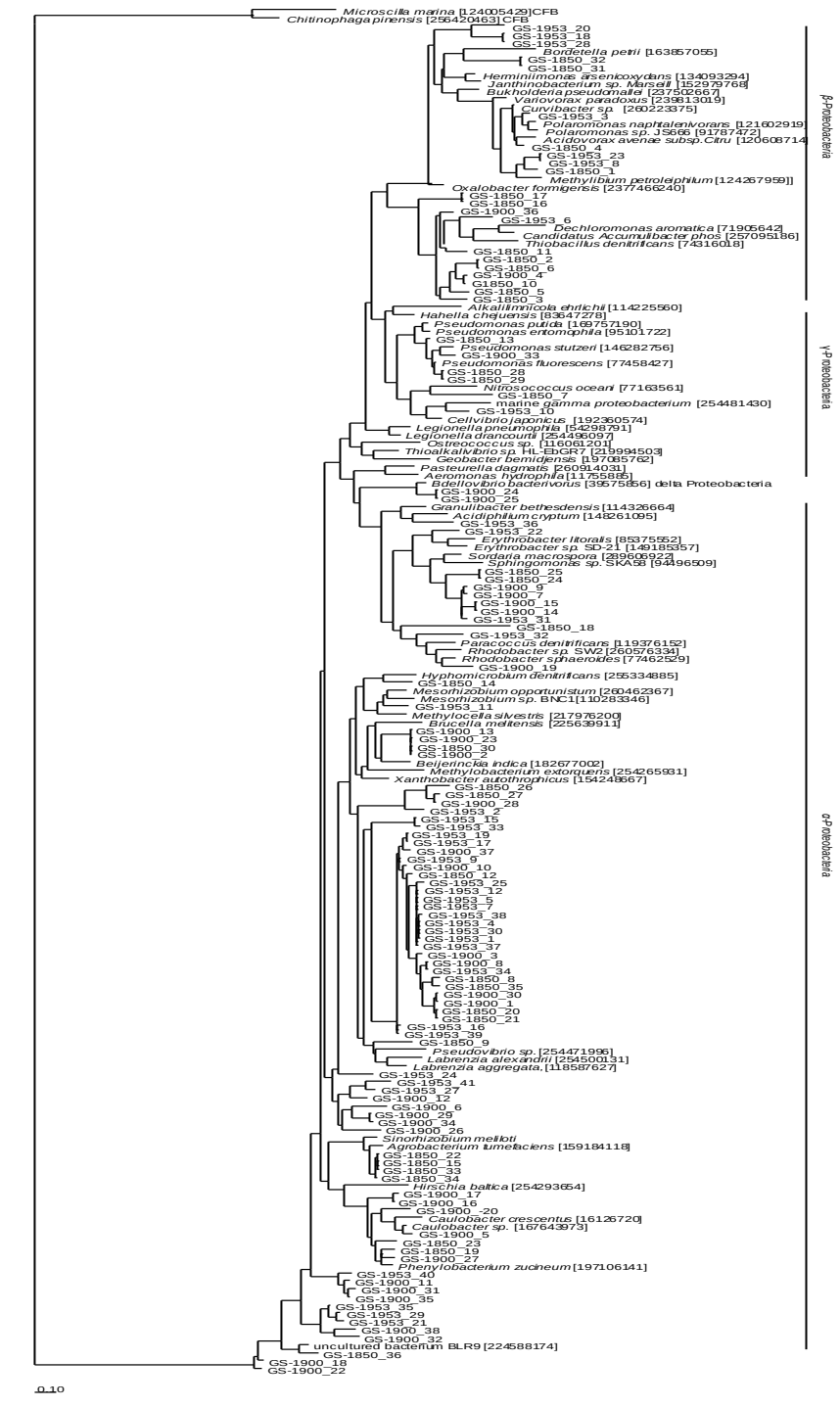

Figure 7.3c: Tree reconstructed using Maximum likelihood (ProML) based on ARB aligned amino acid sequences of *PepN* translated from nucleotide sequences derived from amplicon libraries of *pepN* from glacier grassland soil. Sequences of *Microscilla marina* and *Chitinophaga pinensi* were used as outgroups to root the tree. Bar indicates 10% estimated sequence divergence.

