Institute for Soil Science
University of Hohenheim
Soil Biology
Prof. Dr. Ellen Kandeler

Community Structure and Activity of Nitrate-Reducing Microorganisms in Soils under Global Climate Change

Dissertation

Submitted in fulfilment of the requirements for the degree "Doktor der Agrarwissenschaften"

(Dr. sc. agr.)

to the

Faculty of Agricultural Sciences

presented by

Kathrin Deiglmayr

München

2006

This thesis was accepted as doctoral dissertation in fulfilment of the requirements for the degree "Doktor der Agrarwissenschaften" by the Faculty of Agricultural Sciences at the University of Hohenheim on 18th April 2006.

Date of oral examination: 4th May 2006

Examination Committee

Supervisor and Review:	Prof. Dr. Kandeler
Co-Reviewer:	Prof. Dr. Claupein
Additional examiner:	Prof. Dr. Cadisch
Head of the Committee:	Prof. Dr. Blaich

For

Florian and Antonia

If we knew what is was we were doing, it would not be called research, would it?

Albert Einstein

Acknowledgement

The period of the past three - almost four - years was filled with joy and sorrow, proud and doubt, new insights and old questions. I am very grateful to all the people who accompanied me during this time.

First, I would like to thank **Prof. Kandeler** who readily accepted to supervise my PhD thesis after Prof. Ueli Hartwig had left the University of Hohenheim unexpectedly. I am particularly grateful for the scientific freedom that she gave me, her confidence in my competence and the valuable advice in writing research papers.

I am also grateful to **Prof. Claupein** for accepting to act as co-reviewer. Beyond that, I am indebted to him and **Prof. Piepho** for their insistent requests that guaranteed the financial support of my position at the Institute of Crop Production and Grassland Research.

Without **Dr. Laurent Philippot** (INRA, Dijon, France) this PhD thesis would probably not have been finished yet. He familiarised me with the world of molecular microbiology and gave invaluable support at any time when I despaired of the mysteries of these new methods.

In the laboratory I was greatly assisted by **Dagmar Mezger** who was always perfectly organized and kept track of all the samples. A big thank you for the most reliable work!

Dr. Ulrich Thum was maybe the most encouraging colleague during this time, listening to all the little problems that occur in the daily life of a PhD student. He gave a lot of good advice and assistance in all practical questions.

One statistician should also be mentioned particularly: **Dr. Andreas Büchse.** He introduced me to SAS applications and helped me with creating appropriate models. I have benefitted a lot from these lectures!

Thanks to the friends and colleagues in Hohenheim and Dijon (**Sara Stöber, Barbara Weber, Susan Haase, Oliver Koch, Christian Poll, Dagmar Tscherko, Liliane Rueß, Christoph Dambreville, David Bru, …**) I never felt lost on this long way. Thanks for the talks, cheerful gatherings and the comfort of common trouble!

I am most grateful to **my parents** for their interest in my research and their support in every way. My major thank, however, is addressed to my husband **Florian** and to **Antonia** who put the priorities back in the right order. Life is but a miracle!

List of contents

List of figures

List of tables

List of abbreviations

ANOVA	Analysis of variance
C	Carbon
C_{org}	Organic carbon
CCA	Canonical correspondence analysis
DNA	Deoxyribonucleic acid
DNP	2,4-dinitrophenol
FACE	Free air carbon dioxide enrichment
N	Nitrogen
NAP	Periplasmic nitrate reductase
NAR	Membrane-bound nitrate reductase
OTU	Operational taxonomic unit
PCA	Principal component analysis
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
U	Unit

1 Summary

Since the beginning of the Industrial Revolution, atmospheric carbon dioxide concentrations have been steadily increasing and, thus, contributed to a warming of the climate and altered biogeochemical cycles. To study the response of soil microorganisms to altered environmental conditions under global climate change, the nitrate-reducing community was regarded as a model community in the present thesis. This functional group, which performs the first step in the denitrification pathway, was selected because it is phylogenetically very diverse and, thus, represents microorganisms of various taxa. Simultaneously, denitrification is considered as most sensitive to environmental changes whereby it can serve as an indicator for altered turnover processes in soils. In particular rising levels of atmospheric carbon dioxide as the most important catalyst of temperature rise and the retreat of glaciers in the Alps as one of the most evident consequences of climate change were investigated. In the latter part of the study the main focus was the microbial succession in a glacier foreland, which exhibits a high variety of differently developed soils due to the continuous glacier retreat for 150 years. This ecosystem, thus, represents a static model for dynamic changes. The behaviour of nitrate reducers was investigated in a biphasic approach: (i) at the level of its specific enzyme activity of the nitrate reductase, which was determined via a biochemical method, and (ii) at the level of community structure, which was characterised by RFLP (Restriction Fragment Length Polymorphism)-fingerprints using the functional gene *narG*.

The effect of elevated atmospheric carbon dioxide concentrations on nitrate-reducing micro-organisms was studied in the Swiss FACE (Free Air Carbon dioxide Enrichment) experiment including the rhizosphere of two functional plant types (*Lolium perenne* and *Trifolium repens*), two N fertilisation levels and two sampling dates (June and October 2002). Whereas in June no significant treatment effect was observed, the nitrate reductase activity proved to be significantly reduced under elevated atmospheric carbon dioxide at the autumn sampling date. Simultaneously, elevated enzyme activities were recorded under *Trifolium repens* and high N fertilisation pointing to a control of nitrate reductase activity by nitrate availability at the time of sampling. The community structure of nitrate reducers, however, showed a different response pattern with sampling date and the strongly varying pH of the different experimental plots constituting the main driving factors. With respect to the three experimental factors atmospheric carbon dioxide, plant type and N fertilisation the composition of the nitrate reducers revealed a high stability.

In order to verify the resistance of the community structure of nitrate reducing micro-organisms versus fluctuating nitrate contents in soils a microcosm experiment was performed. Grassland soils were amended with none, high (100 µg NO_3^--N g^{-1} dry soil) and extreme high (300 µg NO_3^--N g^{-1} dry soil) nitrate additions and incubated at 25 °C for three, seven and fourteen days. *narG* RFLP-fingerprints remained unchanged over the whole experimental period indicating high resistance to ecosystem short-term disturbance. However, comparison of the nitrate reductase in the control and under the extreme high nitrate treatment showed a significant increase in the latter at day 3. No further differences were observed at days 7 and 14 which suggest a high resilience of the nitrate reductase activity.

The microbial succession of nitrate-reducing microorganisms was studied in the rhizosphere of *Poa alpina* across the glacier foreland of the Rotmoosferner/Oetz valley. Sampling was performed in August and at the end of the short period of vegetation in September. The nitrate reductase activity increased significantly with progressing successional age, whereas organic carbon together with nitrate concentrations in the soils explained the major part of this effect. The microbial community of nitrate reducers revealed a significant shift across the glacier foreland, with pH and organic carbon representing the most important environmental factors inducing this shift. A detailed analysis of the clone libraries that were constructed for the youngest and the oldest site in the glacier foreland pointed to the tendency of lower diversity in the late succession compared to the young succession. Possibly an increasing selective pressure due to higher densities of microorganisms and, hence, a higher competition for limited resources contributed to the decline in diversity.

In conclusion, the functional group of nitrate reducers responded to changing environmental conditions under global climate change particularly through altered enzyme activities. The amount and the direction of this response depended strongly on the nitrate availability and the organic carbon content in soils. The community structure of nitrate-reducing microorganisms, however, proved to be resilient towards short-term substrate fluctuations. Shifts in the composition of the nitrate-reducing microorganisms occurred only after mid-term to long-term changes in environmental conditions like seasonal fluctuations in temperature and water status or the accumulation of organic carbon in soils and dropping pH, respectively. This indicates that the genetic pool of this specific group of soil microorganisms possesses a high functional stability characterized by a relatively persistent composition and an independent modulation of enzyme activity.

2 Zusammenfassung

Seit dem Beginn der Industriellen Revolution sind die Kohlendioxid-Konzentrationen in der Atmosphäre durch menschliche Aktivitäten stetig angestiegen und haben zu einer Erwärmung des Klimas und veränderten biogeochemischen Kreisläufen beigetragen. Um Reaktionen von Bodenmikroorganismen auf veränderte Umweltbedingungen im globalen Klimawandel zu untersuchen, wurde in der vorliegenden Arbeit die mikrobielle Gemeinschaft der Nitratreduzierer als Modellgemeinschaft betrachtet. Diese funktionelle Gruppe von Bodenmikroorganismen, die den ersten Schritt im Denitrifikationsprozess ausführt, wurde ausgewählt, da sie phylogenetisch sehr divers zusammengesetzt ist und damit Mikroorganismen aus verschiedenen Taxa repräsentiert. Gleichzeitig gilt die Denitrifikation als äußerst empfindlich gegenüber Umweltveränderungen, womit sie als Indikator für veränderte Umsatzprozesse im Boden dienen kann. Im Besonderen wurden die steigenden Konzentrationen an atmosphärischem Kohlendioxid als wichtigster Auslöser des globalen Temperaturanstiegs und der Rückzug der Gletscher in den Alpen als eine der augenscheinlichsten Folgen des Klimawandels untersucht. Bei letzterem Teil der Arbeit galt das Hauptaugenmerk der mikrobiellen Sukzession im Gletschervorfeld, welches durch den kontinuierlichen Gletscherrückzug seit 150 Jahren eine große Spannweite an unterschiedlich weit entwickelten Böden aufweist. Damit stellt dieses Ökosystem ein statisches Modell für dynamische Veränderungen dar.

Die Verhaltensweise der Nitratreduzierer wurde auf zwei verschiedenen Ebenen analysiert: Erstens, auf der Ebene der spezifischen Enzymaktivität der Nitratreduktase, welche durch eine biochemische Messmethode bestimmt wurde, und zweitens, auf der Ebene der Gemeinschaftsstruktur, welche anhand des Funktionsgens *narG* durch RFLP (Restriction Fragment Length Polymorphism)-Fingerprints charakterisiert wurde.

Der Einfluss von erhöhten atmosphärischen Kohlendioxidgehalten auf nitratreduzierende Mikroorganismen wurde im Swiss FACE (Free Air Carbon dioxide Enrichment) Experiment untersucht, wobei die Rhizosphäre von zwei funktionellen Pflanzentypen (*Lolium perenne* und *Trifolium repens*), zwei N-Düngungsniveaus und zwei Zeitpunkte in der Vegetationsperiode (Juni und Oktober 2002) berücksichtigt wurden. Es zeigte sich, dass im Oktober die Nitratreduktase-Aktivität unter erhöhtem atmosphärischem CO_2 signifikant reduziert war. Gleichzeitig wurden unter *Trifolium repens* und der hohen N-Düngungs-

variante erhöhte Enzymaktivitäten gemessen, was darauf hindeutete, dass vor allem die Nitratverfügbarkeit am Termin der Probenahme die Nitratreduktase-Aktivität kontrollierte. Die Gemeinschaftsstruktur der Nitratreduzierer wies dagegen ein anderes Reaktionsmuster auf. Hier stellten sich der Zeitpunkt der Probenahme sowie der stark variierende pH der verschiedenen Versuchsparzellen als wichtige Einflussgrößen dar. Hinsichtlich der drei Versuchsfaktoren atmosphärische CO_2-Konzentration, Pflanzentyp und N-Düngung zeigte die Zusammensetzung der Nitratreduzierer eine hohe Stabilität.

Um die Beständigkeit der Gemeinschaftsstruktur von nitratreduzierenden Mikroorganismen gegenüber schwankenden Nitratgehalten im Boden zu verifizieren, wurde ein Mikrokosmos-Versuch durchgeführt. Dabei wurde Grünlandboden mit hohen (100 µg NO_3^--N g^{-1} Boden) bis extrem hohen (300 µg NO_3^--N g^{-1} Boden) Nitratzugaben versetzt und drei, sieben und vierzehn Tage lang bei 25 °C inkubiert. Die *narG* RFLP-Fingerprints blieben über die gesamte Versuchsdauer unverändert, was auf eine hohe Beständigkeit gegenüber kurzfristigen Störungen des Ökosystems hinweist. Ein Vergleich der Nitratreduktase-Aktivität in der Kontrollvariante und unter extrem hohen Nitratzugaben zeigte jedoch eine signifikante Erhöhung nach drei Tagen in letzterer Variante. Nach sieben und vierzehn Tagen wurden keine Unterschiede mehr festgestellt, was auf eine hohe Resilienz der Nitratreduktase-Aktivität hinweist.

Im Gletschervorfeld des Rotmoosferners (Ötztal) wurde die mikrobielle Sukzession der nitratreduzierenden Mikroorganismen in der Rhizosphäre von *Poa alpina* untersucht. Die Beprobung erfolgte im August und im September am Ende der kurzen Vegetationsperiode. Die Nitratreduktase-Aktivität zeigte einen signifikanten Anstieg mit zunehmendem Sukzessionsalter, wobei in erster Linie der steigende Gehalt an organischem Kohlenstoff zusammen mit der Nitratkonzentration im Boden diesen Effekt erklärte. Die mikrobielle Gemeinschaft der Nitratreduzierer veränderte sich ebenfalls signifikant über das Gletschervorfeld hinweg, wobei der pH und der Gehalt an organischem Kohlenstoff die wichtigsten Einflussgrößen darstellten. Eine detaillierte Analyse der Klonbibliotheken, die jeweils für den jüngsten und ältesten Standort erstellt wurden, deutete darauf hin, dass die Diversität der Nitratreduzierer in der späten Sukzession tendenziell geringer als am Beginn der Sukzession war. Möglicherweise war der zunehmende Selektionsdruck aufgrund höherer Dichten an Mikroorganismen und der daraus resultierenden Konkurrenz um begrenzte Ressourcen für die abnehmende Diversität mitverantwortlich.

Zusammenfassend lässt sich festhalten, dass die funktionelle Gruppe der Nitratreduzierer insbesondere mit veränderter Enzymaktivität auf sich wandelnde Umweltbedingungen im globalen Klimawandel reagierte. Die Höhe und die Richtung dieser Reaktion hingen dabei sehr stark von der Nitratverfügbarkeit und dem Gehalt an organischer Substanz im Boden ab. Die Gemeinschaftsstruktur der Nitratreduzierer hingegen zeigte sich gegenüber kurzfristigen Substratschwankungen beständig. Verschiebungen in der Zusammensetzung der nitratreduzierenden Mikroorganismen traten nur nach mittel- bis langfristigen Änderungen von Umweltbedingungen wie jahreszeitlichen Schwankungen im Temperatur- und Wasserhaushalt beziehungsweise der Anreicherung von organischer Substanz im Boden und sinkendem pH auf. Dies deutet daraufhin, dass diese spezielle Gruppe von Bodenmikroorganismen eine hohe funktionelle Stabilität aufweist, die durch eine relativ beständige Zusammensetzung und einer davon unabhängigen Regulation der Enzymaktivität gekennzeichnet ist.

3 General introduction

3.1 Global climate change and soil microorganisms

Since the early evolution of life one billion years after the Earth's formation living organisms have been causing profound changes in the composition of the atmosphere (Staley and Orians, 2000). Photosynthetic active bacteria gradually changed the atmosphere from a reducing to an oxidising one by releasing oxygen and, thus, created conditions that facilitated the origin of eukaryotic life (Madigan et al., 2003). Eukaryotic organism and particularly man have then contributed increasingly to biogeochemical cycles. Since 1750 anthropogenic activities, particularly burning of fossil fuels and deforestation, have led to a steady increase in atmospheric carbon dioxide concentrations reaching 365 ppmv at present, which has never been recorded before. The radiative forcing of carbon dioxide and other greenhouse gases like methane and nitrous oxides has contributed to a temperature rise of 0.6 ± 0.2 °C since the late 19th century (IPCC, 2001a). Due to the high residence time of CO_2 in the atmosphere this trend is supposed to accelerate in the 21st century. Estimates of atmospheric carbon dioxide concentrations in the year 2100 range between 540 ppmv and 970 ppmv depending on economic growth, technological advances and carbon sequestration by biological and geological processes (IPCC, 2001a). Temperatures are anticipated to rise by 2 to 4.5 °C in the same period. As a result of global warming, deglaciation of pole caps and accelerated shrinking of glaciers have been predicted (IPCC, 2001b). During the observation period 2002-2003 a loss of on average a few decimetres of ice depth per year were reported based on over one hundred glaciers monitored around the globe (Haeberli et al., 2005).

However, there is still considerable uncertainty about the feedback mechanism of terrestrial ecosystems (IPCC, 2001a). Since the capacity of ecosystems to store carbon depends on its net ecosystem productivity, which is the difference between net primary productivity and ecosystem heterotrophic respiration, numerous studies have dealt with the response of plants to elevated atmospheric CO_2 (Kimball, 1983; Bazzaz, 1990; Drake et al., 1997). Most results indicate a positive effect on biomass production by increasing atmospheric CO_2 levels (Ainsworth and Long, 2005). This is attributed to the fact that particularly in C_3 plants the key enzyme of C assimilation, ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco), is unsaturated under ambient CO_2 conditions. Thus, increasing atmospheric carbon dioxide concentrations enhance carbon assimilation of plants. In addition, the decrease in stomatal

conductance and transpiration of plants grown under elevated CO_2 results in a higher water use efficiency, which may promote biomass production under water limiting conditions (Niklaus et al., 1998). Besides, modifications in the tissue quality were observed like higher lignin contents and lower N concentrations, which were, however, often not significant (Norby et al., 2001). Carbon allocation to below-ground increased under elevated atmospheric CO_2 mainly through enhanced root growth (Jongen et al., 1995; Fitter et al., 1997; Zak et al., 2000). Particularly, the density of fine roots was seen to increase under CO_2 enrichment (Rogers et al., 1994). Under unlimiting nitrogen supply, however, carbon allocation to roots was not affected by rising atmospheric CO_2 emphasising the role of N availability for the responses of root growth (Suter et al., 2002). With respect to rhizodepositions and root exudates, controversial results have been reported, which may be partly attributed to differences in the physiological state of the plants under investigation (Pendall et al., 2004; Bazot et al., 2005).

Increasing attention has been paid to the responses of soil microorganisms because of their crucial role in the net balance of C sequestration. Whether soils can act as a sink or as a source for atmospheric CO_2, will depend largely on their heterotrophic respiration of plant residues and soil organic matter. Since natural concentrations of CO_2 in soils are about 50 times higher than in the atmosphere, no direct CO_2 stimulus can be expected, but rather a plant-mediated effect through higher C inputs in soils. In most studies microbial biomass was higher under enriched atmospheric CO_2, although the responses varied widely (Zak et al., 2000; Sonneman and Wolters, 2005). Neutral or negative responses might be explained by increased grazing of protozoa, nematodes and collembola (Lussenhop et al., 1998; Yeates et al., 2003) or by limiting N availability (Diaz et al., 1993). The response of microbial respiration was more consistent and increased in 95 % of all studied ecosystems with a mean response of +28 %, which was attributed to an increased supply of organic substrates for microbial metabolism through greater plant growth under elevated atmospheric CO_2 (Zak et al., 2000).

Only a few studies have considered microbial community composition in the context of global climate change. In general, shifts in community structure were very subtle and rather occurred in specific components of the soil microbiota like e. g. *Pseudomonas* or *Rhizobia* (Marilley et al., 1999; Montealegre et al., 2000; Roussel-Delif et al., 2005). Methods targeting the overall microbial community by PLFA (phospholipid fatty analysis) or the eubacterial

community by 16S DGGE analysis found no or only very little significant differences (Montealegre et al., 2002; Ebersberger et al., 2004).

The activity of soil microorganisms deserves special consideration, since changes in nutrient turnover and particularly N cycling will feedback on plant growth. Studies on important processes of the N cycle, however, showed contrasting results with no clear pattern apparent. Gross N mineralization in soils exposed to enriched atmospheric CO_2 remained constant (Gloser et al., 2000; Richter et al., 2003), increased or decreased depending on the N status of the site (Hungate et al., 1997). Similarly, N immobilization displayed large increases as well as large declines under elevated atmospheric CO_2 (Zak et al., 2000). With regard to nitrification, in most cases a tendency of decreasing activity was reported under elevated atmospheric CO_2 (Barnard et al., 2004). Low oxygen pressures due to higher water contents in the soil and increased heterotrophic respiration might have negatively affected the strictly aerobic nitrifiers. The responses of denitrification processes will be addressed in more detail in the next chapter.

3.2 Effects of global climate change on denitrification

Denitrification is one of the most sensitive soil processes since it is regulated by a complex web of biotic and abiotic factors (Tiedje, 1988). The most important regulator in denitrification is the partial pressure of oxygen. Denitrifiers generally exist in soils as aerobic heterotrophs and switch to nitrate as alternative electron acceptor only if oxygen is limiting. This process can also occur in aerobic soils at microsites, where oxygen consumption exceeds O_2 diffusion, such as in the centre of soil aggregates (Højberg et al., 1994), or in the rhizosphere and other hot spots (Klemedtsson et al., 1987; Højberg et al., 1996). The mechanisms, through which oxygen affects denitrification, are the repression of enzyme synthesis and the inhibition of nitrate-reducing activity by means of a suppressed nitrate transport across the cytoplasmic membrane (Tiedje, 1988; Moir and Wood, 2001). At limiting oxygen concentrations, denitrification rates depend mainly on nitrate availability and carbon resources. The latter not only provides the electrons for the reduction of nitrogenous oxides but also fuels respiration which reduces oxygen concentrations thereby creating anoxic microenvironments. In soils, carbon availability is generally not limiting unless nitrate is present in excess. Thus, denitrification rates are highly dependent on nitrate availability and

show pronounced peaks after fertilizer application, as long as anoxic conditions are given (Clayton et al., 1997; Müller et al., 2004; Šimek et al., 2004).

Denitrification in soils is of interest for several reasons: (i) it leads to a loss of plant available nitrogen, which is one of the most growth-limiting nutrients, (ii) N_2O, a possible end product in the denitrification pathway, is known to contribute to the destruction of the ozone layer and to the greenhouse effect with a radiative force that is 300 times higher than for CO_2 and (iii) it completes the global nitrogen cycle by returning fixed N_2 to the atmosphere. Particularly the first two motives induced scientists to study denitrification under global climate change conditions, since its positive response could accelerate the warming of the planet.

Most studies on the response of denitrification to elevated atmospheric carbon dioxide concentrations both in controlled environments and under field conditions revealed increased rates of activity (Smart et al., 1997; Ineson et al., 1998; Robinson and Conroy, 1999; Carnol et al., 2002; Baggs et al., 2003; Kettunen et al., 2005). They were explained by higher availability of root derived carbon or by higher water saturation in the soils due to higher water use efficiency of plants grown under enriched atmospheric carbon dioxide.

However, contrasting results were reported by Phillips et al. (2001). He observed decreased denitrification rates under carbon dioxide enrichment associated with lower N availability during the summer period, whereas in winter, when plants were less active with lower uptake rates of nitrate, N_2O fluxes increased. In other studies, limiting nitrate concentrations were considered to be responsible for the neutral effect of elevated atmospheric CO_2 on denitrifying processes (Mosier et al., 2002; Martin-Olmedo et al., 2002; Barnard et al., 2004).

3.3 Nitrate-reducing microorganisms as model community

Nitrate-reducing microorganisms perform the first step in the denitrification pathway, i.e. the dissimilatory reduction of nitrate to nitrite. This particular functional group of soil organisms is (i) very diverse including members of the α-, β-, γ-, ε-proteobacteria, high and low GC Gram-positive bacteria and even Archaea, and (ii) one of the largest groups of soil microorganisms involved in the N cycle and estimated to constitute 10 to 50 % of the soil's total bacterial community (Phillipot, 2005).

So far, only those species of nitrate reducers have been known which were cultivable. These were only a minor part of the total nitrate-reducing species. However, new molecular approaches now allow us to determine the diversity of the nitrate reducers based on the functional genes encoding the active site of either the membrane-bound (*narG*) or the periplasmic nitrate reductase (*napA*). The first primers for *narG* were designed by Gregory et al. (2000) based on a nested PCR design. A direct PCR approach was developed more recently and applied to a variety of different soils (Philippot et al., 2002; Chèneby et al., 2003; Mounier et al., 2004). Consistently, a high diversity of nitrate-reducing microorganisms in soils was revealed by constructing clone libraries and sequencing representative recombinants. In order to screen large sample sets a fingerprint technique, the RFLP (Restriction Fragment Length Polymorphism)-Analysis, was employed using the restriction endonuclease *Alu*I.

In a parallel attempt a nested PCR to amplify *napA* was developed (Flanagan et al., 1999). The periplasmic nitrate reductase is phylogenetically less widespread and has been detected only in Gram-negative bacteria up to date. Recent studies on the function of the NAP system point to an important role in redox balancing using nitrate as an ancillary oxidant to dissipate excess reductant, which functions also in the presence of oxygen (Gavira et al., 2002). Studies on enzyme expression revealed that the periplasmic nitrate reductase was predominantly expressed under aerobic growth conditions, whereas under anaerobiosis the NAR system prevailed (Richardson et al., 2001). The significance of the high physiological flexibility of this enzyme is still not completely understood.

The studies presented here focused on the nitrate-reducing community containing the membrane-bound nitrate reductase, because (i) of its wider distribution compared to the NAP system, (ii) many strains possess both dissimilatory nitrate reductases, which reduces the additional information that could be obtained by analysing the molecular marker gene *napA*, and (iii) the existing primers for *napA* require a nested PCR, which we wanted to avoid since it greatly increases the PCR bias.

The method to assess the corresponding enzyme activity, the dissimilatory nitrate reductase, was first published by Abdelmagid and Tabatabai (1987). The main principle of their approach was to incubate waterlogged soil samples with excess nitrate and measure colorimetrically the accumulated nitrite after 24 hours. Nitrite reduction was inhibited by 2,4-dinitrophenol, which is a potent uncoupler of oxydative phosphorylation. The optimal

concentration of this inhibitor, however, varies widely depending on the soil, and has to be determined for every soil type in advance.

To our knowledge, this is the first time that the analysis of the community structure of nitrate-reducing microorganisms and their specific enzyme activity were combined in order to search for the relevant mechanisms governing their responses to environmental changes. As their performance under global climate change could be crucial for future trends we studied their response under a variety of changed environmental variables.

References

Abdelmagid, H. M., and Tabatabai, M. A. (1987) Nitrate reductase activity of soils. Soil Biol. Biochem. 19: 421-427.

Ainsworth, E. A., and Long, S. P. (2005) What have we learned from 15 years of free-air CO_2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO_2. New Phytol. 165: 351-372.

Baggs, E. M., Richter, M., Cadisch, G., and Hartwig, U. A. (2003) Denitrification in grass swards is increased under elevated atmospheric CO_2. Soil Biol. Biochem. 35: 729-732.

Barnard, R., Barthes, L., Le Roux, X., and Leadley, P. W. (2004) Dynamics of nitrifying activities, denitrifying activities and nitrogen in grassland mesocosms as altered by elevated CO_2. New Phytol. 162: 365-376.

Bazot, S., Ulff, L., Blum, H., Nguyen, C., and Robin, C. (2006) Effects of elevated CO_2 concentration on rhizodeposition from *Lolium perenne* grown on soil exposed to 9 years of CO_2 enrichment. Soil Biol. Biochem.: in press.

Bazzaz, F. A. (1990) The response of natural ecosystems to the rising global CO_2 levels. Annu. Rev. Ecol. Syst. 21: 167-196.

Carnol, M., Hogenboom, L., Jach, M. E., Remacle, J., and Ceulemans, R. (2002) Elevated atmospheric CO_2 in open top chambers increases net nitrification and potential denitrification. Global Change Biol. 8: 590-598.

Chèneby, D., Hallet, S., Mondon, A., Martin-Laurent, F., Germon, J. C., and Philippot, L. (2003) Genetic characterization of the nitrate-reducing community based on *narG* nucleotide sequence analysis. Microbial Ecol. 46: 113-121.

Clayton, H., Mc Taggart, I. P., Parker, J., Swan, L., and Smith, K. A. (1997) Nitrous oxide emissions from fertilised grasslands: a two year study of the effects of N fertilizer form and environmental conditions. Biol. Fertil. Soils 25: 252-260.

Diaz, S., Grime, J. P., Harris, J., and McPherson, E. (1993) Evidence of a feedback mechanism limiting plant response to elevated carbon dioxide. Nature 364: 616-617.

Drake, B. G., Gonzalez-Meler, M. A., and Long, S. P. (1997) More efficient plants: a consequence of rising atmospheric CO_2? Annu. Rev. Plant Phys. 48: 609-639.

Ebersberger, D., Wermbter, N., Niklaus, P. A., and Kandeler, E. (2004) Effects of long-term CO_2 enrichment on microbial community structure in calcareous grassland. Plant Soil 264: 313-323.

Fitter, H., Graves, J. D., Wolfenden, J., Self, G. K., Brown, T. K., Bogie, D., and Mansfield, T. A. (1997) Root production and turnover and carbon budgets of two contrasting grasslands under ambient and elevated atmospheric carbon dioxide concentrations. New Phytol. 137: 247-255.

Flanagan, D. A., Gregory, L. G., Carter, J. P., Karakas-Sen, A., Richardson, D. J., and Spiro, S. (1999) Detection of genes for periplasmic nitrate reductase in nitrate respiring bacteria and in community DNA. FEMS Microbiol. Lett. 177: 263-270.

Gavira, M., Roldan, M. D., Castillo, F., and Moreno-Vivian, C. (2002) Regulation of *nap* gene expression and periplasmic nitrate reductase activity in the phototrophic bacterium *Rhodobacter sphaeroides* DSM158. J. Bacteriol. 184: 1693-1702.

Gloser, V., Jezikova, M., Lüscher, A., Frehner, M., Blum, H., Nösberger, J., and Hartwig, U. A. (2000) Soil mineral nitrogen availability was unaffected by elevated atmospheric CO_2 in a four year old experiment (Swiss FACE). Plant Soil 227: 291-299.

Gregory, L. G., Karakas-Sen, A., Richardson, D. J., and Spiro, S. (2000) Detection of genes for membrane-bound nitrate reductase in nitrate-respiring bacteria and in community DNA. FEMS Microbiol. Lett. 183: 275-279.

Haeberli, W., Noetzli, J., Zemp, M., Baumann, S., Frauenfelder, R., and Hoelzle, M. (2005) Glacier mass balance bulletin: Bulletin No. 8 (2002-2003). Compiled by the World Glacier Monitoring Service (WGMS). Retrieved November 4, 2005 from http://www.geo.unizh.ch/wgms.

Højberg, O., Revsbech, N. P., and Tiedje, J. (1994) Denitrification in soil aggregates analyzed with microsensors for nitrous oxide and oxygen. Soil Sci. Soc. Am. J. 58: 1691-1698.

Højberg, O., Binnerup, S. J., and Sørensen, J. (1996) Potential rates of ammonium oxidation, nitrite oxidation, nitrate reduction and denitrification in the young barley rhizosphere. Soil Biol. Biochem. 28: 47-54.

Hungate, B. A., Lund, C., Pearson, H. L., and Chapin, F. S. III. (1997) Elevated CO_2 and nutrient addition alter soil N cycling and N trace gas fluxes with early season wet-up in a Californian annual grassland. Biogeochemistry 37: 89-109.

Ineson, P., Coward, P. A., and Hartwig, U. A. (1998) Soil gas fluxes of N_2O, CH_4 and CO_2 beneath *Lolium perenne* under elevated CO_2: The Swiss free air carbon dioxide enrichment experiment. Plant Soil 198: 89-95.

IPCC (Intergovernmental Panel on Climate Change) (2001a) Climate Change 2001: The scientific basis. Contribution of Working Group I to the third assessment report of the Intergovernmental Panel on Climate Change, Cambridge, University Press.

IPCC (Intergovernmental Panel on Climate Change) (2001b) Climate Change 2001: Impacts, adaptations, and vulnerability. Contribution of Working Group II to the third assessment report of the Intergovernmental Panel on Climate Change, Cambridge, University Press.

Jongen, M., Jones, M. B., Hebeisen, T., Blum, H., and Hendrey, G. R. (1995) The effects of elevated CO_2 concentrations on the root growth of *Lolium perenne* and *Trifolium repens* grown in a FACE system. Global Change Biol. 1: 361-371.

Kettunen, R., Saarnio, S., Martikainen, P., and Silvola, J. (2005) Elevated CO_2 concentrations and nitrogen fertilisation effects on N_2O and CH_4 fluxes and biomass production of *Phleum pratense* on farmed peat soil. Soil Biol. Biochem. 37: 739-750.

Kimball, B. A. (1983) Carbon dioxide and agricultural yield: an assemblage and analysis of 430 prior observations. Agron. J. 75: 779-788.

Klemedtsson, L., Berg, P., Clarholm, M., Schnürer, J., and Roswall, T. (1987) Microbial nitrogen transformations in the root environment of barley. Soil Biol. Biochem. 19: 551-558.

Lussenhop, J., Treonis, A., Curtis, P. S., Teeri, J. A., and Vogel, C. S. (1998) Response of soil biota to elevated atmospheric CO_2 in poplar model systems. Oecologia 113: 247-251.

Madigan, M. T., Martinko, J. M., and Parker, J. (2003) Brock biology of microorganisms (10^{th} edition). Pearson education international, Prentice Hall, London: pp. 1019.

Marilley, L., Hartwig, U. A., and Aragno, M. (1999) Influence of an elevated atmospheric CO_2 content on soil and rhizosphere bacterial communities beneath *Lolium perenne* and *Trifolium repens* under field conditions. Microbial Ecol. 38: 39-49.

Martin-Olmedo, P., Rees, R. M., and Grace, J. (2002) The influence of plants grown under elevated CO_2 and N fertilisation on soil nitrogen dynamics. Global Change Biol. 8: 643-657.

Moir, J. B. W., and Wood, N. J. (2001) Nitrate and nitrite transport in bacteria. Cell. Mol. Life Sci. 58: 215-224.

Montealegre, C. M., van Kessel, C., Blumenthal, J. M., Hur, H. G., Hartwig, U. A., and Sadowsky, M. J. (2000) Elevated atmospheric CO_2 alters microbial structure in a pasture ecosystem. Global Change Biol. 6: 475-482.

Montealegre, C. M., van Kessel, C., Russelle, M. P., and Sadowsky, M. J. (2002) Changes in microbial activity and composition in a pasture ecosystem exposed to elevated atmospheric carbon dioxide. Plant Soil 243: 197-207.

Mosier, A. R., Morgan, J. A., King, J. Y., LeCain, D., and Milchunas, D. G. (2002) Soil-atmosphere exchange of CH_4, CO_2, NO_x and N_2O in the Colorado shortgrass steppe under elevated CO_2. Plant Soil 240: 201-211.

Mounier, E., Hallet, S., Chèneby, D., Benizri, E., Gruet, Y., Nguyen, C., Piutti, S., Robin, C., Slezack-Deschaumes, S., Martin-Laurent, F., Germon, J. C., and Philippot, L. (2004) Influence of maize mucilage on the diversity and activity of the denitrifying community. Environ. Microbiol. 6: 301-312.

Müller, C., Stevens, R. J., Laughlin, R. J., and Jäger, H.-J. (2004) Microbial processes and the site of N_2O production in a temperate grassland soil. Soil Biol. Biochem. 36: 453-461.

Niklaus, P. A., Spinnler, D., and Körner, C. (1998) Soil moisture dynamics of calcareous grassland under elevated CO_2. Oecologia 117: 201-208.

Norby, R. J., Cotrufo, M. F., Ineson, P., O'Neill, E. G., and Canadell, J. G. (2001) Elevated CO_2, litter chemistry and decomposition: a synthesis. Oecologia 127: 153-165.

Pendall, E., Mosier, A. R., and Morgan, J. A. (2004) Rhizodeposition stimulated by elevated CO_2 in a semiarid grassland. New Phytol. 162: 447-458.

Philippot, L., Piutti, S., Martin-Laurent, F., Hallet, S., and Germon, J. C. (2002) Molecular analysis of the nitrate-reducing community from unplanted and maize-planted soils. Appl. Environ. Microbiol. 68: 6121-6128.

Philippot, L. (2005) Tracking nitrate reducers and denitrifiers in the environment. Biochem. Soc. T. 33: 2004-2008.

Phillips, R. L., Whalen, S. C., and Schlesinger, W. H. (2001) Influence of atmospheric CO_2 enrichment on nitrous oxide flux in a temperate forest ecosystem. Global Biogeochem. Cycles 15: 741-751.

Richardson, D. J., Berks, B. C., Russell, D. A., Spiro, S., and Taylor, C. J. (2001) Functional, biochemical and genetic diversity of prokaryotic nitrate reductases. Cell. Mol. Life Sci. 58: 165-178.

Richter, M., Hartwig, U. A., Frossard, E., Nösberger, J., and Cadisch, G. (2003) Gross fluxes of nitrogen in grassland soil exposed to elevated atmospheric pCO_2 for seven years. Soil Biol. Biochem. 35: 1325-1335.

Robinson, D., and Conroy, J. P. (1999) A possible plant-mediated feedback between elevated CO_2, denitrification and the enhanced greenhouse effect. Soil Biol. Biochem. 31: 43-53.

Rogers, H. H., Runion, G. B., and Krupa, S. V. (1994) Plant responses to atmospheric CO_2 enrichment with emphasis on roots and the rhizosphere. Environ. Pollut. 83: 155-189.

Roussel-Delif, L., Tarnawski, S., Hamelin, J., Philippot, L., Aragno, M., and Fromin, N. (2005) Frequency and diversity of nitrate reductase genes among nitrate-dissimilating *Pseudomonas* in the rhizosphere of perennial grasses grown in field conditions. Microbial Ecol. 49: 63-72.

Šimek, M., Elhottová, D., Klimeš, F., and Hopkins, D. W. (2004) Emissions of N_2O and CO_2, denitrification measurements and soil properties in red clover and ryegrass stands. Soil Biol. Biochem. 36: 9-21.

Smart, D. R., Ritchie, K., Stark, J. M., and Bugbee, B. (1997) Evidence that elevated CO_2 levels can indirectly increase rhizosphere denitrifier activity. Appl. Environ. Microbiol. 63: 4621-4624.

Sonneman, I., and Wolters, V. (2005) The micro food web of grassland soils responds to a moderate increase in atmospheric CO_2. Global Change Biol. 11: 1148-1155.

Staley, J. T., and Orians, G. H. (2000) Evolution and the biosphere. In: Earth system science. Jacobson, M. C., Charlson, R. J., Rodhe, H., and Orians, G. H. (eds.). Academic Press, San Diego: pp. 29-61.

Suter, D., Frehner, M., Fischer, B. U., Nösberger, J., and Lüscher, A. (2002) Elevated CO_2 increases carbon allocation to the roots of *Lolium perenne* under free-air CO_2 enrichment but not in a controlled environment. New Phytol. 154: 65-75.

Tiedje, J. M. (1988) Ecology of denitrification and dissimilatory nitrate reduction to ammonium. In: Biology of anaerobic microorganisms. A. J. B. Zehnder (ed.). John Wiley & Sons, New York, NY: pp. 179-244.

Yeates, G. W., Newton, P. C. D., and Ross, D. J. (2003) Significant changes in soil microfauna in grazed pasture under elevated carbon dioxide. Biol. Fert. Soils 38: 319-326.

Zak, D. R., Pregitzer, K. S., King, J. S., and Holmes, W. E. (2000) Elevated atmospheric CO_2, fine roots and the response of soil microorganisms: A review and hypothesis. New Phytol. 147: 201-222.

4 Outline of the thesis

The overall goals of this thesis were (i) to explore responses of nitrate-reducing microorganisms towards environmental changes under global climate change at the level of community structure and enzyme activity, (ii) to identify important variables that drive the modifications and (iii) to deepen our understanding of the link between diversity and function of this specific group. Three independent experiments were performed in order to realize these objectives.

In the first experiment, the impact of long-term elevated atmospheric carbon dioxide on the nitrate-reducing community was examined. The Swiss FACE (Free Air Carbon dioxide Enrichment) experiment offered the opportunity to study the combined effect of enriched carbon dioxide (600 ppmv versus 365 ppmv), plant type (non-leguminous versus leguminous) and nitrogen fertilisation level (56 g m^{-2} a^{-1} versus 14 g m^{-2} a^{-1}) after 10 years of CO_2 fumigation. The FACE technology enabled direct investigations in the field with no modifications of the microclimate as observed in open top chambers. Our investigation was based on previous results, which revealed significantly higher N_2O emissions under enriched atmospheric carbon dioxide. We aimed to verify whether these increased denitrification rates were accompanied by shifts in the community structure and/or in the activity of the nitrate-reducing community. Since the main impact of elevated atmospheric CO_2 was supposed to occur via plants, we analysed the rhizosphere of *Lolium perenne* and *Trifolium repens* grown in monoculture in the Swiss FACE experiment.

As nitrate is one of the major controlling factors of denitrification we tested the susceptibility of the nitrate reducers and their activity towards excessive substrate supplies. We hypothesised that (i) the nitrate-reducing community structure was resistant towards fluctuating nitrate concentrations and (ii) nitrate reducers responded mainly by modulation of their enzyme activity to altered nitrate availabilities in oxygen limited soil environments. Therefore, we amended repacked soil cores with 0, 100 and 300 µg NO_3^--N g^{-1} dry soil and incubated them under anoxic conditions for 3, 7 and 14 days at 25 °C in a dark chamber. Changes in pH, nitrate, nitrite, and ammonia concentrations were monitored and related to measured activities of nitrate reductase. Simultaneously, the structure of the nitrate-reducing community was assessed.

The third experiment addressed one of the most apparent consequences of global climate change, the retreat of glaciers. For more than 150 years the Rotmoosferner, a glacier in the Oetz valley (Austria), has been deglaciating, and left a foreland of 2 km in length. Our specific objectives were (i) to study the succession of nitrate-reducing microorganisms in this newly exposed terrain, (ii) to monitor the corresponding enzyme activity, and (iii) to identify the most important environmental factors such as organic carbon, nitrate, water content and pH governing any changes. The experimental site of the glacier foreland provided, thus, a static model for dynamic processes. Since microbial activity has been found in previous studies to be highest in rhizospheric soil, we focused on the rhizosphere flora of *Poa alpina*, which is a perennial grass and occurred across all successional stages.

The manifold aspects of global climate change included in this work gave a broad view on the response of nitrate-reducing microorganisms to altered environmental conditions as they are forecasted for the 21st century. Simultaneously, they provided detailed insight in the mechanisms governing the community structure and the function of this particular group of soil microorganisms.

5 Structure and activity of the nitrate-reducing community in the rhizosphere of *Lolium perenne* and *Trifolium repens* under long-term elevated atmospheric pCO_2

FEMS Microbiology Ecology 49 (2004): 445-454

K. Deiglmayr (1), L. Philippot (2), U. A. Hartwig (3), E. Kandeler (4)

(1) University of Hohenheim, Institute of Crop Production and Grassland Research, D-70593 Stuttgart, Germany, (2) INRA, Laboratory of Soil Microbiology and Geochemistry, F-21065 Dijon Cedex, France, (3) Academia Engiadina, 7503 Samedan, Switzerland, (4) University of Hohenheim, Institute of Soil Science, D-70593 Stuttgart, Germany

Abstract

In June and October 2002, rhizosphere soil was sampled in monocultures of *Lolium perenne* and *Trifolium repens* at two different nitrogen fertilisation levels (14 g N m^{-2} a^{-1} and 56 g N m^{-2} a^{-1}) and under two pCO_2 atmospheres (360 ppmv and 600 ppmv) at the Swiss FACE (Free Air Carbon dioxide Enrichment) site. Directly extracted soil DNA was analysed via RFLP-PCR by use of degenerated primers for the *narG* gene encoding the active site of the membrane-bound nitrate-reductase. The corresponding enzyme activity of the nitrate reductase was determined colorimetrically after 24 hours of anaerobic incubation. The *narG* RFLP-PCR fingerprints showed that the structure of the nitrate-reducing community was primarily affected by season and pH of the sampling site, whereas CO_2 enrichment, plant species or fertiliser treatment had no apparent effect. In contrast, the nitrate reductase activity responded to N fertilisation, CO_2 enrichment and plant species in October, whereas in June drought stress most likely kept the enzyme activity at a low level in all treatments. Apparently, the respiratory nitrate-reducing community adapted to different treatments primarily by altered enzyme activity.

Introduction

Increasing concentrations of atmospheric pCO_2 have a strong impact on terrestrial ecosystems, leading to higher C assimilation rates in plants and, hence, to greater biomass production. Particularly root growth is stimulated under elevated atmospheric pCO_2 [1-3]. This has been attributed to nutrient limitation inducing plants to invest more carbohydrates into below-ground growth and to release root exudates in order to utilise soil resources more effectively.

Although soil microorganisms are not directly influenced by atmospheric carbon dioxide enrichment because the CO_2 concentration in soil is already more than 50 times greater than in the atmosphere, there may be a plant-mediated influence on soil microorganisms due to altered rhizodeposition and root exudation. In fact, several authors have shown shifts in the composition of soil microbial communities under elevated atmospheric pCO_2, with those bacteria colonising the rhizosphere and the rhizoplane-endorhizosphere being most affected [4, 5]. As microorganisms are responsible for most soil processes, they play a key role in the response of ecosystems to CO_2 enrichment.

Special attention has been focused on the denitrification pathway, which can release the greenhouse gas N_2O as a possible end product and thereby enhance climate change [6]. Denitrification under elevated pCO_2 has therefore been investigated in various studies [7-9]. A significant increase in the denitrifying activity under CO_2 enrichment has consistently been reported for controlled systems as well as under field conditions. Higher denitrification rates under elevated levels of atmospheric pCO_2 may have several causes: (1) Higher growth of fine roots containing large amounts of non-structural carbohydrates, along with enhanced root exudation, may enrich the rhizosphere with easily decomposable carbon sources [10]. (2) Increased soil and microbial respiration may reduce the oxygen content and, hence, create anoxic sites [1]. (3) Higher water use efficiency in plants grown under elevated pCO_2 are likely to increase the soil water content and thus to constrain oxygen diffusion, facilitating the occurrence of anaerobic conditions [11].

Our study focused on the first step in the denitrification pathway, the reduction of nitrate to nitrite, which is catalyzed by a periplasmic or a membrane-bound nitrate reductase. The nitrite produced can be then reduced to gaseous nitrogen by denitrification or to ammonium by DNRA (Dissimilatory Nitrate Reduction to Ammonium), which is of minor importance in soil. For this process both the molecular technique to target the nitrate-reducing community

and the method to study the specific enzyme activity were available. The first step of denitrification is usually catalysed by the membrane-bound nitrate reductase, which is widespread among taxonomically diverse nitrate reducers [12]. Therefore, the functional gene *narG* encoding the catalytic subunit of the membrane-bound nitrate reductase has been used as molecular marker in this study. Adequate primers have been described to amplify a *narG* fragment of approx. 650 bp by polymerase chain reaction and have been successfully applied to environmental samples before [13, 14]. The objective of our study was to test whether the nitrate-reducing community responds to long-term elevated atmospheric pCO_2. We addressed this issue by using the Swiss FACE facility that had been operating for 10 years at the time of sampling. As we expected an indirect impact of atmospheric CO_2 enrichment on microbial community structure via the plants, we focused on the rhizosphere of the two examined grassland species, *Lolium perenne* and *Trifolium repens*.

Material and Methods

Experimental site and soil sampling

To study the long-term effect of elevated pCO_2 on model grassland ecosystems, the Swiss FACE experiment was established in 1993 at the Swiss Federal Institute of Technology (ETH) field station at Eschikon (47°27'N and 8°41'E, 550 m above sea level) near Zurich, Switzerland. The soil was a fertile eutric cambisol with 31 % sand, 38 % silt and 31 % clay in the mineral fraction [15]. The organic matter content varied from 2.7 % to 5.1 % and the pH (KCl extracted) ranged from 4.1 to 7.1 (10 cm top soil), with extreme low values in the third block. Three blocks were set up, each consisting of two rings (18 m diameter), one fumigated with CO_2 to maintain elevated levels of pCO_2 (600 ppmv) and one control ring without fumigation (360 ppmv pCO_2). The CO_2 fumigation was operated during daylight throughout the growing season from March to November at air temperatures above 5 °C. Over the whole experimental period, the 1-min average was 600 ppmv ± 10 % within 90-94 % of the fumigated time for the three rings with elevated pCO_2 [2].

Within each ring, subplots (2.8 m x 1.9 m) were distributed randomly. In this study the following treatments were investigated: *Trifolium repens* cv Milkanova in monoculture and *Lolium perenne* cv Bastion in monoculture, each at low N (14 g N m^{-2} a^{-1}) and high N

fertilisation level (56 g N m^{-2} a^{-1}). Nitrogen was applied as NH_4NO_3 at the start of the growing season and after each cut. In 2002 the above-ground biomass was harvested four times.

The experimental plots were sampled in June and October 2002, directly after defoliation. Eight soil cores (2.5 cm diameter, 10 cm depth) per plot were mixed together to form one composite sample. From each soil sample the rhizospheric soil was recovered by picking out the visible roots. The soil still adhering to the roots after gentle shaking was considered rhizospheric soil and stored at -25 °C prior to further analysis.

DNA extraction and PCR amplification

DNA was extracted from 0.3 g soil using the FastDNA Spin Kit for soil (BIO101, Qbiogene), following the protocol of the manufacturer. The quantity of the DNA extractions was checked using a BioPhotometer (Eppendorff). A *narG* fragment of 650 bp length was amplified using the primers narG1960f and narG2650r [13]. Three independent PCR amplifications were performed for each sample in a total of 50 µl containing 1x PCR buffer, 200 µM of each deoxyribonucleoside triphosphate, 500 pM of each primer, 2 U of *Taq* polymerase, and 10 ng of soil DNA. AmpliWax (Applied Biosystems) was used to facilitate a hot start PCR. The cycling conditions of the PCR were as follows: an initial denaturation step at 95 °C, followed by a "touch down" PCR with a denaturation step at 94 °C for 30 s, primer annealing at 59 °C for 30 s and elongation at 72 °C for 45 s. During the first 9 cycles, the annealing temperature was decreased by 0.5 °C each cycle until it reached 55 °C. The additional 26 cycles were performed at an annealing temperature of 55 °C. Cycling was completed by a final elongation step at 72 °C for 10 min. The size and presence of the amplification products were checked by electrophoresis in a 1.5 % agarose gel.

RFLP analysis and clone library construction

For purification, the *narG* PCR products belonging to the same sample were pooled and then run on a 2 % agarose gel for 3 h at 100 Volt. Gel slices containing the amplified *narG* fragment were excised and DNA was recovered using the Qiaex II kit (Qiagen) as specified by the manufacturer with one slight modification: For higher DNA yield, elution time was extended to 30 min. Purified PCR products were quantified in a 1.5 % agarose gel according to the standardised DNA quantities of the Smart Ladder SF (Eurogentec). Aliquots of same

quantities of the purified *narG* PCR product were digested by *Alu*I restriction enzyme at 38 °C for 12 h and separated by electrophoresis on an 8 % polyacrylamide gel for 15 h at 8 mA. After staining with SYBER green II (Molecular Probes) the *narG* RFLP-fingerprints were scanned with a Phospho Imager.

Aliquots of the purified *narG* PCR products from two samples (June, *Lolium perenne*, low N, 600 ppmv pCO_2 and 360 ppmv pCO_2) were cloned using the pGem-T Easy Vector System (Promega) according to the manufacturer's instructions. Approximately 90 transformants per sample were randomly picked and the inserted *narG* fragment was amplified by transferring small aliquots of cells to PCR mixtures containing the primers T7 and SP6 and thermal cycling. PCR products were digested by the restriction endonuclease *Alu*I as described above. Restriction fragments were resolved by electrophoresis in a 3 % small fragment agarose gel. Recombinants with identical restriction patterns were grouped together into RFLP types and phylogenetic diversity was estimated by Analytic Rarefaction Version 1.3. (Stratigraphy laboratory, University of Georgia).

Sequencing and phylogenetic alignment

Thirty-nine representative recombinants of the various RFLP types were sequenced using the DTCS-1 kit (Beckman Coulter) and a Ceq 2000 XL sequencer (Beckman Coulter) according to the manufacturer's instruction. Vector primers T7 and SP6 were used for sequencing reactions. The deduced protein sequences of *narG* genes were aligned using the CLUSTALX software version V.1.0.1 [16]. The phylogenetic tree based on amino acids alignments (approximately 210-220 amino acids), was constructed by neighbour-joining method with 100 replicate trees. NarG from the Archea *Pyrobaculum aerophilum* was used as outgroup.

Nucleotide accession numbers

The sequences obtained were deposited in the GenBank sequence database under accession numbers AY453347 to AY453384.

Determination of potential nitrate reductase activity

The potential activity of the nitrate reductase was determined by anaerobic incubation of soil following a modified protocol of Kandeler [17]. Briefly, 0.2 g rhizospheric soil was weighed in five replicates into 2.0 ml reaction tubes. 33.3 µg of 2,4-dinitrophenol per g soil (fresh weight) were added to inhibit the nitrite reductase. After 24 h incubation in 1 mM KNO_3 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 x g. The accumulated nitrite in the supernatant was determined by colorimetric reaction.

Statistics

narG RFLP-fingerprints were analysed by the software package Quantity One® (Version 4.2.1) for image analysis, and a band-matching table was generated containing the molecular weight and the trace of each detected band normalised by the molecular weight marker. Based on the band-matching table, bands with similar molecular weights were grouped together in band classes. A cluster analysis was performed on the trace of the respective band class with the statistic software package SAS 8.0. Ward's algorithm was selected for the clustering method. The environmental variables were ranked according to their importance by a canonical correspondence analysis (CCA) in CANOCO (Version 4), a software for canonical community ordination [18]. The statistical significance of the variables was tested by a Monte Carlo permutation test carrying out 1000 permutations restricted by the split-plot design.

The data of the enzyme activity of the nitrate reductase were transformed by natural logarithm and analysed as a split-plot design with repeated measurements using the mixed model procedure in the SAS 8.0 statistical analysis package.

Results

narG fingerprints

Amplification of the *narG* genes with degenerated primers yielded in all samples a band of the expected size (approximately 650 bp, Fig. 5.1). Restriction analysis of the purified *narG* PCR products of rhizosphere soil samples from June and October 2002 showed no difference between the two pCO_2 levels (Fig. 5.2 and Fig. 5.3). In contrast, the *narG* RFLP-fingerprints are grouped according to the sampling date in two separate clusters (Fig. 5.3).