A total of 63 sequences were obtained for the clone library constructed from the amplicons obtained from the DNA of the two grassland soil samples, 30 from AS-C and 33 from AS-ET with 80 – 90% homology to published *PepN* sequences. Most of the obtained sequences (57%) were affiliated to *pepN* genes from α-Proteobacteria with close homology to *Hyphomicrobium denitrificans* [255334885] and *Sphingomonas sp.* SKA58 [94496509]. Thirty-eight percent of the obtained clones were closely related to *PepN* sequences from β-Proteobacteria and they clustered mostly to *Candidatus accumulibacter phos* [257095186], *Bukholderia pseudomallei* [237502667] and *Oxalobacter formigensis* [2377466249. *PepN* sequences of *γ*- Proteobacterial origin were only represented by 1.5% of the sequences. A significant influence of the shift in temperature was not visible. Again the high abundance of *pepN* sequences related to α- and β-Proteobacteria might be explained by the overall high abundance of these groups in the corresponding soils (Borneman et al. 1996; Zhou et al 2003).

To reveal the diversity of *pepN* along a glacial chronosequence, 111 sequences were analyzed from the respective clone libraries. The number of clones analyzed for each part of the chronosequence was comparable (38 for 1953; 37 for 1900 and 36 for 1850). Sequences of almost all clones showed 84 – 95% similarity to known PepN sequences deposited in the NCBI database. Overall, the majority of the clones were affiliated to *PepN* of α-Proteobacteria (73%). Here, one major cluster was observed being closely related to *Labrenzia aggregata* [118587627]. Other smaller clusters showed sequence homology to *Phenylobacterium zucineum* [197106141], *Brucella melitensis* [225639911] and *Sphingomons sp.* SKA58 [94496509]. *PepN* sequences related to β-Proteobacteria were the second most abundant group of clones (19%) with clusters that were closely related to *Thiobacillus denitrificans* [74316018], *Methylibium petroleiphilum* [124267959] and *Bordetella petrii* [163857055]. *PepN* from γ- and δ-Proteobacteria were represented by 5% and 2% of the sequences respectively. The sequences from the site which has been ice free for the longest period showed more diversity compared to the other successional stages of the chronosequence. For glacier chronosequences often the dominance of Actinobacteria and Bacteriodetes has been reported (Nemergut et al., 2007). Therefore in this case also *pepN* sequences of Gram-positive bacteria should be taken into account to get an overall picture on alanine amonipeptidase harboring bacteria.

Potential enzymatic activities

Lowest alanin amonipeptidase activities were detected in the soil samples from the agricultural site (mean: 72.7 ± 0.64 nmol AMC h^{-1} g^{-1} soil), whereas the soil samples from the grassland sites of the glacier fore field ranged from 189 to 851 nmol AMC h^{-1} g^{-1} soil and forest sites from 522 to 985 nmol AMC h^{-1} g^{-1} soil (Table 7.1). These levels are comparable to activities previously reported for agricultural soils (Rasche et al. 2006; Lagomarsino et al. 2012), grassland (Koch et al. 2007, Berner et al. 2011) and forest sites (Enowashu et al. 2009, Schütz et al. 2010). Interestingly those sites where the lowest enzymatic activities were measured, the diversity of *pepN* was lowest according to the collector`s curves. This confirms several ecological theories which state the importance of functional diversity for the corresponding enzymatic activity (Prosser et al., 2007).

Table 7.1: Potential pepN activity measured in samples from different sites.

Soil type	Treatment	Enzyme activity (nmol AMC h^{-1} g^{-1} soil)
Forest	FS-RN	985.21 (62.50)
Forest	FS-C	522.29 (±18.81)
Agricultural	AS-ET	259.83 (±14.94)
Agricultural	AS-C	72.73 (±0.64)
Glacier	GS-1953	189.71(±5.76)
Glacier	GS-1900	295.27 (18.68)
Glacier	GS-1850	851.53 (±22.38)

FS-RN = forest soil-reduced N deposition; FS-C = forest soil-control; AS-ET = agicultural soil-elevated temperature; AS-C = agriculutral-ambient temperature; GS-1953 = glacial soil ice free since 1953; GS-1900 = glacial soil ice free since 1900; GS-1850 = glacial soil ice free since 1850. Values in brackets indicate standard errors (n=5).