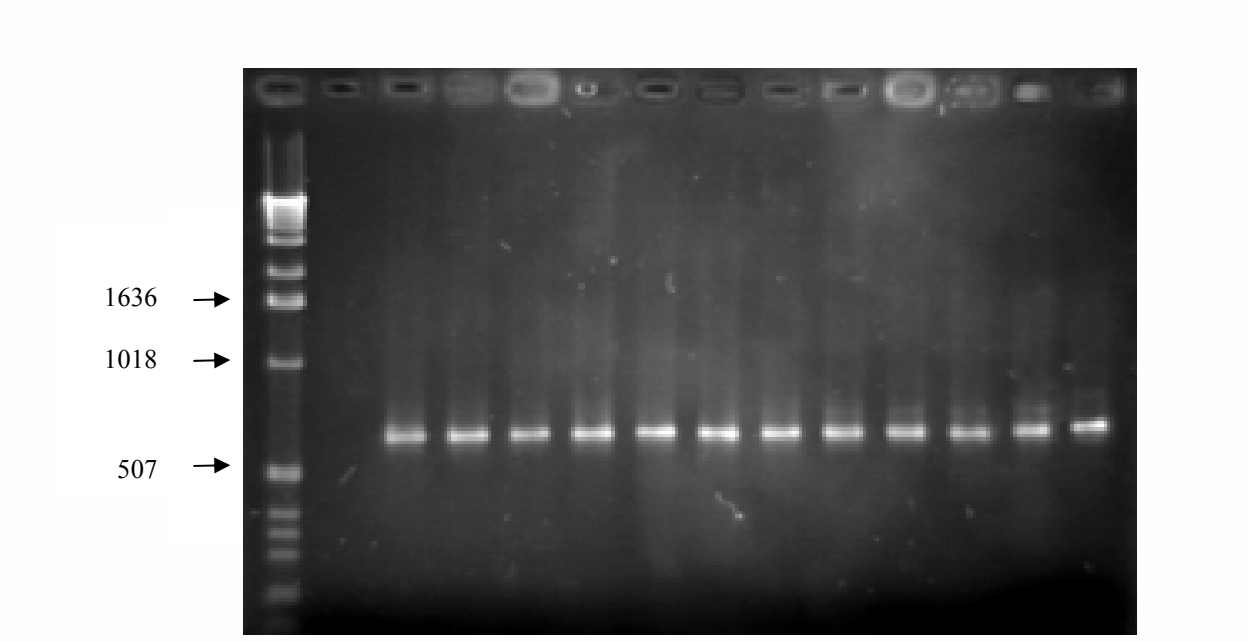

Fig. 5.1: Amplified *narG* products from rhizospheric soil sampled under *Trifolium repens* in June 2002, lane 1: Molecular weight marker 1 kb, lane 2: negative control, lanes 3-8: PCR products from samples obtained under high pCO_2 levels, with low and high N-fertilisation, lanes 9-14: PCR products from samples obtained under low pCO_2 levels, with low and high N-fertilisation.

Whereas a high variation in the composition of the nitrate reducers was recorded within the soils sampled in October, the nitrate-reducing community structure in June appeared to be rather stable over all treatments. Only four *narG* fingerprints obtained from June samples were grouped apart from the others, i.e. three samples from the third ring under elevated pCO_2 and the second replicate of *Trifolium repens* with high nitrogen fertilisation and no CO_2 fumigation (Fig. 5.3, cluster 5). These differences in *narG* RFLP-fingerprints corresponded to lower pH values ($pH \leq 5$).

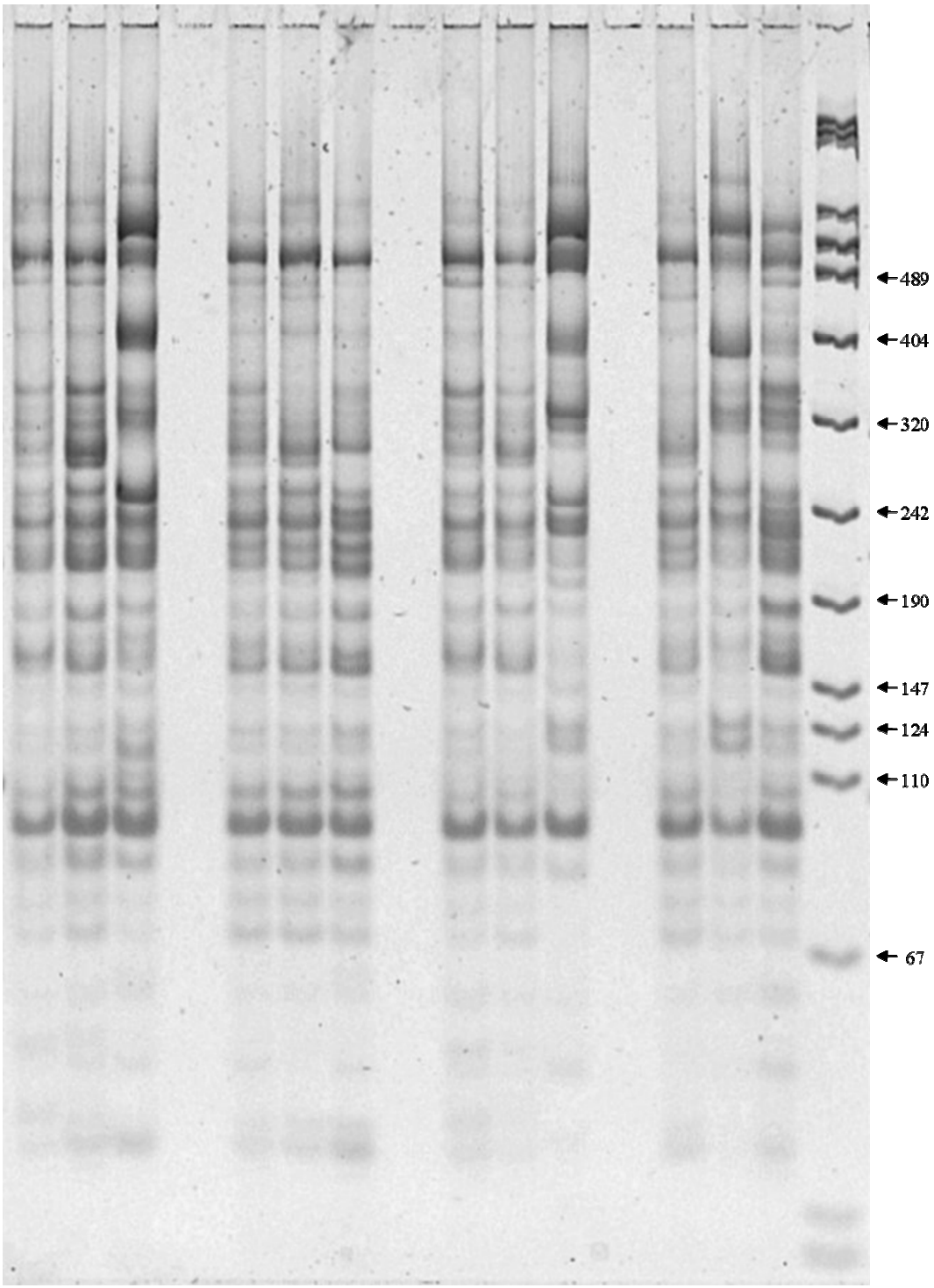

Fig. 5.2: RFLP-fingerprints of the nitrate-reducing community under *Trifolium repens* in June 2002; lanes 1-3: elevated pCO_2, low nitrogen fertilisation, replicates 1, 2, and 3, lanes 4-6: ambient pCO_2, low nitrogen fertilisation, replicates 1, 2, and 3, lanes 7-9: elevated pCO_2, high nitrogen fertilisation, replicates 1, 2, and 3, lanes 10-12: ambient pCO_2, high nitrogen fertilisation, replicates 1, 2, and 3, lanes 13: Molecular weight marker VIII (Roche).

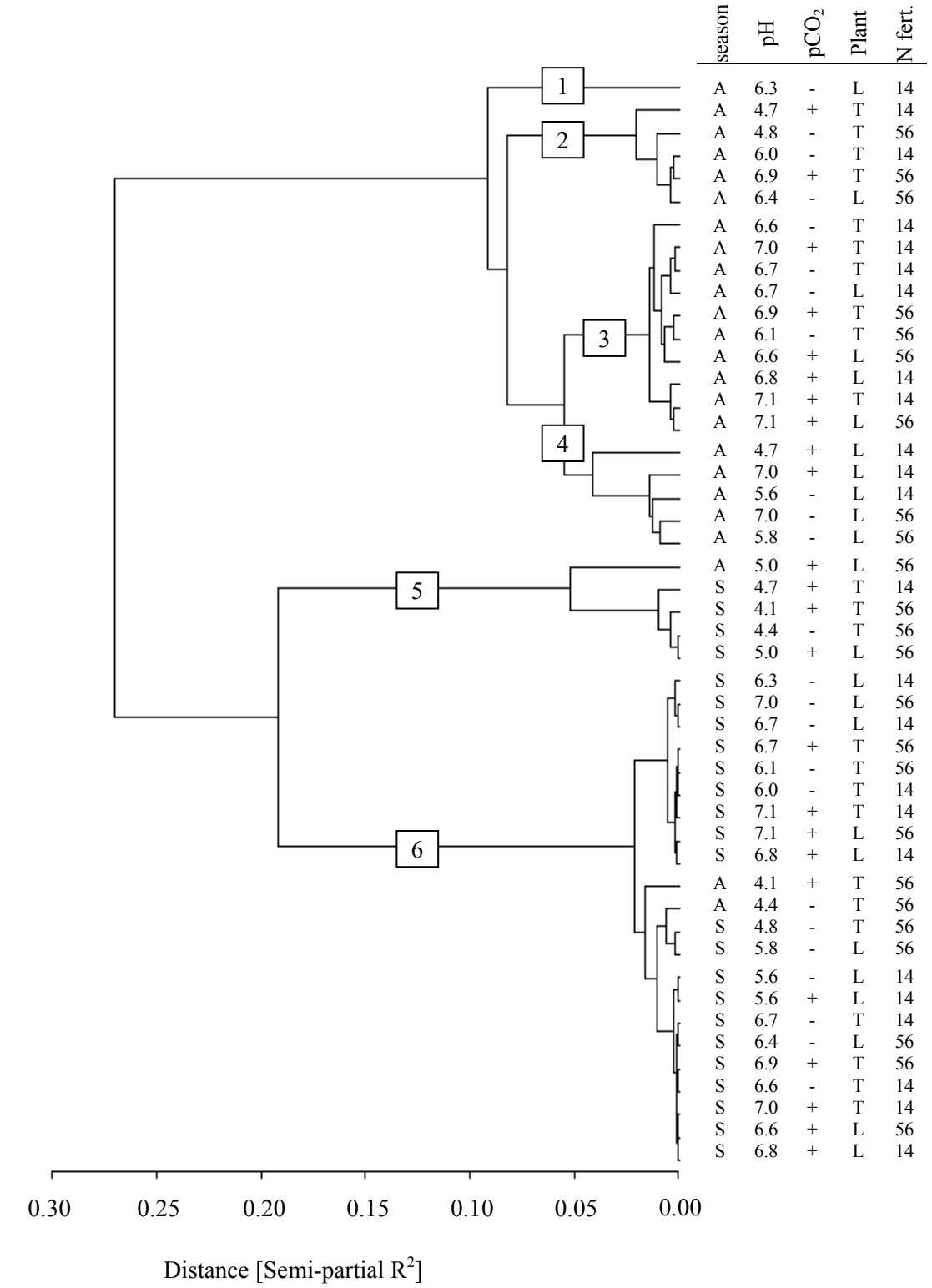

Fig. 5.3: Dendrogram of *narG* RFLP-fingerprints from rhizospheric soil under elevated (+) and ambient (-) pCO_2 sampled under *Lolium perenne* (L) and *Trifolium repens* (T) with two N-fertilisation levels (14=14 g N m^{-2} a^{-1}, 56=56 g N m^{-2} a^{-1}) in June (S=summer) and October (A=autumn) 2002. pH values of the corresponding bulk soil are given in the second column.

Tab. 5.1**:** Ranking environmental variables in importance by their marginal (left) and conditional (right) effects on the nitrate-reducing community, as obtained by forward selection.

Marginal Effects			Conditional Effects			
Variable	λ_1	*P*	Variable	λ_a	*P*	cum (λ_a)
season	0.19	0.005	season	0.19	0.005	0.19
pH	0.18	0.002	pH	0.18	0.002	0.37
N fertilisation	0.06	0.005	pCO_2	0.05	0.191	0.42
plant species	0.04	0.219	plant species	0.03	0.264	0.45
pCO_2	0.03	0.942	N fertilisation	0.03	0.440	0.48

λ_1: fit or eigenvalue with one variable only; λ_a: additional fit or increase in eigenvalue; cum (λ_a): cumulative total of eigenvalues;
P = significance level of the effect, as obtained with a Monte Carlo permutation test under the null model with 1000 random permutations.

The ranking of the environmental variables according to their importance in canonical correspondence analysis confirmed, that mainly season and pH affected the composition of the nitrate-reducing community explaining 37 % of the variance observed within the nitrate-reducing community (Tab. 5.1).

Phylogenetic analysis

To verify the identity of the amplified gene fragments we established a clone library based on two samples, i.e. PCR products from soil sampled under *Lolium perenne* in June 2002 with low nitrogen fertilisation under ambient and elevated pCO_2 levels, respectively. One hundred and sixty recombinants were screened by RFLP and grouped into *narG* RFLP types according to their restriction profile. We obtained 45 different *narG* RFLP types, with one dominant type accounting for almost 30 % of all analysed clones (Fig. 5.4).

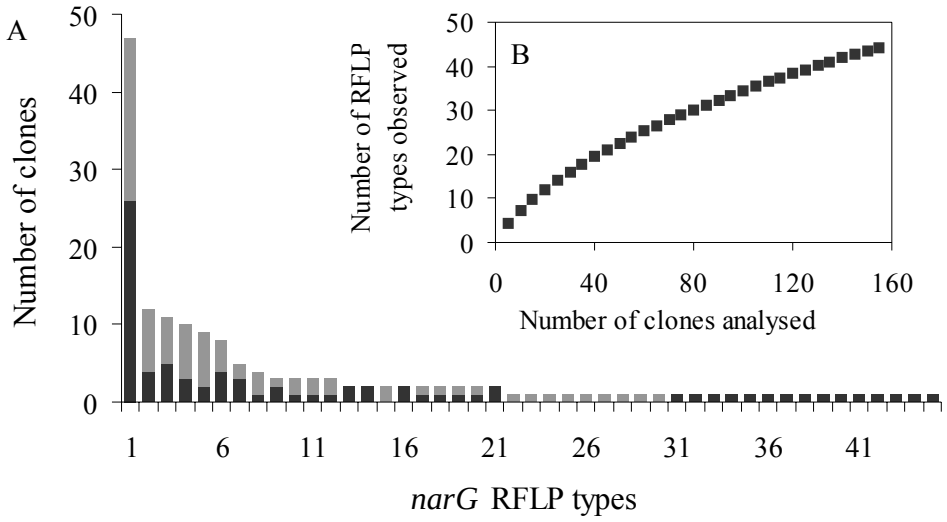

Fig. 5.4: (A) Distribution of *narG* RFLP types in the rhizosphere of *Lolium perenne* in June 2002 with low N-fertilisation under 1) ambient pCO_2 (grey bar) and 2) elevated pCO_2 (black bar); (B) Rarefaction curve of all 160 analysed clones.

Rarefaction analysis estimating the diversity at a given number of studied individuals demonstrated that the 160 clones screened were still insufficient to cover the entire diversity within the nitrate-reducing population; hence, a greater number of analysed clones would detect even higher diversity. Representatives of different RFLP types were sequenced and their identity verified. One clone was dismissed because there was only poor sequence homology to *narG*, but all other clones were identified as *narG* genes. The deduced amino sequences were aligned according to their phylogeny (Fig. 5.5). Twenty-six sequences of known organisms were included in the phylogenetic analysis in addition to the 38 sequences obtained from our clones. Most of the recombinants clustered together either with NarG of *Actinomycetes*, associated to the gram-positive bacteria, or with NarG of *Brucella melitensis biovar suis*, an α-proteobacteria. In addition to this large cluster, two more sequences appeared within the gram-negative bacteria: clone F29 was related to NarG of the β-proteobacteria *Ralstonia* sp. and *Burkholderia pseudomallei*, whereas F23 fell outside the NarG cluster of the β-and γ-proteobacteria. Furthermore, two clones were found to be close to *Thermus thermophilus*.

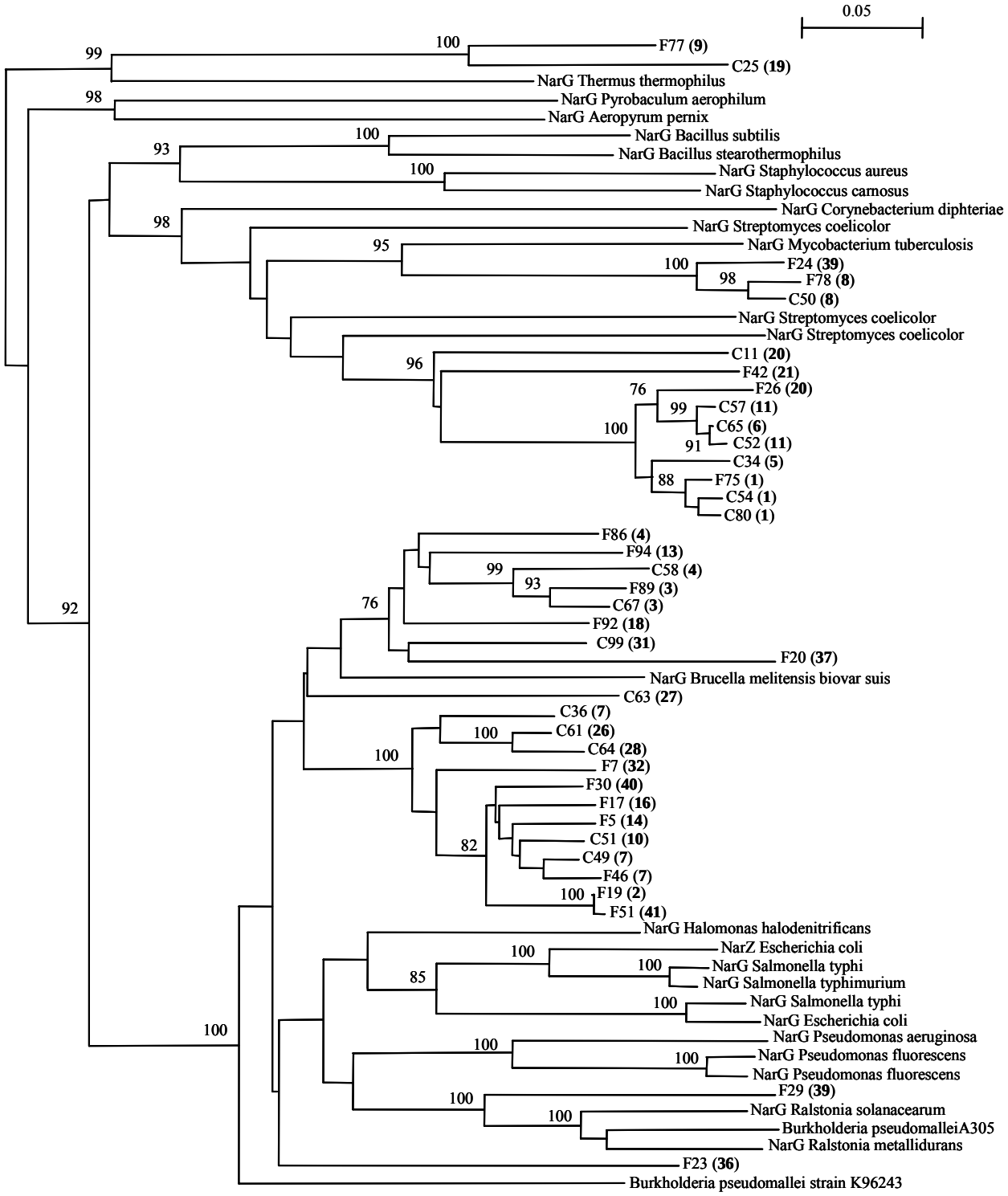

Fig. 5.5: Phylogenetic analysis of deduced protein sequences from 38 *narG* clones. The corresponding RFLP types are indicated in brackets and bold after the clone number. Only bootstrap values above 75 % are given.

Nitrate reductase activity

The results of the potential nitrate reductase activity are presented in Fig. 5.6. In June, the activity in all treatments was at the same level, with spatial variability being very high. Neither CO_2 enrichment nor N fertilisation or plant species had an effect in June. In contrast, enzyme activity in October responded strongly to elevated pCO_2. In the rhizosphere of *Lolium perenne* under low nitrogen fertilisation the nitrate reductase activity was reduced by 84 % (P=0.008), under high nitrogen fertilisation, however, this effect was not significant. Similarly, the decrease in the enzyme activity was less pronounced and statistically not significant in the rhizosphere of *Trifolium repens*.

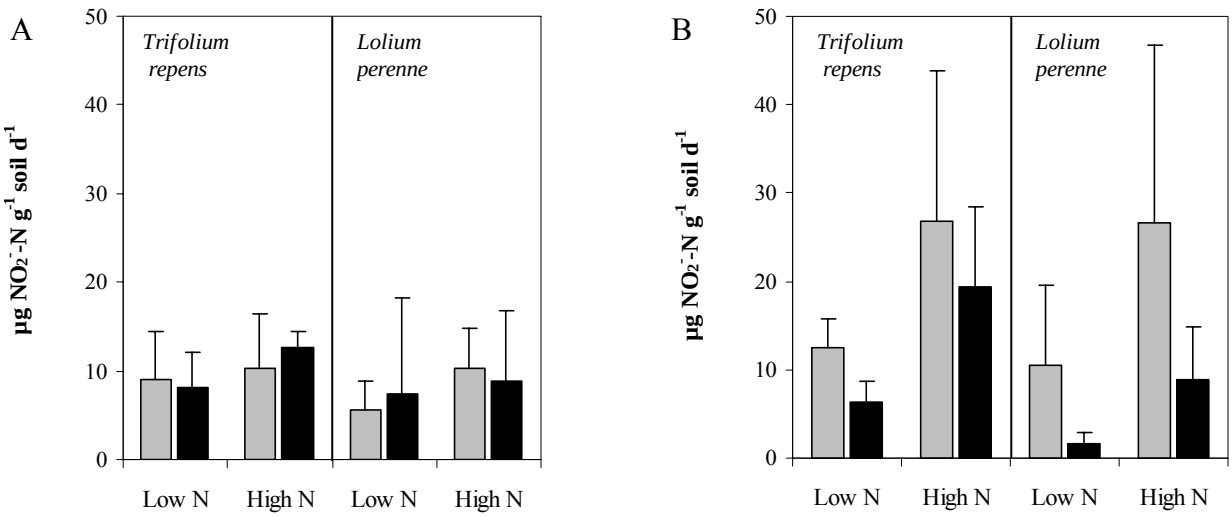

Fig. 5.6: Data of the nitrate reductase activity in µg NO_2^--N g^{-1} dry soil d^{-1} in rhizospheric soil of *Trifolium repens* and *Lolium perenne* under ambient (grey bars) and elevated (black bars) atmospheric pCO_2 sampled in A) June and B) October 2002. Low N corresponds to annual N-fertilisation rates of 14 g N m^{-2} a^{-1} and high N to 56 g N m^{-2} a^{-1}.

Analysis of variance for both dates combined showed that the amount of nitrogen fertiliser was primarily responsible for altering nitrate reductase activity significantly (P=0.016), whereas the plant species and pCO_2 levels had only marginally significant effects, with P=0.059 and P=0.058 (data not shown). Initially, also pH was included in the model as covariable, but as it proved to be of no statistical significance (P=0.498), it was disregarded in all further analysis. Due to the significant interaction of pCO_2 and date (P=0.040), we

calculated the ANOVA statistics for each date separately. In June no significant effect was found (data not shown). In October all three main factors significantly affected nitrate reductase activity, with the N level again being most significant, whereas interactions were negligible (Tab. 5.2).

Tab. 5.2: Results of analysis of variance (procedure MIXED in SAS software package) of nitrate reductase activity in the rhizosphere of *Lolium perenne* and *Trifolium repens* at two N fertilisation levels and under two levels of atmospheric pCO_2 sampled in October 2002.

Source of variance	Significance	*P* value
block	n.s.	0.1543
pCO_2	*	0.0246
plant species	*	0.0361
pCO_2 x plant species	n.s.	0.1914
N fertilisation	**	0.0044
pCO_2 x N fertilisation	n.s.	0.3403
N fertilisation x plant species	n.s.	0.4461
pCO_2 x plant species x N fertilisation	n.s.	0.7417

Discussion

1. Factors controlling the structure of the nitrate-reducing community

In our study, 48 RFLP-fingerprints of *narG* PCR products were analysed with four experimental factors: atmospheric pCO_2, plant species, nitrogen fertilisation and sampling date. Both CO_2 levels yielded similar band patterns (Fig. 5.2 and Fig. 5.3). Elevated atmospheric pCO_2 apparently did not affect the composition of the nitrate-reducing community in the rhizosphere of *Lolium perenne* and *Trifolium repens*. Similar results were obtained recently in the Swiss FACE experiment for *Pseudomonas* possessing the *narG* gene in the rhizosphere of *Lolium perenne* under low N fertilisation [19]. Also, *Rhizobia leguminosarum* biovar. *trifolii* collected from *Trifolium repens* under low N at the Swiss FACE site, showed no response to elevated atmospheric pCO_2 in June 2002 (Stöber, personal communication). These results contradict earlier observations in the Swiss FACE experiment where shifts were detected within the *Rhizobia* strains after 3 years of fumigation [20]. Moreover, Marilley et al. [5] detected major alterations in the composition of the rhizosphere bacterial community, with the frequency of *Pseudomonas* ssp. being reduced under *Trifolium repens* and enhanced under *Lolium perenne* after 5 years of elevated pCO_2. We assume that in the first years of the Swiss FACE, the conversion of ploughed field into perennial grassland provoked a C- and N-sink [21, 22]. Within this labile system, atmospheric CO_2 enrichment had a significant impact on C- and N-dynamics of the soil and hence on soil microorganisms. After 10 years of perennial grassland, however, soil organic matter pools were approximately replenished and effects of the CO_2 treatments on soil microbial communities became negligible.

Our experiment did show, however, that sampling date clearly affected the composition of the nitrate-reducing microorganisms (Tab. 5.1). Seasonal shifts in the structure of microbial communities in soils are well known [23]. They have been attributed partially to changes in water content and soil mineral N-availability [24]. As the period preceding sampling in June was rather hot (>20 °C mean daily air temperature) and almost no rain had fallen for more than two weeks, we assume that the low water potential in the soil matrix determined the homogeneous composition of the *narG* community. In October, however, the composition of *narG* communities in the rhizospheres of *Lolium perenne* and *Trifolium repens* differed much more than in June. As the soil was sampled after a rather wet period (130 mm precipitation

within 3 weeks), the conditions within the rhizosphere were probably more heterogeneous than in June.

After the season, the pH effect was ranked at the second position in the canonical correspondence analysis accounting for 18 % of the variance (P=0.002). Since most microorganisms have a specific pH optimum, as a matter of course also the functional group of nitrate reducers responded to differences in pH. In contrast to other studies, the N fertiliser level had no effect [25]. This may be explained by the rather high supply of nitrogen fertiliser even in plots with low N level. Equally, the plant species did not select for specific microorganisms in their rhizospheres on a larger scale. Even though rhizospheric soil samples obtained from *Trifolium repens* dominated cluster no. 2 and cluster no. 4 contained only samples from the rhizosphere of *Lolium perenne*, plant species was no significant environmental variable in the canonical correspondence analysis.

2. Response of nitrate reductase activity to elevated atmospheric pCO_2

The corresponding enzyme activity – the nitrate reductase activity – responded to variations in atmospheric pCO_2, plant species and N level, yet not in June. During June sampling, the nitrate reductase activity was probably limited by the low water potential in the soil matrix (approx. 18 % water content). N_2O fluxes determined *in situ* in experimental plots of *Lolium perenne* directly after the June sampling confirmed that, at this time, denitrification activity was very low. Only after fertiliser application and rainfall events was N_2O emitted in plots with high N supply [26]. The high spatial variability of the observed nitrate reductase activity is in line with the heterogeneous distribution of denitrifying activity reported for agricultural soils [27, 28].

In October, however, nitrate reductase activity responded strongly to elevated pCO_2. Contrary to our hypothesis that higher amounts of readily available C and lower oxygen potential in the rhizosphere would boost nitrate respiration, we observed a reduced nitrate reductase activity. This finding contrasts with other studies, which show that elevated atmospheric pCO_2 favours potential denitrification, whereby these studies attribute higher denitrification rates to enhanced C availability under high atmospheric pCO_2 levels [7, 29]. Martin-Olmedo et al. [30], however, deduced from stochiometric calculations that root-derived C has only a minor effect on denitrifying activities, as has also been reported by Robinson [8]. With respect to the

oxygen potential, after the wet period preceding the soil sampling, apparently no differences in pO_2 existed between the CO_2 treatments.

On the October sampling date, we rather suggest that nitrate availability was the dominant factor controlling nitrate reductase activity. Apparently, nitrate availability in the rhizosphere of *Lolium perenne* and *Trifolium repens* depended on N-fertilisation rate, but most likely was also affected by CO_2 treatment and plant species. The lowest nitrate reductase activity was detected in the rhizosphere of *Lolium perenne* at elevated atmospheric pCO_2 and low N fertilisation (Fig. 5.6). Since this non-legume plant forms greater root biomass than legumes under N-limiting conditions that are aggravated under elevated pCO_2 [31], N-immobilisation by root-decomposing microorganisms probably reduced nitrate availability, particularly at elevated pCO_2. This conclusion is supported by Richter et al. [32], who detected a trend to increased N immobilisation under CO_2 enrichment in the Swiss FACE experiment for *Lolium perenne*. In contrast, N limitation for the nitrate-reducing community was less pronounced under *Trifolium repens* due to symbiotic N-fixation of this legume. Moreover, roots of *Trifolium repens* have a higher N content compared to *Lolium perenne*, lowering the biosynthetic need for N during decomposition.

3. Relationship between diversity of the nitrate-reducing community and its activity

Linking functional communities and their respective activity is still a very new field in molecular ecology. Nevertheless, this approach is crucial for our understanding of how soil ecosystems function and respond to altered environmental factors. We infer from our data that nitrate-reducers adapt to different CO_2 environments by regulating their enzyme activity. However, in our study, it is unfortunately difficult to relate accurately structure and activity of the nitrate reducing community since: (i) diversity of only a part of the nitrate reducing community was studied because nitrate reducing bacteria having the periplasmic nitrate reductase or having a not yet identified nitrate reductase were not taken into account and (ii) nitrate reductase activity can be influenced not only by the diversity of the corresponding functional community but also by its density which was not determined in this study.

In conclusion, further developments are necessary to target in the future all the bacteria genetically able to reduce nitrate to nitrite but also to determine their density for a better understanding of the relation between structure/density and activity.

Acknowledgements

This work was supported by the National Science Foundation (grant number DEB0120169). We would like to thank Herbert Blum for access to the Swiss FACE facility, Dagmar Mezger and Stéphanie Hallet for technical assistance, Andreas Büchse for his advice in SAS statistics, and Michael Sadowsky for lively discussions.

References

[1] Zak, D. R., Pregitzer, K. S., King, J. S., and Holmes, W. E. (2000) Elevated atmospheric CO_2, fine roots and the response of soil microorganisms: a review and hypothesis. New Phytol. 147: 201-222.

[2] Suter, D., Frehner, M., Fischer, B. U., Nösberger, J., and Lüscher, A. (2002) Elevated CO_2 increases carbon allocation to the roots of *Lolium perenne* under free-air CO_2 enrichment but not in a controlled environment. New Phytol. 154: 65-75.

[3] Fitter, H., Graves, J. D., Wolfenden, J., Self, G. K., Brown, T. K., Bogie, D., and Mansfield, T. A. (1997) Root production and turnover and carbon budgets of two contrasting grasslands under ambient and elevated atmospheric carbon dioxide concentrations. New Phytol. 137: 247-255.

[4] Schortemeyer, M., Hartwig, U. A., Hendrey, G. R., and Sadowsky, M. J. (1996) Microbial community changes in the rhizospheres of white clover and perennial ryegrass exposed to free air carbon dioxide enrichment (FACE). Soil Biol. Biochem. 28: 1717-1724.

[5] Marilley, L., Hartwig, U. A., and Aragno, M. (1999) Influence of an elevated atmospheric CO_2 content on soil and rhizosphere bacterial communities beneath *Lolium perenne* and *Trifolium repens* under field conditions. Microbial Ecol. 38: 39-49.

[6] Lashof, D. A., and Ahuja, D. R. (1990) Relative contributions of greenhouse gas emissions to global warming. Nature 344: 529-531.

[7] Smart, D. R., Ritchie, K., Stark, J. M., and Bugbee, B. (1997) Evidence that elevated CO_2 levels can indirectly increase rhizosphere denitrifier activity. Appl. Environ. Microbiol. 63: 4621-4624.

[8] Robinson, D., and Conroy, J. P. (1999) A possible plant-mediated feedback between elevated CO_2, denitrification and the enhanced greenhouse effect. Soil Biol. Biochem. 31: 43-53.

[9] Baggs, E. M., Richter, M., Cadisch, G., and Hartwig, U. A. (2003) Denitrification in grass swards is increased under elevated atmospheric CO_2. Soil Biol. Biochem. 35: 729-732.

[10] Rogers, H. H., Runion, G. B., and Krupa, S. V. (1994) Plant response to atmospheric CO_2 enrichment with emphasis on roots and rhizosphere. Environ. Pollut. 83: 115-189.

[11] Körner, C. (2000) Biosphere responses to CO_2 enrichment. Ecol. Appl. 10: 1590-1619.

[12] Philippot, L. (2002) Denitrifying genes in bacterial and archeal genomes. Biochim. Biophys. Acta 1577: 355-376.

[13] Philippot, L., Piutti, S., Martin-Laurent, F., Hallet, S., and Germon, J. C. (2002) Molecular analysis of the nitrate-reducing community from unplanted and maize-planted soils. Appl. Environ. Microbiol. 68: 6121-6128.

[14] Chèneby, D., Hallet, S., Mondon, A., Martin-Laurent, F., Germon, J. C., and Philippot, L. (2003) Genetic characterization of the nitrate-reducing community based on *narG* nucleotide sequence analysis. Microbial Ecol. 46: 113-121.

[15] Van Kessel, C., Nitschelm, J., Horwarth, W. R., Harris, D., Walley, F., Lüscher, A., and Hartwig, U. A. (2000) Carbon-13 input and turnover in a pasture soil exposed to long-term elevated atmospheric CO_2. Global Change Biol. 6: 123-135.

[16] Thompson, J. D., Plewniak, F., and Poch, O. (1999) A comprehensive comparison of multiple sequence alignment programs. Nucleic Acids Res. 27: 2682-2690.

[17] Kandeler, E. (1995) Nitrate reductase activity. In: Methods in soil biology. Schinner, F., Öhlinger, R., Kandeler E., and Margesin, R., (eds). Springer Berlin Heidelberg, Germany: pp. 176-179.

[18] ter Braak, C. F. J., and Šmilauer, P. (1998) CANOCO reference manual and user's guide to Canoco for Windows: Software for canonical community ordination (version 4). Microcomputer Power, Ithaca, NY: pp. 352.

[19] Roussel-Delif, L., Tarnawski, S., Hamelin, J., Philippot, L., Aragno, M., and Fromin, N. (2004) Frequency and diversity of nitrate reductase genes among nitrate-dissimilating *Pseudomonas* in the rhizosphere of perennial grasses grown in field conditions. Microbial Ecol. 49: 63-72.

[20] Montealegre, C. M., van Kessel, C., Blumenthal, J. M., Hur, H.-G., Hartwig, U. A., and Sadowsky, M. J. (2000) Elevated atmospheric CO_2 alters microbial population structure in a pasture ecosystem. Global Change Biol. 6: 475-482.

[21] Hartwig, U. A., Lüscher, A., Nösberger, J., and van Kessel, C. (2002) Nitrogen-15 budget in model ecosystems of white clover and perennial ryegrass exposed for four years at elevated atmospheric pCO_2. Global Change Biol. 8: 194-202.

[22] Van Kessel, C., Horwath, W. R., Hartwig, U. A., Harris, D., and Lüscher, A. (2000) Net soil carbon input under ambient and elevated CO_2 concentrations: isotopic evidence after 4 years. Global Change Biol. 6: 435-444.

[23] Buckley, D. H., and Schmidt, T. M. (2003) Diversity and dynamics of microbial communities in soils from agro-ecosystems. Environ. Microbiol. 5: 441-452.

[24] Bardgett, R. D., Lovell, R. D., Hobbs, P. J., and Jarvis, S. C. (1999) Seasonal changes in soil microbial communities along a fertility gradient of temperate grasslands. Soil Biol. Biochem. 31: 1021-1030.

[25] Clegg, C. D., Lovell, R. D. L., and Hobbs, P. J. (2003) The impact of grassland management regime on the community structure of selected bacterial groups in soils. FEMS Microbiol. Ecol. 43: 263-270.

[26] Baggs, E. M., and Blum, H. (2004) CH_4 oxidation and emissions of CH_4 and N_2O from *Lolium perenne* swards under elevated atmospheric CO_2. Soil Biol. Biochem. 36: 713-723.

[27] Murray, R. E., Feig, Y. S., and Tiedje, J. (1995) Spatial heterogeneity in the distribution of denitrifying bacteria associated with denitrification activity zones. Appl. Environ. Microbiol. 61: 2791-2793.

[28] Šimek, M., Elhottová, D., Klimeš, F., and Hopkins, D. W. (2004) Emissions of N_2O and CO_2, denitrification measurements and soil properties in red clover and ryegrass stands. Soil Biol. Biochem. 36: 9-21.

[29] Carnol, M., Hogenboom, L., Jach, M. E., Remacle, J., and Ceulemans, R. (2002) Elevated atmospheric CO_2 in open top chambers increases net nitrification and potential denitrification. Global Change Biol. 8: 590-598.

[30] Martin-Olmedo, P., Rees, R. M., and Grace, J. (2002) The influence of plants grown under elevated CO_2 and N fertilisation on soil nitrogen dynamics. Global Change Biol. 8: 643-657.

[31] Hebeisen, T., Lüscher, A., Zanetti, S., Fischer, B. U., Hartwig, U. A., Frehner, M., Hendrey, G. R., Blum, H., and Nösberger, J. (1997) Growth response of *Trifolium repens* L. and *Lolium perenne* L. as monoculture and bi-species mixture to free air CO_2 enrichment and management. Global Change Biol. 3: 149-160.

[32] Richter, M., Hartwig, U. A., Frossard, E., Nösberger, J., and Cadisch, G. (2003) Gross fluxes of nitrogen in grassland soil exposed to elevated atmospheric pCO_2 for seven years. Soil Biol. Biochem. 35: 1325-1335.

6 Functional stability of the nitrate-reducing community in grassland soils towards high nitrate supply

Submitted to Soil Biology and Biochemistry

K. Deiglmayr (1), L. Philippot (2), E. Kandeler (3)

(1) Institute for Crop Production and Grassland Research, University of Hohenheim, D-70593 Stuttgart, Germany, (2) Laboratory of Soil Microbiology and Geochemistry, INRA-University of Burgundy, F-21065 Dijon Cedex, France, (3) Institute for Soil Science, University of Hohenheim, D-70593 Stuttgart, Germany

Reprinted with permission from Elsevier.

Abstract

To study the effects of short-term fluctuation of nitrate concentrations on the nitrate-reducing community, repacked soil cores were amended with 0, 100 and 300 µg NO_3^--N g^{-1} dry soil and incubated for 3, 7 and 14 days. The nitrate reductase activity was determined by colorimetric measurement of the accumulated nitrite after 24 h of anaerobic incubation. Simultaneously, the community structure of nitrate-reducing microorganisms was characterised by RFLP-PCR using the functional gene *narG*, which encodes the catalytic site of the membrane-bound nitrate reductase. The community structure remained constant over the experimental period indicating that this functional community is characterised by a high resistance towards fluctuating nitrate concentrations. The nitrate reductase activity responded sensitively to anaerobic conditions after onset of the experiment. Decreases in nitrate concentration as well as increasing pH values indicated a very active nitrate-reducing community under nitrate addition. Surprisingly, inhibition of nitrite reductase by 2,4-dinitro-phenol, which is a precondition for the measurement of nitrate reductase activity, could not be achieved in the 100 µg NO_3^--N treatment despite increased concentrations of the inhibitor. However, comparison of the nitrate reductase in the control and the 300 µg NO_3^--N treatment showed a significant increase in the latter at day 3. No further differences were observed at days 7 and 14 which suggest a high resilience of the nitrate reductase activity.

Introduction

Altered environmental conditions might cause either changes in the activity status of soil microorganisms or shifts in their community structure. The nitrate-reducing community, which is responsible for the first step in denitrification, presents a model functional community to study the prevailing effect, since methods to explore the community structure as well as to determine the corresponding enzyme activity have been well established (Philippot et al., 2002; Chèneby et al., 2003; Kandeler, 1995). By analysis of the functional gene *narG*, which encodes the catalytic site of the membrane-bound nitrate reductase, rhizosphere effects as well as effects of pH and season on the structural composition were observed (Philippot et al., 2002; Deiglmayr et al., 2004; Enwall et al., 2005). In contrast, the major factors controlling nitrate reductase activity were total organic carbon content and nitrate availability (Reddy and Reddy, 1998; Deiglmayr et al., 2004). None of the studies up to date revealed any effect of nitrate availability on the structure of nitrate-reducing microorganisms in soils despite its pivotal role in controlling the level of enzyme activity. Therefore this study focused on the effect of extreme nitrate addition to nitrogen limited soils in order to verify the resistance of the nitrate-reducing community structure towards fluctuating nitrate concentrations. We aimed to test the hypothesis that nitrate reducers respond mainly by regulation of their enzyme activity to altered nitrate availability in oxygen limited soil environments, because low growth rates under anaerobiosis don't allow modulation of the community structure.

Material and Methods

Experimental design

For this purpose, repacked soil cores (100 cm^3) were incubated with nitrate added in form of KNO_3^- according to 100 and 300 µg NO_3^--N g^{-1} dry soil, which is equivalent to 21 and 64 mM nitrate in the soil water, respectively. The control was treated in the same way but without any nitrate addition. The soil, a loamy Luvisol with 1.1 % organic carbon and a pH of 5.6, was collected from permanent grassland at the University Hohenheim (0-10 cm) in February 2004. The soil cores, compressed to 1.4 g dry soil cm^{-3} and adjusted to 26 % of water content corresponding to approximately 98 % water filled pore space (WFPS), were kept in a dark chamber at 25 °C. Drying was prevented by moist cloths, which was checked routinely. After

3, 7 and 14 days three replicates of each treatment were sampled; nitrate and ammonia were extracted immediately with 0.0125 M $CaCl_2$ and kept at -20 °C prior analysis.

DNA extraction and PCR amplification

DNA was extracted from 0.3 g soil using the FastDNA Spin Kit for soil (BIO101, Qbiogene), following the protocol of the manufacturer. A *narG* fragment of 650 bp length was amplified using the primers narG1960f and narG2650r (Philippot et al., 2002). Three independent PCR amplifications were performed for each sample in a total of 50 µl containing 1x PCR buffer, 200 µM of each deoxyribonucleoside triphosphate, 1.25 mM of each primer, 2 U of *Taq* polymerase, and 20 ng of soil DNA. The cycling conditions of the PCR were as follows: an initial denaturation step at 95 °C, followed by a pause to facilitate a manual hot start. After adding the *Taq* polymerase the PCR was continued by a "touch down" with a denaturation step at 94 °C for 30 s, primer annealing at 59 °C for 30 s and elongation at 72 °C for 45 s. During the first 9 cycles, the annealing temperature was decreased by 0.5 °C each cycle until it reached 55 °C. The additional 26 cycles were performed at an annealing temperature of 55 °C. Cycling was completed by a final elongation step at 72 °C for 10 min. The size and presence of the amplification products were checked by electrophoresis in a 1.5 % agarose gel.

narG RFLP analysis

Purified PCR products were digested by *Alu*I restriction enzyme at 37 °C for 2 h and separated by electrophoresis on an 8 % polyacrylamide gel for 15 h at 8 mA. After staining with SYBER green II (Molecular Probes) the *narG* RFLP-fingerprints were scanned with a Phospho Imager. To verify the identity of the amplicons, aliquots of purified *narG* PCR products were cloned using the pGEM-T Easy Vector System (Promega) according to the manufacturer's instructions and sequenced using the DTCS-1 kit (Beckman Coulter) and a Ceq 2000 XL sequencer (Beckman Coulter). The sequences obtained were deposited in the GenBank sequence database under accession numbers DQ248877 to DQ248883.

Determination of potential nitrate reductase activity

Nitrate reductase activity was determined by anaerobic incubation of soil following a modified protocol (Deiglmayr et al., 2004). Briefly, 0.2 g soil was weighed in five replicates into 2.0 ml reaction tubes. To inhibit nitrite reductase activity, 7.5 µg of 2,4-dinitrophenol per g soil (fresh weight) were added. After 24 h incubation in 1 mM KNO_3 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 x g. The accumulated nitrite in the supernatant was determined by colorimetric reaction.

narG RFLP fingerprints were analysed by the software package Quantity One® (Version 4.2.1) for image analysis. A principal component analysis (PCA) was performed using CANOCO (Version 4) in order to explore shifts within the *narG* fingerprints (ter Braak and Šmilauer, 1998). The data of the enzyme activity of the nitrate reductase were evaluated by ANOVA in the SAS 8.0 statistical analysis package. Data were log-transformed to obtain a normal distribution as verified by Shapiro-Wilk test (P=0.0008). Significance was accepted at the $P \leq 0.05$ level of probability.

Results and Discussion

Responses of the nitrate-reducing community structure

The *narG* RFLP-fingerprints of the different nitrate treatments had highly similar band patterns over the 14 days of the experiment with only some variations in bands intensity (Fig. 6.1). The diagram of PCA reflected this result by an arbitrary scattering with no separation of the different nitrate treatments (Fig. 6.2). Identity of PCR products used to generate the RFLP-fingerprints has been verified by cloning and sequencing. The deduced amino sequences exhibited identities of 64 % to 87 % with NarG sequences from known strains. Apparently, even extreme additions of nitrate didn't result in a significant response on community level. Yet, we can not exclude that minor changes in community structure occurred, which were not detected by *narG* fingerprint analysis.

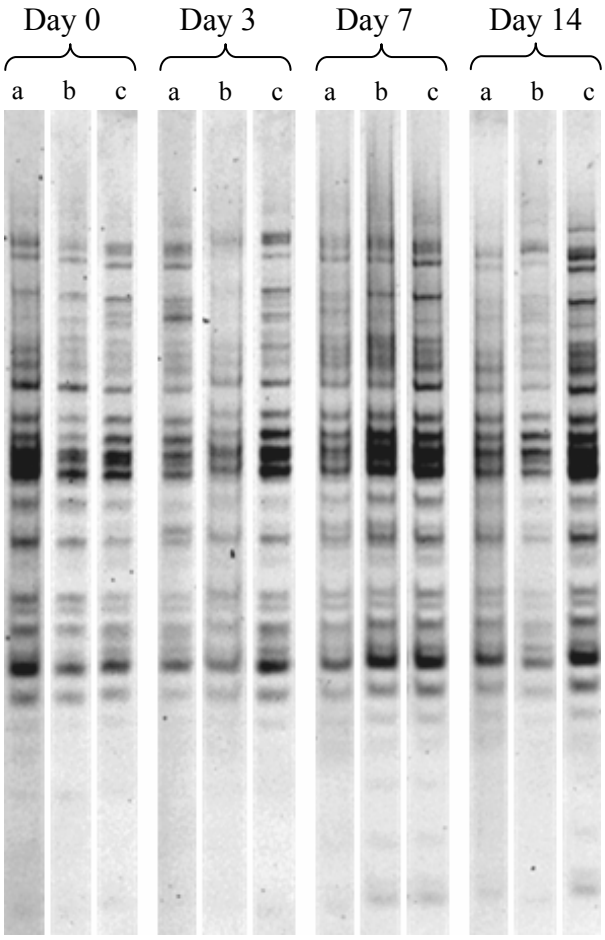

Fig. 6.1: Selected *narG* fingerprints from an incubation experiment with no (a), 100 (b) and 300 (c) µg NO_3^--N g^{-1} dry soil sampled at day 0, 3, 7 and 14.

Possibly, the incubation time of two weeks was too short to see any shifts by genomic DNA analyses. However, Mounier et al. (2004) detected visible changes of *narG* fingerprints within two weeks after mucilage amendment and freezing-thawing resulted in shifts in 16S rDNA patterns after only six days (Sharma, 2006). Moreover, since the incubation time was long enough to observe the reduction of virtually all nitrate added, an extension of incubation time would have been of little benefit. Previous studies on the impact of long-term N-fertilisation on nitrate-reducing microorganisms revealed no changes in the composition of the nitrate-reducing community except if pH changes were associated with the application of different N-fertilisers (Enwall et al., 2005). However, in a study of culturable nitrate-reducers Nijburg et al. (1997) found that NO_3^- availability influenced the community structure in sediments after 69 days of incubation at nitrate concentration exceeding continuously 50 mM.

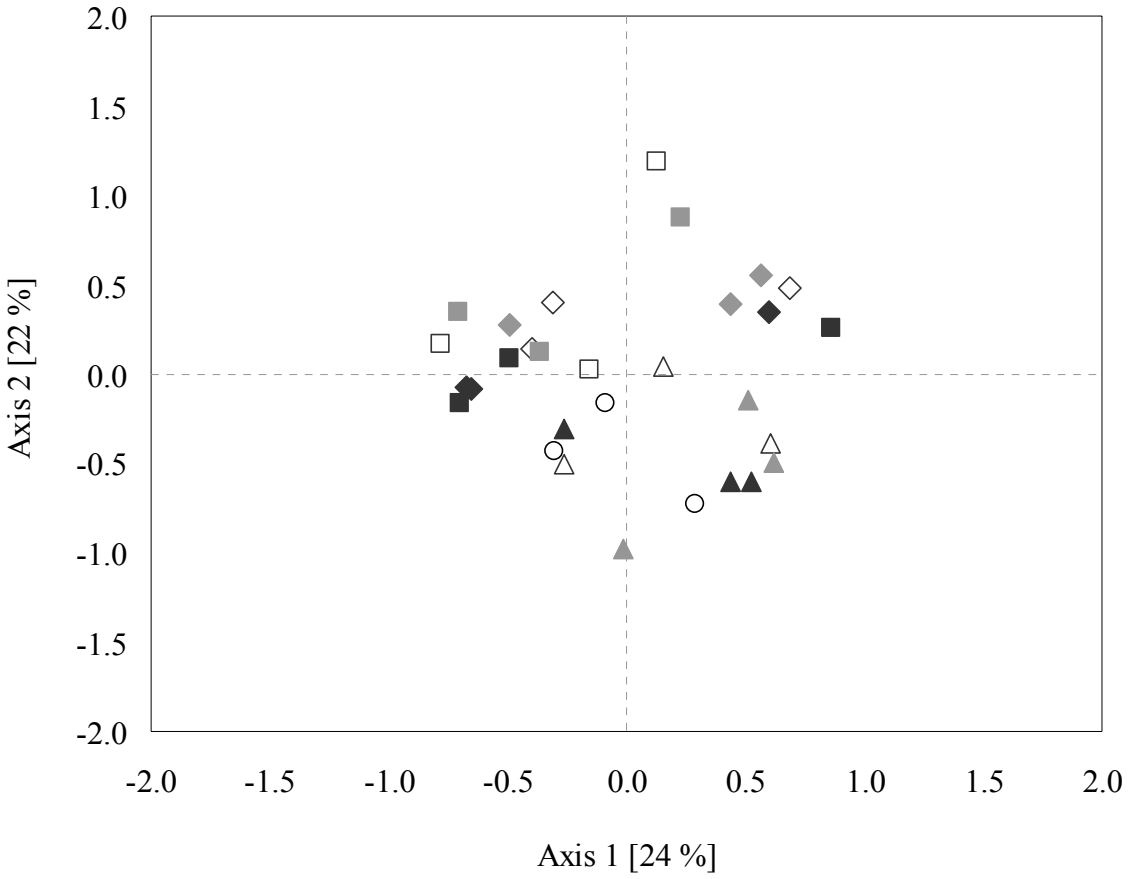

Fig. 6.2: Principal component analysis of *narG* fingerprints from an incubation experiment with no (open symbols), 100 (grey) and 300 (black) µg NO_3^--N g^{-1} dry soil at day 0 (○), day 3 (▲), day 7 (◆) and day 14 (■).

Yet, such conditions are unlikely to occur in agricultural managed lands. Single events of high nitrogen supply as in urine patches of grazing cattle with estimated 510 kg N ha^{-1} (Whitehead, 2000) or fertilizer applications are more frequent, which result in a transient increase of N_20 and/or N_2 emissions under oxygen limiting conditions (e. g. Clayton et al., 1997).

Responses through changes in nitrate reductase activity

In the control treatment the nitrate reductase activity significantly increased after 3 days and declined slightly after two weeks (Fig. 6.3). Under extreme nitrate additions of 300 µg NO_3^--N g^{-1} dry soil higher activities of nitrate reductase than in the control were recorded which were significantly increased (P<0.05) at day 3 of the incubation period. In contrast, with addition of 100 µg NO_3^--N g^{-1} dry soil no immediate response of nitrate reductase activity was observed (Fig. 6.3). After 14 days, however, when all nitrate had disappeared, the nitrate reductase activity approached the values of the control. The underlying causes for these different responses can be enlightened by analysing the monitored soil parameters in detail.

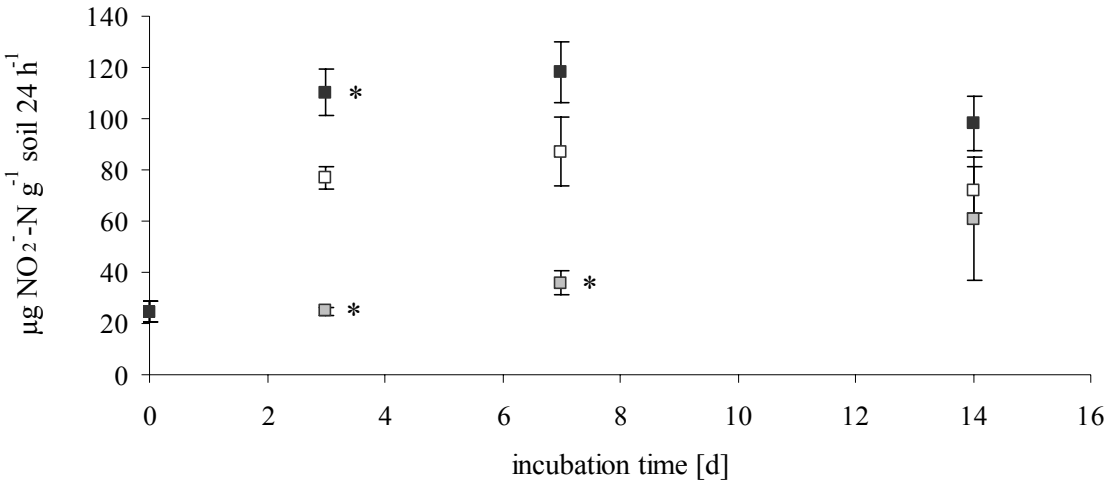

Fig. 6.3: Nitrate reductase activities [µg NO_2^--N g^{-1} 24 h^{-1}] in repacked soil cores amended with no (open symbols), 100 (grey symbols) and 300 (black symbols) µg NO_3^--N g^{-1} dry soil; bars represent means, whiskers indicate standard errors. * shows significant differences ($P\leq$ 0.05) between control and nitrate treatments for a given incubation time.