Environmental changes modified aminopeptidase activity to a great extend: Soils differing in their successional stage after deglaciation (ice-free since 1953, 1900 and 1850) showed increasing potential to degrade aminopeptides (190, 295 and 851 nmol AMC h^{-1} g^{-1} soil), which might be related to increased plant cover at the sites being ice free for a longer period or by different microclimatic conditions at the sites under investigation (Tscherko et al. 2003, 2004 and 2005; Philippot et al. 2011). Excluding the input of atmospheric nitrogen for 16 years reduced aminopeptidase activity in the organic layer of a forest site by 47 % which could be explained mainly by the lower availability of organic substrates after sixteen years of clean rain (Enowashu et al. 2009). The elevation of soil temperature (2.5°C in the soil

depth of 4 cm) in an agricultural site increased the aminopeptidase activity from 73 to 260 nmol AMC h^{-1} g^{-1} soil. This increased potential alanine aminopeptidase activity under elevation of temperature might be related to a higher plant biomass production at the respective sites or a general increase of microbial activity in response to the increased soil temperature. However, it has to be taken into account, that reduced moisture content of surface soils under increased temperature regimes might counteract this effect during periods of low water availability.

Conclusion

A set of degenerate primers specific for alanine aminopeptidase genes (*pepN*) of Gram-negative bacteria was designed *in silico,* validated with selected pure cultures and used to detect the corresponding gene by PCR in DNA extracted from different soil samples. Sequence analysis based on deposited sequences in NCBI indicated a high sequence diversity of *pepN* genes of bacterial origin. Major differences were visible between Gram-positive and Gram-negative bacteria, which made it impossible to find a consensus sequence which allows primer development. In Gram-negative bacteria sequence heterogeneity and also variations in length of *pepN* genes were less pronounced, which made an identification of a consensus sequence possible. However to cover all tested bacteria *in silico,* there was the need to degenerate mainly the reversed primer. The applicability of the designed primer set was confirmed for DNA extracted from all selected pure strains as well as community DNA extracted from different soil samples (no false positive clones obtained). Surprisingly most of the obtained sequences from the gene libraries were closely related to already published sequences (<75 % homology on amino acid level) indicating either that the amplified region is rather conserved, the set of primers is restricted to only selected phyla or the horizontal gene transfer played a major role in *pepN* distribution among Gram-negative bacteria.

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8 Final conclusions and perspectives

Understanding the impact of anthropogenic activities on microbial communities and how this relates to soil functioning poses an important challenge in current investigations in soil microbiology. N deposition resulting mainly from anthropogenic sources posses a threat to forest ecosystems and the processes therein. Over time, forest ecosystems receiving high N deposition are no longer N-limiting but N-saturated. Since ecosystem processes are slow, the recovery of forest following the reduction of N application will equally a slow and long term process. The present thesis used a biphasic approach that provided information on both the level of enzyme activity and the community abundance and diversity to address the impact of reduced N deposition in forest soil profiles. Changes in N availability, soil C quality and supply, and other soil environmental conditions (e.g. pH, soil moisture) in soil horizons affect the activity, size and composition of soil microbial communities. The results obtained in chapters 5 and 6 showed that the overall microbial function (enzyme activity) as well as the size, structure and activity of denitrifier/denitrification community in the forest soil is highly dynamic; remarkable spatial, vertical and seasonal variability were observed due to the variation of the environmental conditions that also greatly affect the level of nitrogen turnover in soil.