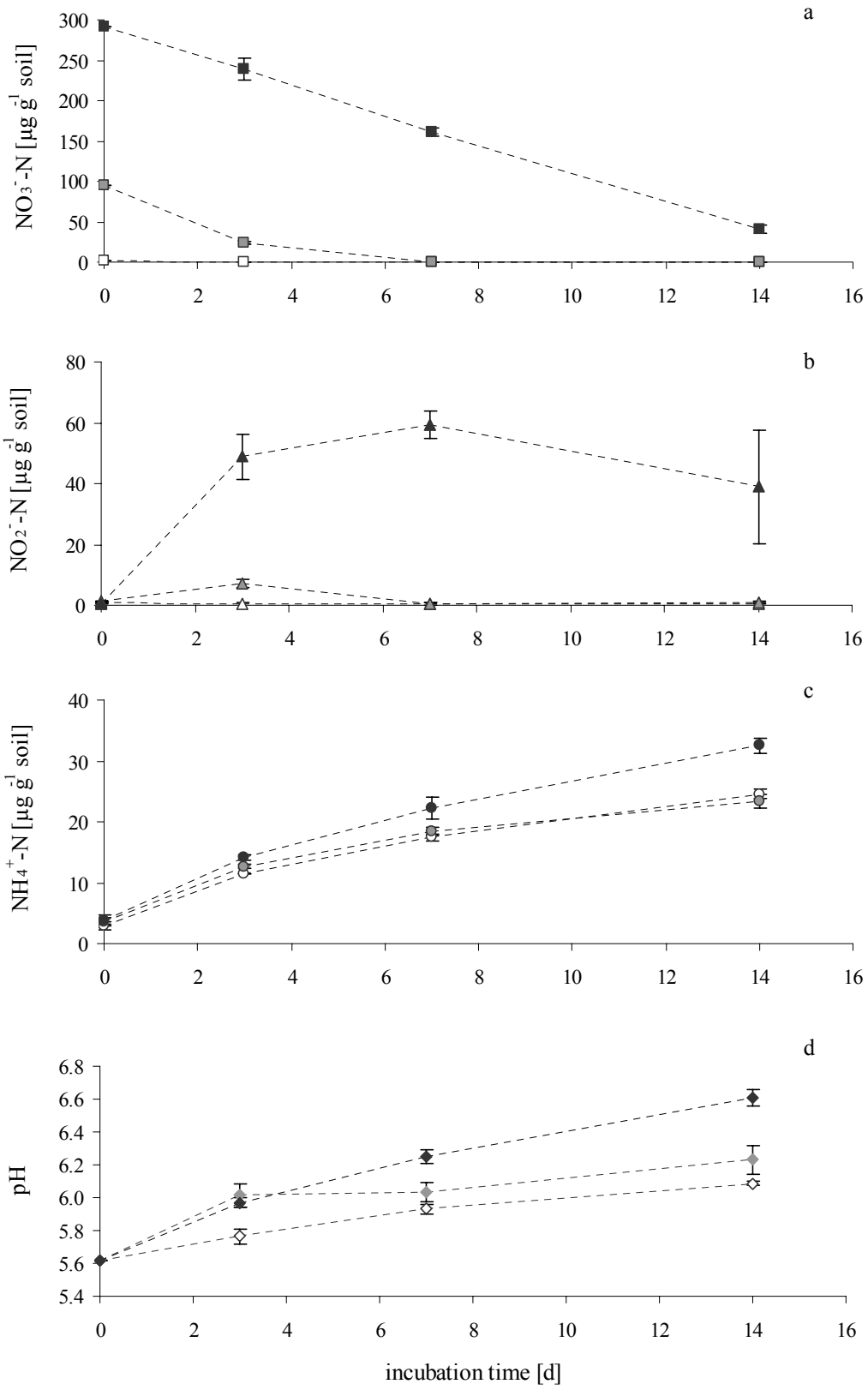

Fig. 6.4: Concentrations of (a) nitrate, (b) nitrite, (c) ammonium and (d) pH values in soils during an incubation experiment with no (open symbols), 100 (grey symbols) and 300 (black symbols) µg NO_3^--N g^{-1} dry soil. Whiskers indicate standard errors.

In the control treatment it is likely that the combined effect of anaerobic conditions, which predominate at WFPS exceeding 70 % (Sexstone et al., 1988; Bateman and Baggs, 2005), and the temperature increase of approximately 20 °C compared to preincubation conditions induced an elevated enzyme synthesis.

The occurrence of anaerobiosis after onset of the experiment was documented by the rapid reduction of nitrate and the accumulation of NH_4^+ that resulted from repressed nitrification of mineralized ammonium (Fig. 6.4 a and c). The lasting effect of increased nitrate reductase activities – though nitrate was not detectable any longer after 3 days - might be explained by the high persistence of this enzyme (Lensi et al., 1991; Dendooven and Anderson, 1994).

The extreme nitrate addition of 300 µg NO_3^--N g^{-1} dry soil induced a significant increase in enzyme activity compared to the control, which was obviously limited by nitrate concentrations below 2 µg g^{-1} dry soil. However, after seven days the differences between control and extreme nitrate treatment were no longer significant pointing to a high resilience of the nitrate reductase activity towards nitrate addition. We can not exclude the possibility that the relatively low response to nitrate addition might be associated with osmotic stress or a toxic effect on some denitrifier strains through the high accumulation of nitrite, which was observed in this treatment (Tiedje, 1988). Apparently, nitrite reduction was completely inhibited during the first 3 days of incubation, since the accumulated amount of nitrite was equivalent to the decrease in nitrate. Similar high accumulations of nitrite were observed by Ellis et al. (1998) and attributed to a long lag phase for the synthesis of nitrite reductase. In our experiment this explanation is unlikely since under addition of 100 µg NO_3^--N g^{-1} dry soil no such lag phase was observed. Polcyn and Lucinski (2003) rather argue that under high nitrate additions the competition for electrons between nitrate and nitrite reductase is responsible for nitrite accumulation since nitrate reduction is energetically more advantageous and, hence, the limited electron-flow is preferentially directed to nitrate reductase.

In the 100 µg NO_3^--N treatment the low nitrate reductase activity conflicted with the rapid decrease of nitrate concentration (Fig. 6.3 and 6.4a). This could be explained by the inability of 2,4-dinitrophenol (DNP) to inhibit nitrite reduction in our enzyme assay, which resulted in an underestimation of nitrate reductase activity. Although the DNP concentration used effectively repressed nitrite reduction in the other treatments, we tested up to 10 fold increased concentrations of DNP, but the result remained unchanged (data not shown). Why did the method fail in only this treatment? We suggest that the very high activity of nitrite

reductase, which reduced almost all NO_2^- produced during the first three days of the incubation, interfered with the principle of measurement, which is based on the relative differences in sensitivity of nitrate and nitrite reductase towards DNP. Only after 14 days when levels of nitrite reductase, which possesses only a short persistence in soils (Dendooven and Anderson, 1994), were presumably low again, the measured activities of the nitrate reductase approached the values of the control.

In conclusion, the composition of the nitrate-reducing community carrying the membrane-bound nitrate reductase was not influenced by fluctuating nitrate concentrations. Even extreme nitrate additions did not induce shifts in the structure of the nitrate-reducing community. Apparently, the present nitrate-reducing community had a high capacity to reduce the additional nitrate without growth of one particular competitive population. That points to a high resistance towards changes in nitrate availability. Decreasing nitrate concentrations and the rising pH point to a highly active nitrate-reducing community. Measurements of nitrate reductase activity only partly supported this observation but failed under high N supply of 100 µg NO_3^--N g^{-1} dry soil most likely due to incomplete inhibition of nitrite reduction during the enzyme assay. However, comparison between the control and the 300 µg NO_3^--N treatment revealed a significant short-term effect of nitrate addition and a high resilience of the nitrate reductase activity since no differences were observed at day 7. These results indicate a high functional stability of the nitrate-reducing community towards fluctuating nitrate concentrations characterised by a resistant community structure and a resilient nitrate reductase activity.

Acknowledgement

We would like to thank Dagmar Mezger for assistance with the lab analysis, Martin Zahner (Ihinger Hof) for mineral nitrogen analysis, and Stéphanie Hallet for the sequence analysis. The discussions with Dagmar Tscherko were very fruitful and improved the manuscript substantially. The financial support provided by the COST Action 856 for a Short Term Scientific Mission in the Laboratory of Soil Microbiology and Geochemistry at the INRA/Dijon is also gratefully acknowledged.

References

Bateman, E. J., and Baggs, E. M. (2005) Contributions of nitrification and denitrification to N_2O emissions from soils at different water-filled pore space. Biol. Fertil. Soils 41: 379-388.

Chèneby, D., Hallet, S., Mondon, A., Martin-Laurent, F., Germon, J. C., and Philippot, L. (2003) Genetic characterization of the nitrate reducing community based on *narG* nucleotide sequence analysis. Microbial Ecol. 46: 113-121.

Clayton, H., Mc Taggart, I. P., Parker, J., Swan, L., and Smith, K. A. (1997) Nitrous oxide emissions from fertilised grasslands: a two year study of the effects of N fertilizer form and environmental conditions. Biol. Fertil. Soils 25: 252-260.

Deiglmayr, K., Philippot, H., Hartwig, U. A., and Kandeler, E. (2004) Structure and activity of the nitrate-reducing community in the rhizosphere of *Lolium perenne* and *Trifolium repens* under long-term elevated pCO_2. FEMS Microbiol. Ecol. 49: 445-454.

Dendooven, L., and Anderson, J. M. (1994) Dynamics of reduction enzymes involved in the denitrification process in pasture soil. Soil Biol. Biochem. 26: 1501-1506.

Ellis, S., Howe, M. T., Goulding, K. W. T., Mugglestone, M. A., and Dendooven, L. (1998) Carbon and nitrogen dynamics in a grassland soil with varying pH: effect of pH on the denitrification potential and dynamics of the reduction enzymes. Soil Biol. Biochem. 30: 359-367.

Enwall, K., Philippot, L., and Hallin, S. (2005) Activity and composition of the denitrifying community respond differently to long-term fertilization. Appl. Environ. Microbiol. 71: 8335-8343.

Kandeler, E. (1995) Nitrate reductase activity. In: Methods in soil biology. Schinner, F., Öhlinger, R., Kandeler E., and Margesin, R., (eds). Springer Berlin Heidelberg, Germany: pp. 176-179.

Lensi, R., Lescure, C., Steinberg, C., Savoie, J.-M., and Faurie, G. (1991) Dynamics of residual enzyme activities, denitrification potential, and physico-chemical properties in a gamma-sterilized soil. Soil Biol. Biochem. 23: 367-373.

Mounier, E., Hallet, S., Chèneby, D., Benizri, E., Gruet, Y., Nguyen, C., Piutti, S., Robin, C., Slezack-Deschaumes, S., Martin-Laurent, F., Germon, J. C., and Philippot, L. (2004)

Influence of maize mucilage on the diversity and activity of the denitrifying community. Environ. Microbiol. 6: 301-312.

Nijburg, J. W., Coolen, M. J. L., Gerards, S., Klein Gunnewiek, P. J. A., and Laanbroek, H. J. (1997) Effects of nitrate availability and the presence of *Glyceria maxima* on the composition and activity of the dissimilatory nitrate-reducing bacterial community. Appl. Environ. Microbiol. 63: 931-937.

Philippot, L., Piutti, S., Martin-Laurent, F., Hallet, S., and Germon, J. C. (2002) Molecular analysis of the nitrate-reducing community from unplanted and maize-planted soils. Appl. Environ. Microbiol. 68: 6121-6128.

Polcyn, W., and Lucinski, R. (2003) Aerobic and anaerobic nitrate and nitrite reduction in free-living cells of *Bradyrhizobium* sp. (*Lupinus*). FEMS Microbiol. Lett. 226: 331-337.

Reddy, R. U., and Reddy, M. S. (1998) Dissimilatory nitrate reductase activity in soils of Northern Telangana of Andhra Pradesh and its relationship with various soil properties. Ann. Agr. Res. 19: 250-253.

Sexstone, A. J., Parkin, T. B., and Tiedje, J. M. (1988) Denitrification response to soil wetting in aggregated and unaggregated soil. Soil Biol. Biochem. 20: 767-769.

Sharma, S., Szele, Z., Schilling, R., Munch, J. C., and Schloter, M. (2006) Influence of freeze-thaw on the structure and function of microbial communities and denitrifying population in soil. Appl. Environ. Microbiol. 72: 2148-2154.

ter Braak, C. F. J., and Šmilauer, P. (1998) CANOCO reference manual and user's guide to Canoco for Windows: Software for canonical community ordination (version 4). Microcomputer Power, Ithaca, NY: pp. 352.

Tiedje, J. M. (1988) Ecology of denitrification and dissimilatory nitrate reduction to ammonium. In: Biology of anaerobic microorganisms. A. J. B. Zehnder (ed.). John Wiley & Sons, New York, NY: pp. 179-244.

Whitehead, D. C. (2000) Nutrient elements in grasslands – Soil-plant-animal relationships. CABI Publishing, New York: pp 369.

7 Microbial succession of nitrate-reducing bacteria in the rhizosphere of *Poa alpina* across a glacier foreland in the Central Alps

Submitted to Environmental Microbiology

K. Deiglmayr (1), L. Philippot (2), D. Tscherko (3), E. Kandeler (3)

(1) Institute of Crop Production and Grassland Research, University of Hohenheim, D-70593 Stuttgart, Germany, (2) Laboratory of Soil Microbiology and Geochemistry, INRA-University of Burgundy, F-21065 Dijon Cedex, France, (3) Institute of Soil Science, University of Hohenheim, D-70593 Stuttgart, Germany

The definitive version is available at www.blackwell-synergy.com

Abstract

Changes in community structure and activity of the dissimilatory nitrate-reducing community were investigated across a glacier foreland in the Central Alps to gain insight into the successional pattern of this functional group and the driving environmental factors. Bulk soil and rhizosphere soil of *Poa alpina* was sampled in five replicates in August during the flowering stage and in September after the first snowfalls along a gradient from 25 to 129 years after deglaciation and at a reference site outside the glacier foreland (>2000 years deglaciated). In a laboratory based assay, nitrate reductase activity was determined colorimetrically after 24 hours of anaerobic incubation. In selected rhizosphere soil samples, the community structure of nitrate-reducing microorganisms was analysed by RFLP-PCR using degenerate primers for the *narG* gene encoding the active site of the membrane-bound nitrate reductase. Clone libraries of the early (25 years) and late (129 years) succession were constructed and representative clones sequenced. The activity of the nitrate-reducing community increased significantly with age mainly due to higher carbon and nitrate availability in the late succession. The community structure, however, only showed a small shift over the 100 years of soil formation with pH explaining a major part (19 %) of the observed variance. Clone library analysis of the early and late succession pointed to a trend of declining diversity with progressing age. Presumably, the pressure of competition on the nitrate reducers was relatively low in the early successional stage due to minor densities of microorganisms compared to the late stage; hence, a higher diversity could persist in this sparse environment. These results suggest that the nitrate reductase activity is regulated by environmental factors other than those shaping the genetic structure of the nitrate reducing community.

Introduction

Alpine ecosystems are extremely sensitive to climatic fluctuations. Thus, slightly increasing temperatures since the 'Little Ice Age' around 1820 to 1850 led to a continuous retreat of glaciers and the existence of new barren terrain. The successively colonized glacier forelands offer a chronosequence with different stages of primary succession in close vicinity and are, hence, ideal subjects to study successional processes (Raffl, 1999; Kaufmann, 2001).

In the sparse environments of these glacier forelands, soil microorganisms are particularly essential for plant growth as they play a key role in nutrient cycling. In fact, microbial and invertebrate communities colonize new terrain before establishment of plant species (Hodkinson et al., 2002). In this phase, nitrogen, phosphorus and other nutrients accumulate and facilitate succeeding plant growth. In the course of primary succession, autotrophic plant growth results in the input of organic carbon by litter and root deposits and, hence, stimulates the microbial biomass and its activity (Ohtonen et al., 1999). Most directly the rhizosphere flora benefits from these inputs (Bardgett and Walker, 2004; Tscherko et al., 2004).

In contrast to the succession of plant assemblies, the evolution of soil microbial diversity in glacier forelands has attracted increasing attention only in the last few years. By PLFA analysis of microbial communities in the rhizosphere, differences between pioneer, transition and mature stages could be observed (Tscherko et al., 2004). Sigler et al. (2002) detected shifts in the bacterial community structure from 0 to 100 year-old soils by molecular fingerprinting of bulk soil samples. Equally, the soil fungal community assembly has been studied in detail showing significant differences between a recently deglaciated site and a successional site that has been ice-free over more than 100 years (Jumpponen, 2003).

This work focuses on the functional group of nitrate-reducing microorganisms. Respiratory nitrate reduction catalyzed by a membrane-bound or periplasmatic nitrate reductase is the first step in the denitrification pathway and in the dissimilatory reduction of nitrate to ammonia (DNRA). Since DNRA is of minor importance in soils, the major part of the reduced nitrate is returned to the atmosphere in form of N_2O or N_2. Thus, nitrate-reduction represents an important process of open nutrient cycling. According to Odum's theory of succession, open cycles of mineral nutrients are characteristic for young developing ecosystems, whereas in mature systems closed cycles are prevalent (Odum, 1969). In the context of climate change, denitrification is of particular significance as the greenhouse gas N_2O, which is also a natural

catalyst of stratospheric ozone degradation, can be emitted as possible end product (Lashof and Ahuja, 1990).

The main objective of this study was to monitor changes in diversity and activity of nitrate-reducing micro-organisms in the course of primary succession and to search for the principal environmental variables such as organic carbon, nitrate, water content and pH driving these changes. To study the community structure of nitrate-reducing microorganisms, RFLP (Restriction Fragment Length Polymorphism)-analysis of the functional gene *narG,* which codes for the catalytic unit of the membrane-bound nitrate reductase has been well established (Philippot et al., 2002; Chèneby et al., 2003; Deiglmayr et al., 2004; Mounier et al., 2004; Patra et al., 2005). By biochemical measurement of the nitrate reductase activity, the corresponding enzyme activity of this functional community can be determined (Kandeler, 1995). In this way, structure and activity of the nitrate reducers can be studied in parallel. Since major changes in community structure were previously found in the rhizosphere (Tscherko et al., 2004), we focused on the nitrate-reducing community of the rhizosphere flora of *Poa alpina* occurring across all successional stages.

Experimental Procedures

Study site

The study was carried out at the glacier foreland of the Rotmoosferner (46°50’ N, 11°03’ E) in the Ötz valley (Austria) at an altitude of 2280-2450 m above sea level (Kaufmann, 2001). Since 1858 the Rotmoosferner has been retreating and left a mainly level valley 2 km in length ascending only in the younger parts of the foreland (<50 years) (Kaufmann, 2001). The well-preserved chronosequence has been described in detail regarding the soil formation, the vegetational gradient, the invertebrate succession and the total soil microbial communities (Erschbamer et al., 1999; Raffl, 1999; Kaufmann, 2001; Tscherko et al., 2004). The parent material of the soils is mainly neoglacial moraine till and fluvio-glacial sands (Tscherko et al., 2004). The texture of the soils is pure sand to silty sand. Organic carbon, pH, NO_3^- and NH_4^+ contents were determined following standard methods and results are shown in Tab. 7.1.

Soil sampling

The soil was sampled in mid of August 2004 at the flowering stage of *Poa alpina* and in the end of September 2004 after the first snowfalls. Seven successional sites deglaciated for 25 to 129 years (Rüdiger Kaufmann, personal communication) were selected within the orographic right side of the glacier foreland. One site outside the glacier foreland served as reference (>2000 years old). For each of the five replicates 3-5 plants of *Poa alpina* were digged out, shaken and the soil still adhering to the roots was considered rhizospheric soil. The bulk soil was sampled in 0-10 cm depth with as little roots as possible. The rhizospheric and bulk soil samples were sieved through a 2 mm sieve directly on site and stored at – 20 °C prior to analysis.

Determination of potential nitrate reductase activity

The potential activity of the nitrate reductase was determined by anaerobic incubation of soil following a modified protocol (Kandeler, 1995). Briefly, 0.2 g rhizospheric soil was weighed in five replicates into 2.0 ml reaction tubes. To inhibit nitrite reduction, 167 µg of 2,4-dinitrophenol per g soil (fresh weight) were added. After 24 h incubation in 1 mM KNO_3 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 x g. The accumulated nitrite in the supernatant was determined by colorimetric reaction.

DNA extraction and PCR amplification

Based on the results of the nitrate reductase activity, the investigation on the community structure of the nitrate-reducing community was restricted to five successional sites within the glacier foreland. To exclude heterogeneity based on different vegetation, only rhizospheric soil of *Poa alpina* was included in the analysis. DNA was extracted from 0.3 g soil using the FastDNA Spin Kit for soil (BIO101, Qbiogene), following the protocol of the manufacturer. The quantity of the DNA extractions was checked using a BioPhotometer (Eppendorff). A *narG* fragment of 650 bp length was amplified using the primers narG1960f and narG2650r (Philippot et al., 2002). Three independent PCR amplifications were performed for each sample in a total of 50 µl containing 1x PCR buffer, 200 µM of each deoxyribonucleoside triphosphate, 1.25 mM of each primer, 2 U of *Taq* polymerase, and 20 ng of soil DNA.

To increase amplification efficencies, 1 µg of T4 gene 32 protein (BioLabs) was added per reaction volume (Kreader, 1996). The cycling conditions of the PCR were as follows: an initial denaturation step at 95 °C, followed by a pause to facilitate a manual hot start. After adding the *Taq* polymerase the PCR was continued by a "touch down" with a denaturation step at 94 °C for 30 s, primer annealing at 59 °C for 30 s and elongation at 72 °C for 45 s. During the first 9 cycles, the annealing temperature was decreased by 0.5 °C each cycle until it reached 55 °C. The additional 26 cycles were performed at an annealing temperature of 55 °C. Cycling was completed by a final elongation step at 72 °C for 10 min. The size and presence of the amplification products were checked by electrophoresis in a 1.5 % agarose gel.

RFLP analysis and clone library construction

For purification, the *narG* PCR products belonging to the same sample were pooled and then run on a 2 % agarose gel for 3 h at 100 Volt. Gel slices containing the amplified *narG* fragment were excised and DNA was recovered using the Qiaex II kit (Qiagen) as specified by the manufacturer. Purified PCR products were quantified in a 1.5 % agarose gel according to the standardised DNA quantities of the Smart Ladder SF (Eurogentec). Aliquots of same quantities of the purified *narG* PCR product were digested by *Alu*I restriction enzyme at 37 °C for 2 h and separated by electrophoresis on an 8 % polyacrylamide gel for 15 h at 8 mA. After staining with SYBER green II (Molecular Probes) the *narG* RFLP-fingerprints were scanned with a Phospho Imager.

For construction of clone libraries, aliquots of the purified *narG* PCR products from the five replicate samples of early (25 years) and late (129 years) successional site of each of the two sampling dates were pooled together and cloned using the pGEM-T Easy Vector System (Promega) according to the manufacturer's instructions. Approximately 55 transformants per clone library were randomly picked and the inserted *narG* fragment was amplified by transferring small aliquots of cells to PCR mixtures containing the primers T7 and SP6 and thermal cycling. PCR products were digested by the restriction endonuclease *Alu*I as described above. Restriction fragments were resolved by electrophoresis in a 3 % small fragment agarose gel. Recombinants with identical restriction patterns were grouped together into OTUs (operational taxonomic units).

Sequencing and phylogenetic alignment

Fourty-one representative recombinants of the various RFLP types were sequenced using the DTCS-1 kit (Beckman Coulter) and a Ceq 2000 XL sequencer (Beckman Coulter) according to the manufacturer's instruction. Vector primers T7 and SP6 were used for sequencing reactions. The deduced protein sequences of *narG* genes were aligned using the CLUSTALX software version V.1.0.1 (Thompson et al., 1999). The phylogenetic tree based on amino acids alignments (approximately 210-220 amino acids), was constructed by neighbour-joining method with 100 replicate trees. NarG from the Archea *Pyrobaculum aerophilum* was used as outgroup.

Nucleotide accession numbers

The sequences obtained were deposited in the GenBank sequence database under accession numbers DQ233258 to DQ233296.

Statistics

The data of the enzyme activity of the nitrate reductase were evaluated by ANOVA using the mixed model procedure in the SAS 8.0 statistical analysis package. Statistics were performed for the dataset including and excluding the reference site. The normal distribution of untransformed data was verified by Shapiro-Wilk test (P<0.0001). To improve the fit of the model, heterogeneous group variances were allowed for the different successional stages. For covariates NO_3^-, organic carbon, water content and pH were included in the model. Significance was accepted at the $P \leq 0.05$ level of probability. In order to estimate the proportions of explained variance for the various factors, homogeneous group variances had to be accepted.

narG RFLP-fingerprints were analysed by the software package Quantity One® (Version 4.2.1) for image analysis. A band-matching table was generated containing the molecular weight and the relative intensity of each detected band in reference to the mean intensity within each lane. Based on the band-matching table and the visual control of the gels, bands with comparable molecular weights were summarised in band classes.

To evaluate shifts in community structure among successional sites and sampling dates, discriminant function analysis via multidimensional scaling was applied, according to Tiunov and Scheu (2000). In short, based on the square matrix of nonparametric Gamma correlation a multidimensional scaling analysis was performed in order to compress the total of information given in the band-matching table to only a few dimensions. To reassure not to lose any significant information, the optimal number of dimensions *n* was determined by comparing actual stress values with the theoretical exponential function of stress. Subsequently, the coordinates of the samples in *n*-dimensional space were used for discriminant function analysis with successional age and sampling date as grouping variables. Four canonical roots proved to contribute significantly to the discriminatory power of the model. Squared Mahalanobis distances were calculated to determine significant differences between group centroids. The calculations were performed using the STATISTICA software package (Version 6.0, StatSoft®).

The impact of environmental variables on the diversity of nitrate-reducing microorganisms was analysed by redundancy analysis using CANOCO (Version 4), a software for canonical community ordination (ter Braak and Šmilauer, 1998). The data of band intensities were log-transformed. As the environmental gradient proved to be rather short across the glacier foreland, the linear response model was chosen (Lepš and Šmilauer, 2003). The statistical significance of the environmental variables was tested by a Monte Carlo permutation test carrying out 1000 non restricted permutations.

For comparison of the phylotype richness of the early and late succession, various diversity parameters were calculated by addressing OTUs as representatives for different species. In order to examine how exhaustively the total diversity was captured in the clone libraries, expected species accumulation curves (i. e. sample-based rarefaction curves) were computed using EstimateS (Version 7.5, R. K. Colwell, http://purl.oclc.org/estimates). The 95% confidence intervals were calculated using the analytical formulas of Colwell et al. (2004).

According to Chao and Shen (2003), the nonparametric estimator of the Shannon diversity index $\hat{H}$ accounting for unseen species was calculated based on Horvitz-Thompson estimator and sample coverage method. For its good discriminant abilities Fisher's α recommended by Magurran (1988) as the standard diversity statistic was also included in the statistical analysis. This index is based on the assumption of a log series distribution of the species abundances.

The total species richness was approximated by two different nonparametric estimators. First, S_{Chao1} was calculated according to the classical formula $S_{Chao1} = S_{obs} + \frac{n_1^2}{2n_2}$,

where S_{obs} is the number of observed species, n_1 is the number of singletons (species captured once), and n_2 is the number of doubletons (species captured twice) (Hughes et al., 2001). The 95 % confidence interval was applied using a log-transformation as suggested by Chao (1987). For validation the abundance-based coverage estimator S_{ACE} was considered additionally incorporating data of all OTUs with fewer than 10 individuals (Hughes et al., 2001).

Tab. 7.1: Soil characteristics of bulk and rhizospheric soil under *Poa alpina* in the glacier foreland of the Rotmoosferner sampled in August 2004.

Successional age	Sample type	C_{org} [%]	pH	NO_3^--N [µg g^{-1} soil]	NH_4^+-N [µg g^{-1} soil]
25 years	Bulk soil	0.12 *(0.01)*	7.83 *(0.01)*	0.12 *(0.04)*	0.20 *(0.01)*
	Rhizosphere	0.37 *(0.10)*	7.53 *(0.01)*	0.33 *(0.06)*[a]	0.64 *(0.10)*
40 years	Bulk soil	0.17 *(0.04)*	7.46 *(0.07)*	0.25 *(0.06)*	0.23 *(0.04)*
	Rhizosphere	0.64 *(0.14)*	7.23 *(0.07)*	1.76 *(0.23)*	1.17 *(0.27)*
44 years	Bulk soil	0.29 *(0.05)*	7.43 *(0.07)*	0.46 *(0.16)*	0.72 *(0.22)*
	Rhizosphere	2.58 *(0.94)*	6.75 *(0.16)*	2.82 *(0.45)*	6.20 *(2.56)*
52 years	Bulk soil	0.66 *(0.17)*	7.03 *(0.24)*	0.55 *(0.27)*	1.60 *(0.47)*
	Rhizosphere	3.79 *(1.11)*	6.45 *(0.08)*	3.04 *(0.72)*	11.60 *(4.02)*
57 years	Bulk soil	0.33 *(0.05)*	7.20 *(0.22)*	0.45 *(0.10)*	0.82 *(0.28)*
	Rhizosphere	1.39 *(0.40)*	6.24 *(0.14)*	1.28 *(0.26)*[a]	2.92 *(0.73)*
72 years	Bulk soil	0.37 *(0.14)*	7.29 *(0.36)*	0.20 *(0.09)*	0.90 *(0.37)*
	Rhizosphere	3.02 *(1.29)*	5.49 *(0.12)*[a]	0.85 *(0.20)*	8.80 *(4.41)*
129 years	Bulk soil	1.93 *(0.72)*	6.36 *(0.70)*	0.37 *(0.14)*	4.46 *(1.78)*
	Rhizosphere	9.67 *(3.33)*	5.56 *(n. d.)*	1.67 *(0.31)*[a]	17.42 *(1.75)*
>2000 years	Bulk soil	5.47 *(1.27)*	4.50 *(0.56)*	0.17 *(0.08)*	10.21 *(3.24)*
	Rhizosphere	17.34 *(1.45)*	n. d.	0.36 *(0.05)*[a]	25.28 *(0.68)*

Values are given as means (± standard error)
[a] missing values, only a part of the replicates is included in the mean

Results

Nitrate reductase activity

The nitrate reductase activity increased highly significantly (P=0.0002) with progressing succession with a maximum activity in the late succession, whereas the reference site showed much lower enzyme activities (Fig. 7.1, Tab. 7.2). The nitrate reductase activities in the rhizospheric soil were significantly higher than in the bulk soil (P=0.0037), particularly in the earlier part of the glacier foreland with up to 23 fold higher enzyme activities in the rhizosphere (August, 44 years after deglaciation). The significant interaction of age and rhizosphere effect (P=0.0125) pointed to a decrease in rhizosphere effect with progressing age. No significant effect of sampling date on the enzyme activity was observed. Step by step the covariates organic carbon, nitrate, pH and water content were included in the statistical model according to their significance. Organic carbon and nitrate availability affected the nitrate reductase activity most significantly explaining 51.0 % and 10.8 % of the observed variance thereby reducing the proportion of variance explained by successional age from 37.5 % to 6.3 % (Tab. 7.2).

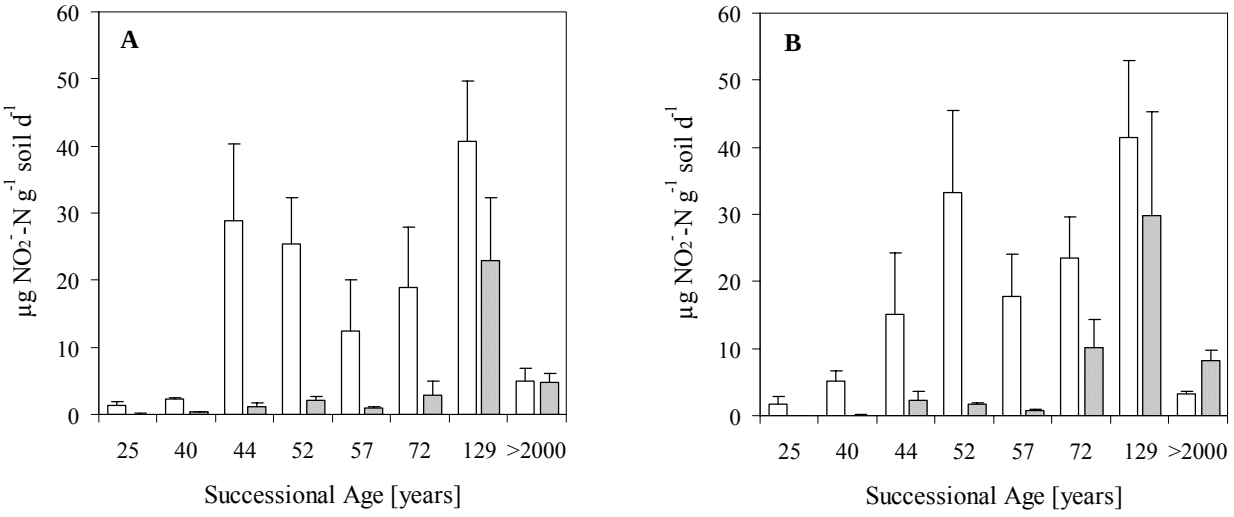

Fig. 7.1: Nitrate reductase activity in the rhizosphere of *Poa alpina* (white) and the bulk soil (striped) from seven successional sites in the glacier foreland of the Rotmoosferner and one reference site sampled in August (A) and September (B) 2004; bars are means, whiskers indicate standard error.

Tab. 7.2: ANOVA of the nitrate reductase activities in the bulk soil and rhizosphere soil of *Poa alpina* across the glacier foreland of the Rotmoosferner (reference site excluded).

Source	F value	*P*	Proportion of explained variance	
			without covariates [%]	including covariates [%]
Age	5.60	0.0002	37.5	6.3
Date	1.29	0.2603	0.2	0.0
Age × Date	0.85	0.5410	1.0	1.0
Rhizosphere	8.98	0.0037	16.3	0.0
Age × Rhizosphere	3.15	0.0125	6.5	1.6
Date × Rhizosphere	0.73	0.3951	0.2	0.0
Age × Date × Rhizosphere	0.73	0.6264	0.9	0.6
C_{org}	34.19	<0.0001	-	51.0
NO_3	9.68	0.0031	-	10.8
pH	6.40	0.0219	-	0.0
H_2O	1.12	0.3031	-	6.1
Residual			37.4	22.6

The data presented here refer on the statistics without consideration of the reference site, since its converse response masked the successional effects within the glacier foreland. By including the reference site, the effect of successional age could only marginally be explained by environmental covariates with nitrate availability exceeding organic carbon in significance.

RFLP-fingerprints

NarG RFLP-fingerprints of the rhizosphere soil samples of August revealed slight shifts in the nitrate-reducing community structure across the five successional sites of the glacier foreland (Fig. 7.2 A). In particular in the lower part of the gel several bands (39 bp, 42 bp, 49 bp, 87 bp, 171 bp) decreased in relative intensity with progressing age. The same patterns could be observed at the sampling date in September (Fig. 7.2 B).

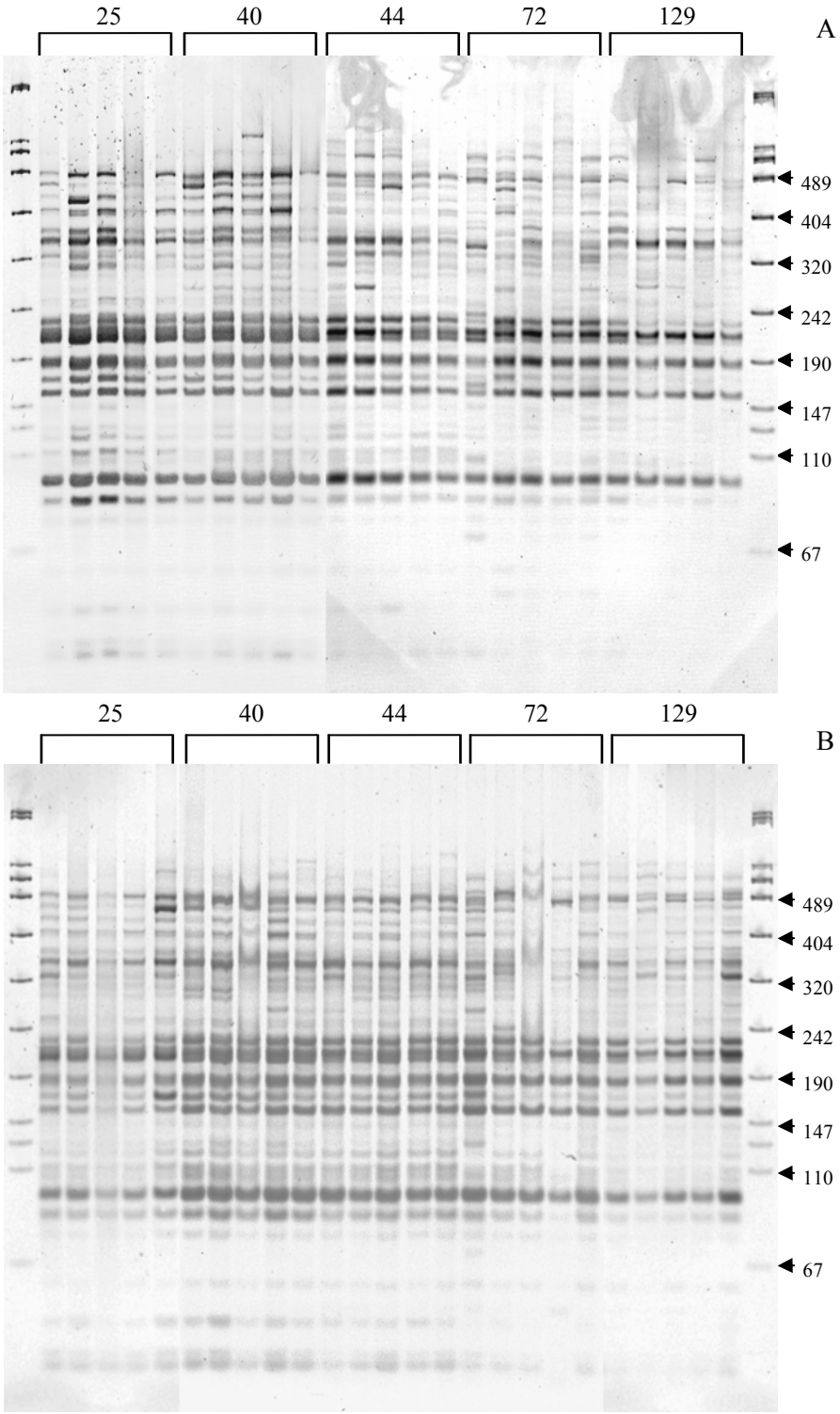

Fig. 7.2: RFLP-fingerprints of the nitrate-reducing community under *Poa alpina* from five successional sites in the glacier foreland of the Rotmoosferner sampled in (A) August and (B) September 2004; lanes 2-6: 25 years after deglaciation; lanes 7-11: 40 years after deglaciation; lanes 12-16: 44 years after deglaciation; lanes 17-21: 72 years after deglaciation; lanes 22-26; 129 years after deglaciation; lane 1 and 27: molecular weight marker VIII (Roche).

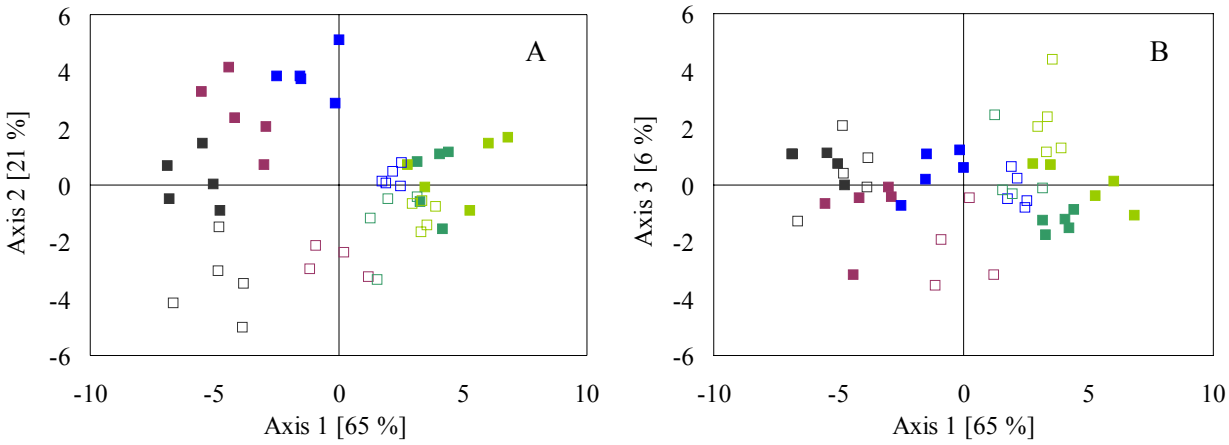

Fig. 7.3: Ordination diagram of discriminant analysis via multidimensional scaling of *narG* RFLP-fingerprints from five successional sites in the glacier foreland of the Rotmoosferner sampled in August (filled symbols) and September (open symbols) 2004: sites deglaciated for 25 years (light green), for 40 years (dark green), for 44 years (blue), for 72 years (red) and for 129 years (black). (A) Axis 1 plotted versus axis 2, (B) axis 1 plotted versus axis 3.

The discriminant analysis via multidimensional scaling including seven dimensions reflected very clearly the transition from late to young succession (Fig. 7.3 A and B). Four axes proved to add significantly to the discrimination between groups. Primarily along the first axis which accounted for 65 % of the discriminatory power, the different successional ages were separated. Along the second axis the samples were split into the two sampling dates. Based on the squared Mahalonobis Distance all five successional sites and the two sampling dates could be distinguished significantly, except for the two youngest sites (25 and 40 years) in August and the 40 and 44 years old sites in September (data not shown).

In order to search for the driving factors of the changes in community structure a redundancy analysis was performed. In this ordination method the axes are constrained to be linear combinations of environmental variables. The first two axes explained 30 % of the species variability, which corresponded to 78 % of the total species-environment relation (Fig. 7.4). The successional age accounted for 22 % of the observed variability in community structure followed in importance by pH, H_2O, C_{org} and NO_3^- (Tab. 7.3). Treating the environmental variables pH, C_{org}, H_2O and NO_3^- as covariates the additional effects of age and date amounted to 5 % and 3 %, respectively. All environmental variables in sum explained 39 % of the observed variability in community structure.

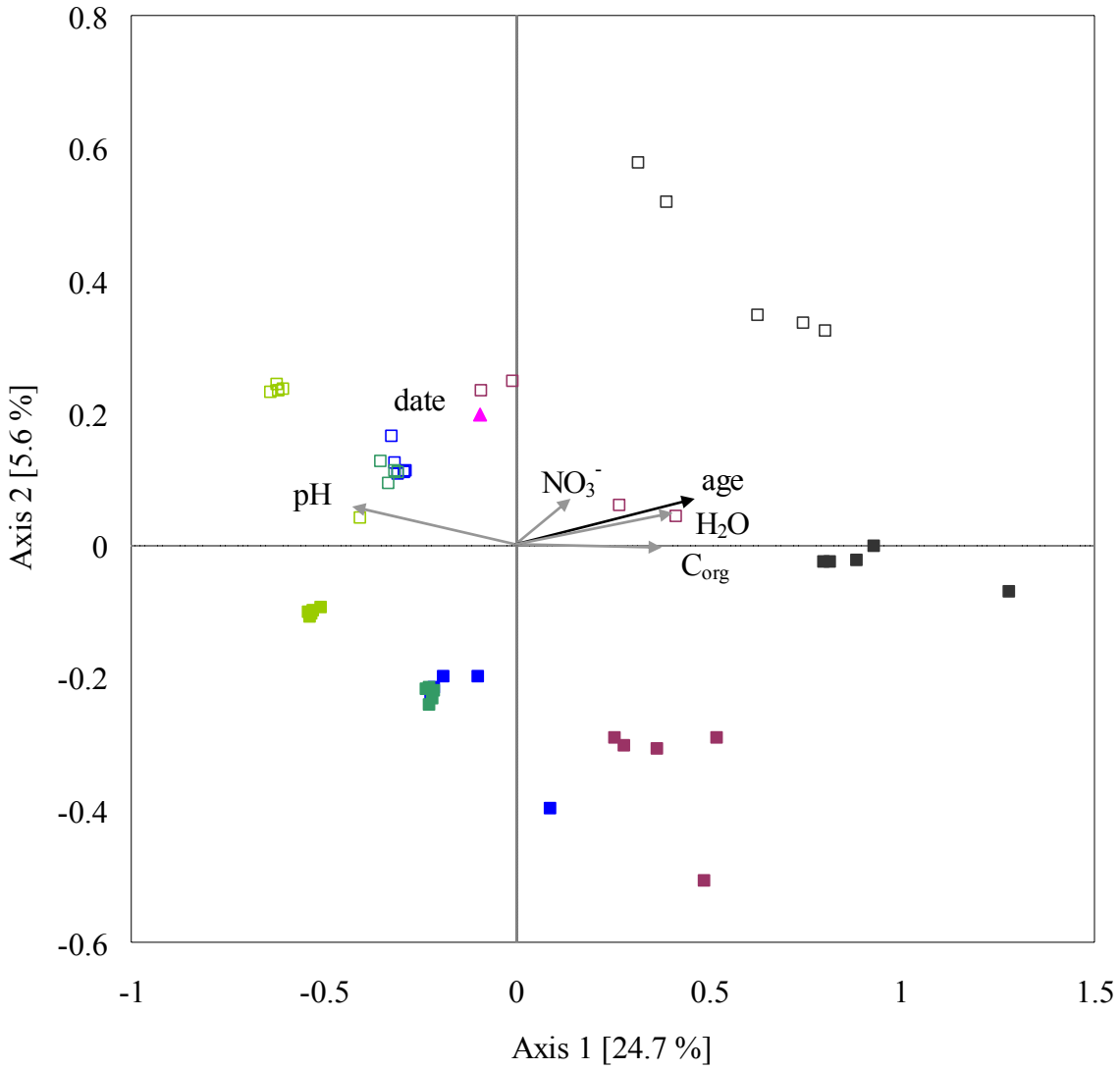

Fig. 7.4: Ordination diagram of redundancy analysis of *narG* RFLP-fingerprints from five successional sites in the glacier foreland of the Rotmoosferner sampled in August (filled symbols) and September (open symbols) 2004: sites deglaciated for 25 years (light green), for 40 years (dark green), for 44 years (blue), for 72 years (red) and for 129 years (black); arrows indicate quantitative environmental variables, the qualitative environmental variable date is shown with its centroid (▲).

Tab. 7.3: Ranking environmental variables in importance by their marginal (left) and conditional (right) effects on the nitrate-reducing community, as obtained by manual forward selection.

Marginal effects			Conditional Effects after forward selection			
Variable	λ_1	*P*	Variable	λ_a	*P*	cum (λ_a)
Age	0.22	0.001	pH	0.19	0.001	0.189
pH	0.19	0.001	C_{org}	0.06	0.001	0.244
H_2O	0.17	0.001	H_2O	0.04	0.005	0.279
C_{org}	0.15	0.001	NO_3^-	0.03	0.010	0.309
NO_3^-	0.05	0.022	Age	0.05	0.002	0.356
Date	0.05	0.012	Date	0.03	0.005	0.389

λ_1: fit or eigenvalue with one variable only; λ_a: additional fit or increase in eigenvalue; cum (λ_a): cumulative total of eigenvalues;
P = significance level of the effect, as obtained with Monte Carlo permutation test with 1000 random permutations.

Clone libraries

To investigate the diversity of the nitrate-reducing community in more detail, clone libraries of the early and late succession at both sampling dates were constructed. Within the total of 221 clones, 84 different RFLP patterns (with 34 represented by at least two clones) were detected and grouped into operational taxonomic units (OTUs) (Fig. 7.5). In the late succession, one prominent OTU accounted for 22 % of the clones, whereas only 7 % of the early succession's clones were found in this group. The most frequent OTU in the early succession comprised 10 % of the total of clones analysed. This OTU was detected only once in the late succession. Several other frequent OTUs were found only in either the early or the late succession.

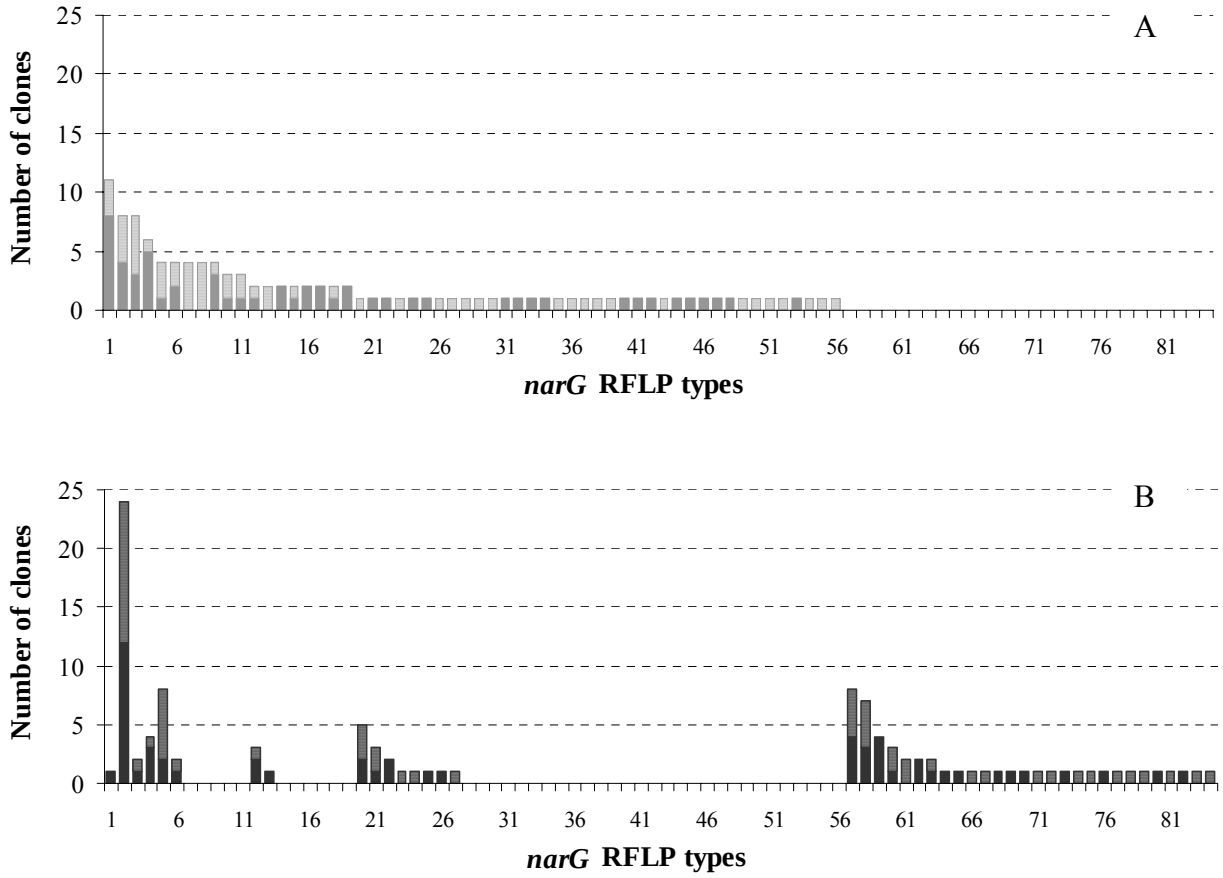

Fig. 7.5: Distribution of *narG* RFLP types in clone libraries of the pooled PCR products from (A) early and (B) late succession sampled in August (filled) and September (lined) 2004.

For all statistical analysis the two sampling dates were pooled together. To check the coverage of diversity by the screened clone libraries, expected species accumulation curves were calculated (Fig. 7.6). These curves present - according to sample-based rarefaction curves - the expected number of observed OTUs at a certain sampling intensity. As the curves did not reach saturation, it must be concluded that further screening would still reveal higher diversity.

To estimate the total species richness, S_{Chao1} and S_{ACE} were calculated according to Chao (1987) (Tab. 7.4). Both estimators indicated that the nitrate-reducing community in the early succession tended to be more diverse than in the late succession. This latter conclusion was also confirmed by the diversity indices Shannon's $\hat{H}$ and Fisher's α (Tab. 7.4).

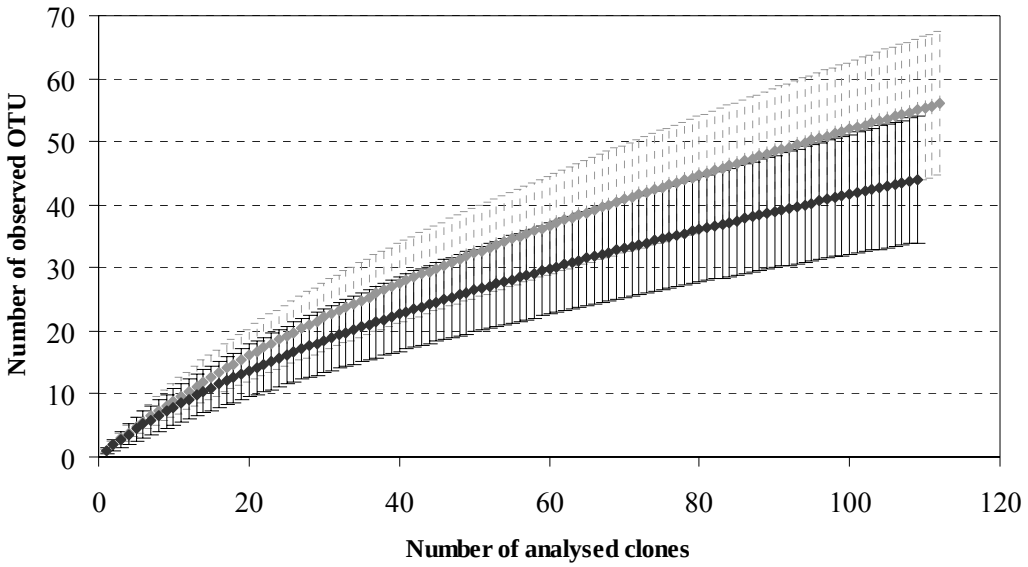

Fig. 7.6: Species accumulation curves of *narG* RFLP types in clone libraries from (grey) early and (black) late succession, sampling dates are pooled together; error bars indicate the 95 % confidence interval.