In the first study, analysis of functional genes provided new insights into the relationship between structure and function of the nitrate reducers and denitrification communities as influenced by reduction in N deposition. One of the outstanding findings of this study was the evidence that denitrifiers capable of nitrous oxide reduction might be enriched in deeper soil layers based on increases in the *nosZ*/16S rRNA gene and nosZ/*nirK* ratios with soil depth. This was probably as a consequence of the differences in water content and pH within studied horizons which were low and only a slight increase in pH in the mineral layer could be seen in comparison to the upper layers. Reducing N deposition only had a minor effect on the abundance and activity of the nitrate reducers and denitrifier communities in the different soil profiles. The decline in the size of 16S rRNA and bacterial denitrifier/denitrification gene pool observed through the soil profile was predominantly a function of both, decreasing amount and quality of organic matter contentThe variables depth and sampling dates had a stronger impact than reduced N deposition which could be explained by low level of nitrogen oxides respiration and the dominance of microbial NH_4^+ turnover in the internal N cycle.

The functioning of microorganisms in forest ecosystems in particular depends primarily on the activities of extracellular enzymes. Enzymatic assays were used in the second study to evaluate the overall microbial functional response to reduced N deposition within the Solling forest soil profiles. There was stronger though differential (stimulation, suppression) response of N cycling enzymes under reduced N deposition compared to C cycling enzymes. This was probably due to the stimulation of internal N cycling under the reduction of N. However, the pattern of substrate decomposition was not affected, that is, the decomposition of easily available substrates (e.g. cellulose) versus recalcitrant substrates (e.g. lignin). This seemed to be associated with a different nutrient distribution pattern according to reduced N deposition. Again, resource allocation and spatial and temporal availability of substrates were the main drivers regulating the decrease in microbial activity with soil depth.

Microbial communities respond rapidly to changes in their habitat. There are many abiotic and biotic environmental factors that induce shifts in microbial communities. These shifts appear to be due to interaction of different factors at the same time making our understanding of regulatory mechanisms of microbial communities more difficult. N cycling enzymes in the second study were more affected by reduced N deposition and more obvious was the influence on peptidases with alanine aminopeptidase showing the highest activities. This was then the bases for the third study where a new set of *pepN* primers was designed encoding the alanine aminopeptidase enzyme. Using enzymatic assays, PCR-based analyses and cloning, the function and diversity of alanine aminopeptidase gene communities of three different soils undergoing environmental and climate changes were shown. Gene library constructions revealed a high diversity of *pepN* sequences affiliated to α–Proteobacteria. Diversity data could be linked to potential enzymatic activities of alanine aminopeptidase, revealing lowest diversity of *pepN* in soil samples that displayed lowest activity levels. This confirms the importance of diversity studies for ecosystem function especially involving the decomposition of complex molecules.

Great advances in microbiological and molecular research on diverse microbial communities in complex ecosystems have been made and provide new opportunities to link community structure and ecosystem processes. The measurement of potential enzyme activities and functional gene based approach presented in this thesis was valuable and well suited to study the specific soil processes of denitrification and proteolysis. The knowledge of these two aspects put together will be needed to address future challenges of N deposition and to develop appropriate guidelines to improve forest ecosystem management.

From the study site, the level of nitrate concentration is being controlled by the forest management practices (reduction in N deposition) which present a partial solution. However, for the future, studies in this thesis could be extended to (1) assess the regulatory factors that contribute to the expression of denitrification genes and especially N_2O fluxes in relation to soil depth. This would be necessary to confirm whether the risk of N_2O release from mineral soil layers can be neglected and therefore important for the mitigation of N_2O emissions in view of green house gases; (2) real-time-PCR could be used to evaluate the abundance of *pepN* and peptide degrading bacteria. This therefore emphasizes the importance of organismal biology to improve our understanding of regulation of proteolytic activity.

In addition, the biases and drawbacks of PCR and specificity and sensitivity primers designed could be minimized with the application more diligent methods employing bacterial artificial chromosomes (BAC) and evaluating enzymes by immunological approaches will greatly afford our understanding of microbial processes which will greatly improve future studies on specific N transformation pathways that contribute to N emissions to the environment.

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Enowashu, E., Engel, M., Schloter, M., Rasche, F., Kandeler, E. (2009). Abundance and diversity of peptide degrading bacteria in soils. German Society of Soil Science Conference, Bonn, Germany.

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