Tab. 7.4: Diversity indices and estimators of total species richness for the pooled clone libraries of late and early succession in the glacier foreland of the Rotmoosferner.

		Diversity Indices		Total Species Richness Estimators	
Source	S_{obs}	Shannon's $\hat{H}$	Fisher's α	S_{Chao1}	S_{ACE}
Early succession	56 (112)[a]	4.12 (3.78, 4.47)[b]	44.57 (32.90, 56.24)[b]	141.6 (90.2, 270.1)[b]	144.8 (97.9, 244.3)[b]
Late succession	44 (109)[a]	3.62 (3.10, 4.13)[b]	27.43 (19.32, 35.53)[b]	109.3 (67.1, 228.8)[b]	108.9 (72.4, 192.2)[b]

S_{obs}= Number of observed OTUs
[a] Number of analysed clones
[b] lower and upper limits of the 95 % confidence interval

Phylogenetic analyis

Representatives of the different OTUs comprising at least two clones were sequenced and the deduced amino sequences phylogenetically aligned (Fig. 7.7). Two sequences were found to have no sequence homology to *narG* and were thus dimissed. In addition to the remaining 39 sequences obtained from the glacier foreland 54 sequences of known organisms were included in the phylogenetic analysis. One sequence representing four clones of the early succession was related to NarG of *Geobacter metallireducens*. All other clones clustered together with NarG either from *Actinobacteria* (Cluster 1) or from *Proteobacteria* (Cluster 2). In the late succession 79 % and 21 % of all identified clones were found in Cluster 1 and 2, respectively (Tab. 7.5). In contrast, a different percentage of the clones within the two clusters was found in the early succession: 51 and 44 % in Cluster 1 and 2, respectively. Hence, the ratio of clones belonging to Cluster 1 to those of Cluster 2 increased from 1.2 to 3.7 from early to late succession.

Tab. 7.5: Affiliation of clones from early and late succession in the glacier foreland of the Rotmoosferner.

	Early succession		Late succession	
Affiliation	No. of clones	Proportion [%]	No. of clones	Proportion [%]
Cluster 1 (Actinobacteria)	43	51	67	79
Cluster 2 (Proteobacteria)	37	44	18	21
Geobacter association	4	5	0	0
Ratio Cluster 1: Cluster 2		1.2		3.7

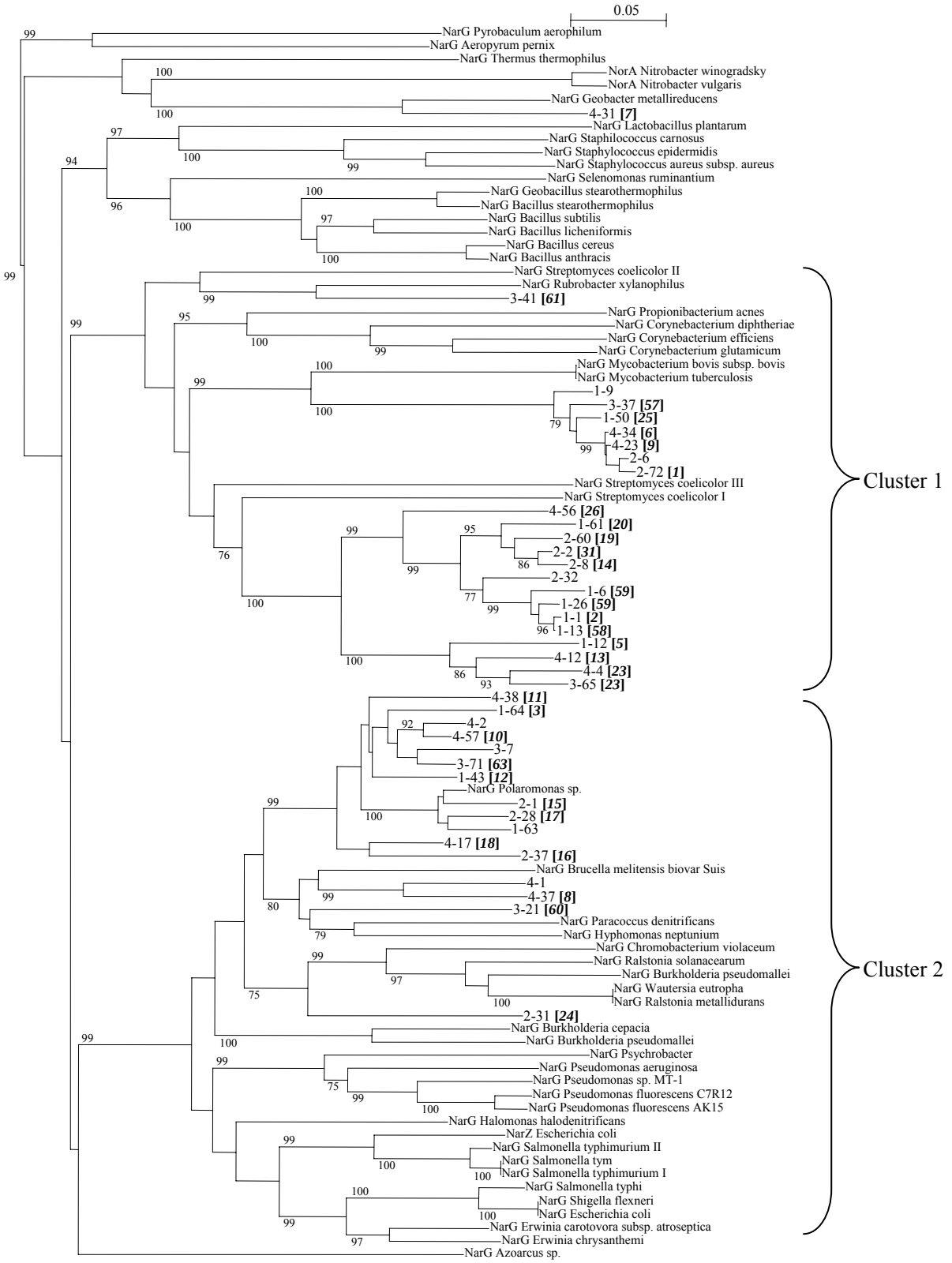

Fig. 7.7: Phylogenetic analysis of deduced NarG amino acid sequences from 39 *narG* clones. The corresponding OTUs are indicated in brackets and bold after the clone number. Only bootstrap values above 75 are given.

Discussion

Environmental factors regulating nitrate reductase activity

The data of nitrate reductase activity revealed a significant increase with successional age as it has been demonstrated for other enzyme activities (Ohtonen et al., 1999; Tscherko et al., 2003; Sigler and Zeyer, 2002). The stimulation of nitrate reductase activity could be explained by increasing contents of organic carbon and varying nitrate resources with progressing age (Tab. 7.2). The remaining significant effect of successional age may be attributed to the increasing vegetation cover, which prevents the soil surface from heat stress and dryness and, hence, improves the environmental conditions for soil microorganisms (Tscherko et al., 2005). Possibly the quality of organic carbon that also alters during primary succession contributed to the observed effect of successional age additionally. One should also consider that due to our sample design restricted to one chronosequence with no true replicates of successional stages, the effects of age and position in the glacier foreland could not be distinguished from one another. In this respect, the temperature gradient along the valley as well as varying sun exposure at the different sites may be included in the observed "age effect" (Kaufmann, 2001). Additionally, coarse parent material of the glacial drift as it prevailed at the 57 years old site, may have hampered soil development and led to lower enzyme activity rates. At the reference site, nitrification was possibly repressed due to the low pH of 4.5 that is critical for ammonia-oxidizing bacteria (Stienstra et al., 1994). Hence, the low nitrate contents restricted the nitrate reductase activity to the observed low levels. This finding confirms Odum's theory, that in mature successional stages closed nutrient cycles dominate (Odum, 1969). In the glacier foreland the relatively high nitrate reductase activities point to rather open nitrogen cycles with possible losses due to emissions of N_2O and N_2.

The strong effect of the rhizosphere on the enzyme activity observed in the younger part of the glacier foreland resulted from the very low concentrations of organic carbon of the bulk soil. Under these sparse conditions the extra carbon entering the soil in the rhizosphere of *Poa alpina* through root exudates, lysates and decomposing fine roots apparently affected the respiratory nitrate reduction substantially. As in the late succession organic carbon contents in the bulk soil increased due to higher litter inputs, the rhizosphere effect declined. Beside the higher availability of organic carbon in the rhizosphere, the low partial oxygen pressure in the proximity of roots resulting from respiration by roots and rhizoflora may have favoured nitrate reduction additionally (Klemedtsson et al., 1987; Højberg and Sørensen, 1993).

Analysis of the covariates stressed the pivotal role of organic carbon together with nitrate availability on the nitrate reductase activity during primary succession. As organic carbon significantly accumulated with successional age due to increasing plant biomass production and low decomposition rates, it may have affected the nitrate reductase in several ways: (i) by providing the substrate for a higher microbial biomass and activity, resulting in higher oxygen consumption (Ohtonen et al., 1999; Tscherko et al., 2003), and (ii) by enhancing the water retention capacity of the soils, which may have led to a decrease in oxygen diffusion. In the consequence anaerobic sites probably occurred more frequently under higher organic matter contents inducing nitrate-reducing microorganisms to switch to anaerobic respiration and use nitrate as alternative electron acceptor. The nitrate availability was very low across all successional stages and, hence, limited the nitrate reductase activity where organic carbon was abundant.

Shifts in community structure across the glacier foreland

The *narG* RFLP-fingerprints showed slight changes across the glacier foreland. The discriminant analysis via nonmetric multidimensional scaling indicated that the different successional stages and dates could be separated from one another and that the shift in community composition followed a directional pattern as it is characteristic for successional series (Odum, 1969). In the same glacier foreland, Tscherko et al. (2004) observed a similar splitting into pioneer, transient and mature successional stages by multidimensional scaling of PLFA data from rhizosphere microorganisms of *Poa alpina* (Tscherko et al., 2004). By ranking environmental variables according to their importance, the redundancy analysis revealed that the environmental variable explaining most of the variance was successional age followed by pH, water content and organic carbon. Since these soil characteristics are correlated among themselves, it is impossible to correctly attribute the partial effects to these various variables. By selecting first pH, organic carbon, water content and nitrate and thus treating them as covariates the additional successional age effect was reduced to 5 %. We concluded that apparently these four environmental variables explained the major part of the successional age effect. Particularly, pH had a pivotal impact on the nitrate-reducing community. The significance of pH as driver of microbial community structure has been discussed by numerous authors (O'Donnell et al., 2001; Deiglmayr et al., 2004; Stres et al., 2004; Enwall et al., 2005). The relatively low impact of NO_3^- on the nitrate-reducing

community is congruent to previous results, where nitrate fertilisation did not alter the community composition of nitrate reducers (Deiglmayr et al., 2004).

The detailed study of the early and late succession by clone library analysis revealed further insight into these differences. Although clones related to NarG from *Actinobacteria* and *Proteobacteria* were prevalent in all clone libraries which is in accordance with inventories from other soil types (McCaig et al., 1999; Philippot et al., 2002; Buckley and Schmidt, 2003; Chèneby et al., 2003; Mounier et al., 2004), a shift in the composition of OTUs was observed from early to late succession. Accordingly to McCaig et al. (1999), who reported a slightly higher proportion of *Actinobacteria* in improved grassland soils, clones related to NarG of *Actinobacteria* became more dominant in the late succession. Our observation is also confirmed by PLFA analysis of the rhizosphere flora of *Poa alpina* where an increasing ratio of Gram+/Gram- bacteria with progressing age was stated (Tscherko et al., 2004). We suggest that at high densities of soil microorganisms with elevated intra- and interspecific competition selection favours K-strategists, i.e. microorganisms that can survive and reproduce with limited resources like *Actinobacteria* (Bottomley, 1998). Their ability to use a wide variety and also complex substrates such as lignin may be an additional competitive advantage. At low densities of soil microorganisms, however, r-strategists that can reproduce rapidly regardless of efficiency are more successful (Boyce, 1984; quoted by Ricklefs and Miller, 2000). This hypothesis is supported by observations of Sigler and Zeyer (2004), who found a higher proportion of rapidly colonizing bacteria in recently deglaciated soils than in older soils.

The tendency of declining diversity in the late succession could be attributed to higher competition for carbon resources due to a rising density of heterotrophic microorganisms and, hence, a higher selective pressure. PLFA analysis of the rhizosphere flora indicates that with progressing age the rhizosphere selects for a specific rhizosphere flora whereas in the early succession rhizosphere and bulk soil harbour the same microbial communities (Tscherko et al., 2004). Accordingly, DGGE and RISA analysis of microbial communities at two Swiss glacier forelands confirm that the highest diversity was found in the pioneer stage (Sigler and Zeyer, 2002; Sigler et al., 2002). The authors argued that noncompetitive conditions prevail in the early succession due to relatively high nutrient availability in relation to low population density. Zhou et al. (2002) stressed the importance of spatial isolation of soil particles, which are not interconnected by free water, for the occurrence of high microbial diversity in low

carbon soils as competition between species is excluded under these conditions. In the glacier foreland of the Rotmoosferner differences in water contents between the early and the late succession accounted for more than 10 % (data not shown), which certainly attributed to variable grades of spatial isolation between soil particles. Additionally, the low pH in the late succession possibly selected for adapted bacteria thereby reducing the diversity (Stres et al., 2004).

Conclusion

Whereas the community structure showed only slight shifts which were rather bound to long-term soil characteristics like pH and organic carbon, the nitrate reductase activity increased significantly with successional age and responded sensitively to the substrate availability of C_{org} and NO_3^-. The source of the higher nitrate reductase activity in the late succession might be either a higher density of the nitrate-reducing community and/or an upregulated enzyme synthesis. To unravel the underlying mechanism, further studies are required to quantify the nitrate-reducing bacteria and their expression of nitrate reductase along the successional gradient and to relate this to the enzyme activity. In addition, since nitrate reduction is only the first step of the denitrification cascade, it will be of interest to extent this study to bacteria reducing soluble nitrogen oxides to N_2O or N_2 for a better understanding of the N cycle in alpine ecosystems.

Acknowledgement

We would like to thank Meinhard Strobel for his support at the alpine research center (Obergurgl, Ötz valley), Dagmar Mezger, Stéphanie Hallet and David Bru for technical assistance with the laboratory analysis, and Blaž Stres and Andreas Büchse for their good advice on the statistics. The financial support provided by the COST Action 856 for a Short Term Scientific Mission in the Laboratory of Soil Microbiology and Geochemistry at the INRA/Dijon is also gratefully acknowledged.

References

Bardgett, R. D., and Walker, L. R. (2004) Impact of colonizer plant species on the development of decomposer microbial communities following deglaciation. Soil Biol. Biochem. 36: 555-559.

Bottomley, P. J. (1998) Microbial Ecology. In: Principles and applications of soil microbiology. Sylvia, D. M., Fuhrmann, J. J., Hartel, P. G., and Zuberer, D. A. (eds.). Upper Saddle River, New Jersey, USA. Prentice Hall: pp. 550.

Buckley, D. H., and Schmidt, T. M. (2003) Diversity and dynamics of microbial communities in soils from agroecosystems. Environ. Microbiol. 5: 441-452.

Chao, A., and Shen, T.-J. (2003) Nonparametric estimation of Shannon's index of diversity when there are unseen species in sample. Environ. Ecol. Stat. 10**:** 429-443.

Chao, A. (1987) Estimating the population size for capture-recapture data with unequal catchability. Biometrics 43: 783-791.

Chèneby, D., Hallet, S., Mondon, A., Martin-Laurent, F., Germon, J. C., and Philippot, L. (2003) Genetic characterization of the nitrate reducing community based on *narG* nucleotide sequence analysis. Microbial Ecol. 46: 113-121.

Colwell, R. K., Mao, C. X., and Chang, J. (2004) Interpolating, extrapolating, and comparing incidence-based species accumulation curves. Ecology 85: 2717-2727.

Deiglmayr, K., Philippot, H., Hartwig, U. A., and Kandeler, E. (2004) Structure and activity of the nitrate-reducing community in the rhizosphere of *Lolium perenne* and *Trifolium repens* under long-term elevated pCO_2. FEMS Microbiol. Ecol. 49**:** 445-454.

Enwall, K., Philippot, L., and Hallin, S. (2005) Activity and composition of the denitrifying community respond differently to long-term fertilization. Appl. Environ. Microbiol. 71: 8335-8343.

Erschbamer, B., Bitterlich, W., and Raffl, C. (1999) Die Vegetation als Indikator für die Bodenbildung im Gletschervorfeld des Rotmoosferners (Obergurgl, Ötztal, Nordtirol). Ber. nat.-med. Verein Innsbruck 86: 107-122.

Hodkinson, I. D., Webb, N. R., and Coulson, S. J. (2002) Primary community assembly on land – the missing stages: why are the heterotrophic organisms always there first? J. Ecol. 90: 569-577.

Højberg, O., and Sørensen, J. (1993) Microgradients of microbial oxygen consumption in a barley rhizosphere model system. Appl. Environ. Microbiol. 59: 431-437.

Hughes, J. B., Hellmann, J. J., Ricketts, T. H., and Bohannan, B. J. M. (2001) Counting the uncountable: statistical approaches to estimating microbial diversity. Appl. Environ. Microbiol. 67: 4399-4406.

Jumpponen, A. (2003) Soil fungal community assembly in a primary successional glacier forefront ecosystem as inferred from rDNA sequence analysis. New Phytol. 158: 569-578.

Kandeler, E. (1995) Nitrate reductase activity. In: Methods in soil biology. Schinner, F., Öhlinger, R., Kandeler E., and Margesin, R., (eds). Springer Berlin Heidelberg, Germany: pp. 176-179.

Kaufmann, R. (2001) Invertebrate succession on an alpine glacier foreland. Ecology 82: 2261-2278.

Klemedtsson, L., Berg, P., Clarholm, M., Schnürer, J., and Roswall, T. (1987) Microbial nitrogen transformations in the root environment of barley. Soil Biol. Biochem. 19: 551-558.

Kreader, C. A. (1996) Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. Appl. Environ. Microbiol. 62: 1102-1106.

Lashof, D. A., and Ahuja, D. R. (1990) Relative contributions of greenhouse gas emissions to global warming. Nature 344: 529-531.

Lepš, J., and Šmilauer, P. (2003) Multivariate analysis of ecological data using CANOCO. Cambridge University Press, United Kingdom: pp. 269.

McCaig, E. A., Glover, L. A., and Prosser, J. I. (1999) Molecular analysis of bacterial community structure and diversity in unimproved and improved upland grass pastures. Appl. Environ. Microbiol. 65: 1721-1730.

Magurran, A. E. (1988) Ecological diversity and its measurement. Princeton University Press, New Jersey: pp. 179.

Mounier, E., Hallet, S., Chèneby, D., Benizri, E., Gruet, Y., Nguyen, C., Piutti, S., Robin, C., Slezack-Deschaumes, S., Martin-Laurent, F., Germon, J. C., and Philippot, L. (2004) Influence of maize mucilage on the diversity and activity of the denitrifying community. Environ. Microbiol. 6: 301-312.

O'Donnell, A. G., Seasman, M., Macrae, A., Waite, I., and Davies, J. T. (2001) Plants and fertilizers as drivers of change in microbial community structure and function in soils. Plant Soil 232**:** 135-145.

Odum, E. P. (1969) The strategy of ecosystem development. Science 164: 262-270.

Ohtonen, R., Fritze, H., Pennanen, T., Jumpponen, A., and Trappe, J. (1999) Ecosystem properties and microbial community changes in primary succession on a glacier forefront. Oecologia 119: 239-246.

Patra, A. K., Abbadie, L., Clay-Josserand, A., Degrange, V., Grayston, S. J., Loiseau, P., Louault, F., Mahmood, S., Nazaret, S., Philippot, L., Poly, E., Prosser, J. I., Richaume, A., and Le Roux, X. (2005) Effects of grazing on microbial functional groups involved in soil N dynamics. Ecol. Monogr. 75: 65-80.

Philippot, L., Piutti, S., Martin-Laurent, F., Hallet, S., and Germon, J. C. (2002) Molecular analysis of the nitrate-reducing community from unplanted and maize-planted soils. Appl. Environ. Microbiol. 68: 6121-6128.

Raffl, C. (1999) Vegetationsgradienten und Sukzessionsmuster in einem zentralalpinen Gletschervorfeld (Ötztaler Alpen, Tirol). Diploma thesis. University of Innsbruck, Austria: pp. 88.

Ricklefs, R. E., and Miller, G. L. (2000) Ecology (4th ed.). W. H. Freeman and Company, New York: pp. 822.

Sigler, W. V., Crivii, S., and Zeyer, J. (2002) Bacterial succession in glacial forefield soils characterized by community structure, activity and opportunistic growth dynamics. Microbial Ecol. 44: 306-316.

Sigler, W. V., and Zeyer, J. (2002) Microbial diversity and activity along the forefields of two receding glaciers. Microbial Ecol. 43: 397-407.

Sigler, W. V., and Zeyer, J. (2004) Colony-forming analysis of bacterial community succession in deglaciated soils indicates pioneer stress-tolerant opportunists. Microbial Ecol. 48: 316-323.

Stres, B., Mahne, I., Avguštin, G., and Tiedje, J. M. (2004) Nitrous oxide reductase (*nosZ*) gene fragments differ between native and cultivated Michigan soils. Appl. Environ. Microbiol. 70: 301-309.

Stienstra, A. W., Klein Gunnewiek, P., and Laanbroek, H. J. (1994) Repression of nitrification in soils under a climax grassland vegetation. FEMS Microbiol. Ecol. 14: 42-52.

ter Braak, C. F. J., and Šmilauer, P. (1998) CANOCO reference manual and user's guide to Canoco for Windows: Software for canonical community ordination (version 4). Microcomputer Power, Ithaca, NY: pp. 352.

Thompson, J. D., Plewniak, F., and Poch, O. (1999) A comprehensive comparison of multiple sequence alignment programs. Nucleic Acids Res. 27: 2682-2690.

Tiunov, A. V., and Scheu, S. (2000) Microfungal communities in soil, litter and cast of *Lumbricus terrestris* L. (Lumbricidae): a laboratory experiment. Appl. Soil. Ecol. 14: 17-26.

Tscherko, D., Rustemeier, J., Richter, A., Wanek, W., and Kandeler, E. (2003) Functional diversity of the soil microflora in primary succession across two glacier forelands in the Central Alps. Eur. J. Soil. Sci. 54: 685-696.

Tscherko, D., Hammesfahr, U., Marx, M.-C., and Kandeler, E. (2004) Shifts in rhizosphere microbial communities and enzyme activity of *Poa alpina* across an alpine chronosequence. Soil Biol. Biochem. 36: 1685-1698.

Tscherko, D., Hammesfahr, U., Zeltner, G., Kandeler, E., and Böcker, R. (2005) Plant succession and rhizosphere microbial communities in a recently deglaciated alpine terrain. Basic Appl. Ecol. 6: 367-383.

Zhou, J., Xia, B., Treves, D. S., Wu, L.-Y., Marsh, T. L., O'Neill, Palumbo, A. V., and Tiedje, J. M. (2002) Spatial and resource factors influencing high microbial diversity in soil. Appl. Environ. Microbiol. 68: 326-334.

8 Final conclusions and perspectives

Linking the structure of soil microbial communities with its function in soil processes is a big challenge for current research in soil microbiology. In the present thesis the dissimilatory nitrate-reducing community was investigated as a model community using a biphasic approach that provided information on both the level of enzyme activity and the community structure. The combined knowledge of these two aspects will be needed to address future challenges of global climate change and to develop appropriate guidelines for the improved management of agricultural ecosystems.

Responses of nitrate reductase activity

By measurement of the nitrate reductase activity valuable information about the potential enzyme activity of the nitrate-reducing microorganisms in soils could be obtained. The following responses of nitrate reductase activity were observed:

- In the Swiss FACE experiment sensitive and significant responses of the nitrate reductase activity to nitrogen supply, elevated atmospheric CO_2 and functional plant type were detected, which were mainly attributed to differences in nitrate availability. Recent results on root exudations of *Lolium perenne* collected at the same sampling dates and in the same plots point to a possible contribution of reduced root exudation to the decline in nitrate reductase activity under elevated atmospheric CO_2 (Bazot et al., 2006).
- The incubation experiment confirmed the fast reaction rate of the nitrate reductase activity with regard to nitrate supply showing a significant difference between the control and the treatment of 300 µg NO_3^--N g^{-1} soil after three days. Simultaneously, a high resilience of the enzyme activity could be deduced since differences between the control and the nitrate amended treatments disappeared within seven days. However, under addition of 100 µg NO_3^--N g^{-1} soil the nitrate reductase activity was most likely underestimated, which could possibly be explained by the inability of 2,4-dinitrophenol to inhibit nitrite reduction in the enzyme assay.
- The data obtained from the glacier foreland revealed significant effects of successional age and rhizosphere on the level of enzyme activity. The predominant environmental factors governing this response were the concentration of organic carbon, which varied over a large range across the glacier foreland and was closely related to the water content

of the soil samples, and the nitrate availability, which was rather low and limited the response of the nitrate reductase activity, where carbon was abundant.

The consistent pattern found identified the short-term availability of nitrate and carbon as one of the most important factors controlling the nitrate reductase activity. Estimates of future developments of nitrate reductase activity under global climate change, therefore, will depend strongly on the extent of nitrate and carbon availability. The latter might possibly be enhanced under elevated atmospheric carbon dioxide concentrations depending on plant species, physiological state and nutrient supply. Levels of nitrate concentrations, however, can at least partially be controlled by management practices and represent, thus, a possible approach to reduce the increased denitrification risks under global climate change in agricultural managed fields.

Some drawbacks of the methodical approach also have to be considered:

- The determination of nitrate reductase activity is a potential measurement, which does not provide any information about *in situ* activities. In order to obtain these relevant data for modelling future climate scenarios, other methods have to be employed using e. g. ^{15}N stable isotopes.
- Under high nitrite reducing activities the nitrite reductase seemed not to be inhibited by 2,4-dinitrophenol in the enzyme assay. Further research is needed to reveal the mechanism of nitrite reductase inhibition.

Responses of nitrate-reducing community structure

The community structure of nitrate-reducing microorganisms proved to be very stable in the face of short-term fluctuations in nitrate availability. The observed shifts in community structure were rather based on long-term changes of the physical and chemical environment. The most important environmental variable affecting the composition of the nitrate-reducing microorganisms proved to be pH. Organic carbon content was an additional driver of shifts in the community composition of the nitrate reducers in the primary successional soils of the glacier foreland. However, not only the quantity of soil organic carbon, but also the organic carbon quality might be an important factor in determining the composition of nitrate-reducers. A shift towards an *Actinomycetes* dominated rhizosphere flora with increasing

successional age could perhaps be linked to the capacity of *Actinomycetes* to metabolize complex substrates such as lignin.

In conclusion, future trends in global climate change will probably not result in large shifts of the nitrate-reducing community. However, minor changes in the composition of the nitrate-reducing community have to be expected, particularly in sensitive environments like glacier forelands.

Certainly, when interpreting molecular data one has to keep in mind the limitations of this technique. Methodical biases can occur through all steps of the procedure starting with DNA extraction. Martin-Laurent et al. (2001) have revealed that the phylotype abundance and the community structure of the eubacterial community varied with different DNA recovery methods. Another inherent bias lies within the primer design as the universality and the specificity of the primer sequence depends strongly on the existing database. Up to date less than 1 % of the total existing microbial diversity is comprised in the ribosomal database project (Forney et al., 2004). For functional genes the available sequences are even more limited. During the polymerase chain reaction preferential binding of the primers can result in overamplification of specific target sequences (Polz and Cavanaugh, 1998). Last but not least by fingerprinting methods (e.g. DGGE, RFLP, SSCP) developed for fast screening of large sample sets only dominant phylotypes can be detected, whereas many smaller populations remain unseen.

Perspectives

In order to improve our understanding of the behaviour of denitrifying communities in the face of global climate change future studies should extend to the complete series of functional genes involved in the denitrification pathway. Therein included are *narG* encoding the catalytic subunit of the membrane-bound nitrate reductase and its periplasmic counterpart *napA*, the copper-containing (*nirK*) and cytochrome cd1-containing (*nirS*) nitrite reductases, and *norB/norZ* and *nosZ* as marker genes for the nitric oxide and nitrous oxide reductases. The microarray technology, based on the DNA-DNA hybridization principle, offers a great opportunity to rapidly screen for a vast number of phylotypes and to obtain even quantitative information (Wu et al., 2001; Taroncher-Oldenburg et al., 2003). However, probe development faces the same drawbacks as primer design in regard of specificity and is

restricted additionally by the requirements of similar melting temperatures. Information on changes in density of the various functional communities can be acquired by quantitative real-time PCR, which is established already for the major part of genes involved in the denitrification pathway.

Since the presence of denitrifying genes in soils does not imply that the respective microorganisms are active, further methods are needed in order to link community structure and activity closer. The biphasic approach applied in this thesis needs to be completed by the missing intermediates. Targeting the mRNA and the corresponding enzymes by immunological approaches will be one step in this direction (Philippot and Hallin, 2005). If we want to recommend appropriate policies how to reach lower denitrification rates in order to reduce the emissions of greenhouse gases, we will need to deepen our understanding of the factors governing the activity of denitrifying microorganisms.

References

Bazot, S., Ulff, L., Blum, H., Nguyen, C., and Robin, C. (2006) Effects of elevated CO_2 concentration on rhizodeposition from *Lolium perenne* grown on soil exposed to 9 years of CO_2 enrichment. Soil Biol. Biochem.: in press.

Forney, L. J., Zhou, X., and Brown, C. J. (2004) Molecular microbial ecology: land of the one-eyed king. Curr. Opin. Microbiol. 7: 210-220.

Martin-Laurent, F., Philippot, L., Hallet, S., Chaussod, R., Germon, J. C., Soulas, G., and Catroux, G. (2001) DNA extraction from soil: old bias for new microbial diversity analysis methods. Appl. Environ. Microbiol. 67: 2354-2359.

Philippot, L., and Hallin, S. (2005) Finding the missing link between diversity and activity using denitrifying bacteria as a model functional community. Curr. Opin. Microbiol. 8: 234-239.

Polz, M. F., and Cavanaugh, C. M. (1998) Bias in template-to-product ratios in multitemplate PCR. Appl. Environ. Microbiol. 64: 3724-3730.

Taroncher-Oldenburg, G., Griner, E. M., Francis, C. A., Ward, B. B. (2003) Oligonucleotide microarray for the study of functional gene diversity in the nitrogen cycle in the environment. Appl. Environ. Microbiol. 69: 1159-1171.

Wu, L., Thompson, D. K., Li, G., Hurt, R. A., Tiedje, J. M., and Zhou, J. (2001) Development and evaluation of functional gene arrays for detection of selected genes in the environment. Appl. Environ. Microbiol. 67: 5780-5790.

9 Literature cited

Abdelmagid, H. M., and Tabatabai, M. A. (1987) Nitrate reductase activity of soils. Soil Biol. Biochem. 19: 421-427.

Ainsworth, E. A., and Long, S. P. (2005) What have we learned from 15 years of free-air CO_2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO_2. New Phytol. 165: 351-372.

Baggs, E. M., Richter, M., Cadisch, G., and Hartwig, U. A. (2003) Denitrification in grass swards is increased under elevated atmospheric CO_2. Soil Biol. Biochem. 35: 729-732.

Baggs, E. M., and Blum, H. (2004) CH_4 oxidation and emissions of CH_4 and N_2O from *Lolium perenne* swards under elevated atmospheric CO_2. Soil Biol. Biochem. 36: 713-723.

Bardgett, R. D., Lovell, R. D., Hobbs, P. J., and Jarvis, S. C. (1999) Seasonal changes in soil microbial communities along a fertility gradient of temperate grasslands. Soil Biol. Biochem. 31: 1021-1030.

Bardgett, R. D., and Walker, L. R. (2004) Impact of colonizer plant species on the development of decomposer microbial communities following deglaciation. Soil Biol. Biochem. 36: 555-559.

Barnard, R., Barthes, L., Le Roux, X., and Leadley, P. W. (2004) Dynamics of nitrifying activities, denitrifying activities and nitrogen in grassland mesocosms as altered by elevated CO_2. New Phytol. 162: 365-376.

Bateman, E. J., and Baggs, E. M. (2005) Contributions of nitrification and denitrification to N_2O emissions from soils at different water-filled pore space. Biol. Fertil. Soils 41: 379-388.

Bazot, S., Ulff, L., Blum, H., Nguyen, C., and Robin, C. (2006) Effects of elevated CO_2 concentration on rhizodeposition from *Lolium perenne* grown on soil exposed to 9 years of CO_2 enrichment. Soil Biol. Biochem.: in press.

Bazzaz, F. A. (1990) The response of natural ecosystems to the rising global CO_2 levels. Annu. Rev. Ecol. Syst. 21: 167-196.

Bottomley, P. J. (1998) Microbial Ecology. In: Principles and applications of soil microbiology. Sylvia, D. M., Fuhrmann, J. J., Hartel, P. G., and Zuberer, D. A. (eds.). Upper Saddle River, New Jersey, USA. Prentice Hall: pp. 550.

Buckley, D. H., and Schmidt, T. M. (2003) Diversity and dynamics of microbial communities in soils from agro-ecosystems. Environ. Microbiol. 5: 441-452.

Carnol, M., Hogenboom, L., Jach, M. E., Remacle, J., and Ceulemans, R. (2002) Elevated atmospheric CO_2 in open top chambers increases net nitrification and potential denitrification. Global Change Biol. 8: 590-598.

Chao, A. (1987) Estimating the population size for capture-recapture data with unequal catchability. Biometrics 43: 783-791.

Chao, A., and Shen, T.-J. (2003) Nonparametric estimation of Shannon's index of diversity when there are unseen species in sample. Environ. Ecol. Stat. 10**:** 429-443.

Chèneby, D., Hallet, S., Mondon, A., Martin-Laurent, F., Germon, J. C., and Philippot, L. (2003) Genetic characterization of the nitrate-reducing community based on *narG* nucleotide sequence analysis. Microbial Ecol. 46: 113-121.

Clayton, H., Mc Taggart, I. P., Parker, J., Swan, L., and Smith, K. A. (1997) Nitrous oxide emissions from fertilised grasslands: a two year study of the effects of N fertilizer form and environmental conditions. Biol. Fertil. Soils 25: 252-260.

Clegg, C. D., Lovell, R. D. L., and Hobbs, P. J. (2003) The impact of grassland management regime on the community structure of selected bacterial groups in soils. FEMS Microbiol. Ecol. 43: 263-270.

Colwell, R. K., Mao, C. X., and Chang, J. (2004) Interpolating, extrapolating, and comparing incidence-based species accumulation curves. Ecology 85: 2717-2727.

Deiglmayr, K., Philippot, H., Hartwig, U. A., and Kandeler, E. (2004) Structure and activity of the nitrate-reducing community in the rhizosphere of *Lolium perenne* and *Trifolium repens* under long-term elevated pCO_2. FEMS Microbiol. Ecol. 49: 445-454.

Dendooven, L., and Anderson, J. M. (1994) Dynamics of reduction enzymes involved in the denitrification process in pasture soil. Soil Biol. Biochem. 26: 1501-1506.

Diaz, S., Grime, J. P., Harris, J., and McPherson, E. (1993) Evidence of a feedback mechanism limiting plant response to elevated carbon dioxide. Nature 364: 616-617.

Drake, B. G., Gonzalez-Meler, M. A., and Long, S. P. (1997) More efficient plants: a consequence of rising atmospheric CO_2? Annu. Rev. Plant Phys. 48: 609-639.

Ebersberger, D., Wermbter, N., Niklaus, P. A., and Kandeler, E. (2004) Effects of long-term CO_2 enrichment on microbial community structure in calcareous grassland. Plant Soil 264: 313-323.

Ellis, S., Howe, M. T., Goulding, K. W. T., Mugglestone, M. A., and Dendooven, L. (1998) Carbon and nitrogen dynamics in a grassland soil with varying pH: effect of pH on the denitrification potential and dynamics of the reduction enzymes. Soil Biol. Biochem. 30: 359-367.

Enwall, K., Philippot, L., and Hallin, S. (2005) Activity and composition of the denitrifying community respond differently to long-term fertilization. Appl. Environ. Microbiol. 71: 8335-8343.

Erschbamer, B., Bitterlich, W., and Raffl, C. (1999) Die Vegetation als Indikator für die Bodenbildung im Gletschervorfeld des Rotmoosferners (Obergurgl, Ötztal, Nordtirol). Ber. nat.-med. Verein Innsbruck 86: 107-122.

Fitter, H., Graves, J. D., Wolfenden, J., Self, G. K., Brown, T. K., Bogie, D., and Mansfield, T. A. (1997) Root production and turnover and carbon budgets of two contrasting grasslands under ambient and elevated atmospheric carbon dioxide concentrations. New Phytol. 137: 247-255.

Flanagan, D. A., Gregory, L. G., Carter, J. P., Karakas-Sen, A., Richardson, D. J., and Spiro, S. (1999) Detection of genes for periplasmic nitrate reductase in nitrate respiring bacteria and in community DNA. FEMS Microbiol. Lett. 177: 263-270.

Forney, L. J., Zhou, X., and Brown, C. J. (2004) Molecular microbial ecology: land of the one-eyed king. Curr. Opin. Microbiol. 7: 210-220.

Gavira, M., Roldan, M. D., Castillo, F., and Moreno-Vivian, C. (2002) Regulation of *nap* gene expression and periplasmic nitrate reductase activity in the phototrophic bacterium *Rhodobacter sphaeroides* DSM158. J. Bacteriol. 184: 1693-1702.

Gloser, V., Jezikova, M., Lüscher, A., Frehner, M., Blum, H., Nösberger, J., and Hartwig, U. A. (2000) Soil mineral nitrogen availability was unaffected by elevated atmospheric CO_2 in a four year old experiment (Swiss FACE). Plant Soil 227: 291-299.

Gregory, L. G., Karakas-Sen, A., Richardson, D. J., and Spiro, S. (2000) Detection of genes for membrane-bound nitrate reductase in nitrate-respiring bacteria and in community DNA. FEMS Microbiol. Lett. 183: 275-279.

Haeberli, W., Noetzli, J., Zemp, M., Baumann, S., Frauenfelder, R., and Hoelzle, M. (2005) Glacier mass balance bulletin: Bulletin No. 8 (2002-2003). Compiled by the World Glacier Monitoring Service (WGMS). Retrieved November 4, 2005 from http://www.geo.unizh.ch/wgms.

Hartwig, U. A., Lüscher, A., Nösberger, J., and van Kessel, C. (2002) Nitrogen-15 budget in model ecosystems of white clover and perennial ryegrass exposed for four years at elevated atmospheric pCO_2. Global Change Biol. 8: 194-202.

Hebeisen, T., Lüscher, A., Zanetti, S., Fischer, B. U., Hartwig, U. A., Frehner, M., Hendrey, G. R., Blum, H., and Nösberger, J. (1997) Growth response of *Trifolium repens* L. and *Lolium perenne* L. as monoculture and bi-species mixture to free air CO_2 enrichment and management. Global Change Biol. 3: 149-160.

Hodkinson, I. D., Webb, N. R., and Coulson, S. J. (2002) Primary community assembly on land – the missing stages: why are the heterotrophic organisms always there first? J. Ecol. 90: 569-577.

Højberg, O., and Sørensen, J. (1993) Microgradients of microbial oxygen consumption in a barley rhizosphere model system. Appl. Environ. Microbiol. 59: 431-437.

Højberg, O., Revsbech, N. P., and Tiedje, J. (1994) Denitrification in soil aggregates analyzed with microsensors for nitrous oxide and oxygen. Soil Sci. Soc. Am. J. 58: 1691-1698.

Højberg, O., Binnerup, S. J., and Sørensen, J. (1996) Potential rates of ammonium oxidation, nitrite oxidation, nitrate reduction and denitrification in the young barley rhizosphere. Soil Biol. Biochem. 28: 47-54.

Hughes, J. B., Hellmann, J. J., Ricketts, T. H., and Bohannan, B. J. M. (2001) Counting the uncountable: statistical approaches to estimating microbial diversity. Appl. Environ. Microbiol. 67: 4399-4406.

Hungate, B. A., Lund, C., Pearson, H. L., and Chapin, F. S. III. (1997) Elevated CO_2 and nutrient addition alter soil N cycling and N trace gas fluxes with early season wet-up in a Californian annual grassland. Biogeochemistry 37: 89-109.

Ineson, P., Coward, P. A., and Hartwig, U. A. (1998) Soil gas fluxes of N_2O, CH_4 and CO_2 beneath *Lolium perenne* under elevated CO_2: The Swiss free air carbon dioxide enrichment experiment. Plant Soil 198: 89-95.

IPCC (Intergovernmental Panel on Climate Change) (2001a) Climate Change 2001: The scientific basis. Contribution of Working Group I to the third assessment report of the Intergovernmental Panel on Climate Change, Cambridge, University Press.

IPCC (Intergovernmental Panel on Climate Change) (2001b) Climate Change 2001: Impacts, adaptations, and vulnerability. Contribution of Working Group II to the third assessment report of the Intergovernmental Panel on Climate Change, Cambridge, University Press.

Jongen, M., Jones, M. B., Hebeisen, T., Blum, H., and Hendrey, G. R. (1995) The effects of elevated CO_2 concentrations on the root growth of *Lolium perenne* and *Trifolium repens* grown in a FACE system. Global Change Biol. 1: 361-371.

Jumpponen, A. (2003) Soil fungal community assembly in a primary successional glacier forefront ecosystem as inferred from rDNA sequence analysis. New Phytol. 158: 569-578.

Kandeler, E. (1995) Nitrate reductase activity. In: Methods in soil biology. Schinner, F., Öhlinger, R., Kandeler E., and Margesin, R., (eds). Springer Berlin Heidelberg, Germany: pp. 176-179.

Kaufmann, R. (2001) Invertebrate succession on an alpine glacier foreland. Ecology 82: 2261-2278.

Kettunen, R., Saarnio, S., Martikainen, P., and Silvola, J. (2005) Elevated CO_2 concentrations and nitrogen fertilisation effects on N_2O and CH_4 fluxes and biomass production of *Phleum pratense* on farmed peat soil. Soil Biol. Biochem. 37: 739-750.

Kimball, B. A. (1983) Carbon dioxide and agricultural yield: an assemblage and analysis of 430 prior observations. Agron. J. 75: 779-788.

Klemedtsson, L., Berg, P., Clarholm, M., Schnürer, J., and Roswall, T. (1987) Microbial nitrogen transformations in the root environment of barley. Soil Biol. Biochem. 19: 551-558.

Körner, C. (2000) Biosphere responses to CO_2 enrichment. Ecol. Appl. 10: 1590-1619.

Kreader, C. A. (1996) Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. Appl. Environ. Microbiol. 62: 1102-1106.

Lashof, D. A., and Ahuja, D. R. (1990) Relative contributions of greenhouse gas emissions to global warming. Nature 344: 529-531.

Lensi, R., Lescure, C., Steinberg, C., Savoie, J.-M., and Faurie, G. (1991) Dynamics of residual enzyme activities, denitrification potential, and physico-chemical properties in a gamma-sterilized soil. Soil Biol. Biochem. 23: 367-373.

Lepš, J., and Šmilauer, P. (2003) Multivariate analysis of ecological data using CANOCO. Cambridge University Press, United Kingdom: pp. 269.

Lussenhop, J., Treonis, A., Curtis, P. S., Teeri, J. A., and Vogel, C. S. (1998) Response of soil biota to elevated atmospheric CO_2 in poplar model systems. Oecologia 113: 247-251.

Madigan, M. T., Martinko, J. M., and Parker, J. (2003) Brock biology of microorganisms (10^{th} edition). Pearson education international, Prentice Hall, London: pp. 1019.

Magurran, A. E. (1988) Ecological diversity and its measurement. Princeton University Press, New Jersey: pp. 179.

Marilley, L., Hartwig, U. A., and Aragno, M. (1999) Influence of an elevated atmospheric CO_2 content on soil and rhizosphere bacterial communities beneath *Lolium perenne* and *Trifolium repens* under field conditions. Microbial Ecol. 38: 39-49.

Martin-Laurent, F., Philippot, L., Hallet, S., Chaussod, R., Germon, J. C., Soulas, G., and Catroux, G. (2001) DNA extraction from soil: old bias for new microbial diversity analysis methods. Appl. Environ. Microbiol. 67: 2354-2359.

Martin-Olmedo, P., Rees, R. M., and Grace, J. (2002) The influence of plants grown under elevated CO_2 and N fertilisation on soil nitrogen dynamics. Global Change Biol. 8: 643-657.

McCaig, E. A., Glover, L. A., and Prosser, J. I. (1999) Molecular analysis of bacterial community structure and diversity in unimproved and improved upland grass pastures. Appl. Environ. Microbiol. 65: 1721-1730.

Moir, J. B. W., and Wood, N. J. (2001) Nitrate and nitrite transport in bacteria. Cell. Mol. Life Sci. 58: 215-224.

Montealegre, C. M., van Kessel, C., Blumenthal, J. M., Hur, H. G., Hartwig, U. A., and Sadowsky, M. J. (2000) Elevated atmospheric CO_2 alters microbial structure in a pasture ecosystem. Global Change Biol. 6: 475-482.

Montealegre, C. M., van Kessel, C., Russelle, M. P., and Sadowsky, M. J. (2002) Changes in microbial activity and composition in a pasture ecosystem exposed to elevated atmospheric carbon dioxide. Plant Soil 243: 197-207.

Mosier, A. R., Morgan, J. A., King, J. Y., LeCain, D., and Milchunas, D. G. (2002) Soil-atmosphere exchange of CH_4, CO_2, NO_x and N_2O in the Colorado shortgrass steppe under elevated CO_2. Plant Soil 240: 201-211.

Mounier, E., Hallet, S., Chèneby, D., Benizri, E., Gruet, Y., Nguyen, C., Piutti, S., Robin, C., Slezack-Deschaumes, S., Martin-Laurent, F., Germon, J. C., and Philippot, L. (2004) Influence of maize mucilage on the diversity and activity of the denitrifying community. Environ. Microbiol. 6: 301-312.

Müller, C., Stevens, R. J., Laughlin, R. J., and Jäger, H.-J. (2004) Microbial processes and the site of N_2O production in a temperate grassland soil. Soil Biol. Biochem. 36: 453-461.

Murray, R. E., Feig, Y. S., and Tiedje, J. (1995) Spatial heterogeneity in the distribution of denitrifying bacteria associated with denitrification activity zones. Appl. Environ. Microbiol. 61: 2791-2793.

Niklaus, P. A., Spinnler, D., and Körner, C. (1998) Soil moisture dynamics of calcareous grassland under elevated CO_2. Oecologia 117: 201-208.

Nijburg, J. W., Coolen, M. J. L., Gerards, S., Klein Gunnewiek, P. J. A., and Laanbroek, H. J. (1997) Effects of nitrate availability and the presence of *Glyceria maxima* on the composition and activity of the dissimilatory nitrate-reducing bacterial community. Appl. Environ. Microbiol. 63: 931-937.

Norby, R. J., Cotrufo, M. F., Ineson, P., O'Neill, E. G., and Canadell, J. G. (2001) Elevated CO_2, litter chemistry and decomposition: a synthesis. Oecologia 127: 153-165.

O'Donnell, A. G., Seasman, M., Macrae, A., Waite, I., and Davies, J. T. (2001) Plants and fertilizers as drivers of change in microbial community structure and function in soils. Plant Soil 232**:** 135-145.

Odum, E. P. (1969) The strategy of ecosystem development. Science 164: 262-270.

Ohtonen, R., Fritze, H., Pennanen, T., Jumpponen, A., and Trappe, J. (1999) Ecosystem properties and microbial community changes in primary succession on a glacier forefront. Oecologia 119: 239-246.

Patra, A. K., Abbadie, L., Clay-Josserand, A., Degrange, V., Grayston, S. J., Loiseau, P., Louault, F., Mahmood, S., Nazaret, S., Philippot, L., Poly, E., Prosser, J. I., Richaume, A., and Le Roux, X. (2005) Effects of grazing on microbial functional groups involved in soil N dynamics. Ecol. Monogr. 75: 65-80.

Pendall, E., Mosier, A. R., and Morgan, J. A. (2004) Rhizodeposition stimulated by elevated CO_2 in a semiarid grassland. New Phytol. 162: 447-458.

Philippot, L. (2002) Denitrifying genes in bacterial and archeal genomes. Biochim. Biophys. Acta 1577: 355-376.

Philippot, L., Piutti, S., Martin-Laurent, F., Hallet, S., and Germon, J. C. (2002) Molecular analysis of the nitrate-reducing community from unplanted and maize-planted soils. Appl. Environ. Microbiol. 68: 6121-6128.

Philippot, L. (2005) Tracking nitrate reducers and denitrifiers in the environment. Biochem. Soc. T. 33: 2004-2008.

Philippot, L., and Hallin, S. (2005) Finding the missing link between diversity and activity using denitrifying bacteria as a model functional community. Curr. Opin. Microbiol. 8: 234-239.

Phillips, R. L., Whalen, S. C., and Schlesinger, W. H. (2001) Influence of atmospheric CO_2 enrichment on nitrous oxide flux in a temperate forest ecosystem. Global Biogeochem. Cycles 15: 741-751.

Polcyn, W., and Lucinski, R. (2003) Aerobic and anaerobic nitrate and nitrite reduction in free-living cells of *Bradyrhizobium* sp. (*Lupinus*). FEMS Microbiol. Lett. 226: 331-337.

Polz, M. F., and Cavanaugh, C. M. (1998) Bias in template-to-product ratios in multitemplate PCR. Appl. Environ. Microbiol. 64: 3724-3730.

Raffl, C. (1999) Vegetationsgradienten und Sukzessionsmuster in einem zentralalpinen Gletschervorfeld (Ötztaler Alpen, Tirol). Diploma thesis. University of Innsbruck, Austria: pp. 88.

Reddy, R. U., and Reddy, M. S. (1998) Dissimilatory nitrate reductase activity in soils of Northern Telangana of Andhra Pradesh and its relationship with various soil properties. Ann. Agr. Res. 19: 250-253.

Richardson, D. J., Berks, B. C., Russell, D. A., Spiro, S., and Taylor, C. J. (2001) Functional, biochemical and genetic diversity of prokaryotic nitrate reductases. Cell. Mol. Life Sci. 58: 165-178.

Richter, M., Hartwig, U. A., Frossard, E., Nösberger, J., and Cadisch, G. (2003) Gross fluxes of nitrogen in grassland soil exposed to elevated atmospheric pCO_2 for seven years. Soil Biol. Biochem. 35: 1325-1335.

Ricklefs, R. E., and Miller, G. L. (2000) Ecology (4^{th} ed.). W. H. Freeman and Company, New York: pp. 822.

Robinson, D., and Conroy, J. P. (1999) A possible plant-mediated feedback between elevated CO_2, denitrification and the enhanced greenhouse effect. Soil Biol. Biochem. 31: 43-53.

Rogers, H. H., Runion, G. B., and Krupa, S. V. (1994) Plant responses to atmospheric CO_2 enrichment with emphasis on roots and the rhizosphere. Environ. Pollut. 83: 155-189.

Roussel-Delif, L., Tarnawski, S., Hamelin, J., Philippot, L., Aragno, M., and Fromin, N. (2005) Frequency and diversity of nitrate reductase genes among nitrate-dissimilating *Pseudomonas* in the rhizosphere of perennial grasses grown in field conditions. Microbial Ecol. 49: 63-72.

Schortemeyer, M., Hartwig, U. A., Hendrey, G. R., and Sadowsky, M. J. (1996) Microbial community changes in the rhizospheres of white clover and perennial ryegrass exposed to free air carbon dioxide enrichment (FACE). Soil Biol. Biochem. 28: 1717-1724.

Sexstone, A. J., Parkin, T. B., and Tiedje, J. M. (1988) Denitrification response to soil wetting in aggregated and unaggregated soil. Soil Biol. Biochem. 20: 767-769.

Sharma, S., Szele, Z., Schilling, R., Munch, J. C., and Schloter, M. (2006) Influence of freeze-thaw on the structure and function of microbial communities and denitrifying population in soil. Appl. Environ. Microbiol. 72: 2148-2154..

Sigler, W. V., Crivii, S., and Zeyer, J. (2002) Bacterial succession in glacial forefield soils characterized by community structure, activity and opportunistic growth dynamics. Microbial Ecol. 44: 306-316.

Sigler, W. V., and Zeyer, J. (2002) Microbial diversity and activity along the forefields of two receding glaciers. Microbial Ecol. 43: 397-407.

Sigler, W. V., and Zeyer, J. (2004) Colony-forming analysis of bacterial community succession in deglaciated soils indicates pioneer stress-tolerant opportunists. Microbial Ecol. 48: 316-323.

Šimek, M., Elhottová, D., Klimeš, F., and Hopkins, D. W. (2004) Emissions of N_2O and CO_2, denitrification measurements and soil properties in red clover and ryegrass stands. Soil Biol. Biochem. 36: 9-21.

Smart, D. R., Ritchie, K., Stark, J. M., and Bugbee, B. (1997) Evidence that elevated CO_2 levels can indirectly increase rhizosphere denitrifier activity. Appl. Environ. Microbiol. 63: 4621-4624.

Sonneman, I., and Wolters, V. (2005) The micro food web of grassland soils responds to a moderate increase in atmospheric CO_2. Global Change Biol. 11: 1148-1155.

Staley, J. T., and Orians, G. H. (2000) Evolution and the biosphere. In: Earth system science. Jacobson, M. C., Charlson, R. J., Rodhe, H., and Orians, G. H. (eds.). Academic Press, San Diego: pp. 29-61.

Stienstra, A. W., Klein Gunnewiek, P., and Laanbroek, H. J. (1994) Repression of nitrification in soils under a climax grassland vegetation. FEMS Microbiol. Ecol. 14: 42-52.

Stres, B., Mahne, I., Avguštin, G., and Tiedje, J. M. (2004) Nitrous oxide reductase (*nosZ*) gene fragments differ between native and cultivated Michigan soils. Appl. Environ. Microbiol. 70: 301-309.

Suter, D., Frehner, M., Fischer, B. U., Nösberger, J., and Lüscher, A. (2002) Elevated CO_2 increases carbon allocation to the roots of *Lolium perenne* under free-air CO_2 enrichment but not in a controlled environment. New Phytol. 154: 65-75.

ter Braak, C. F. J., and Šmilauer, P. (1998) CANOCO reference manual and user's guide to Canoco for Windows: Software for canonical community ordination (version 4). Microcomputer Power, Ithaca, NY: pp. 352.

Taroncher-Oldenburg, G., Griner, E. M., Francis, C. A., Ward, B. B. (2003) Oligonucleotide microarray for the study of functional gene diversity in the nitrogen cycle in the environment. Appl. Environ. Microbiol. 69: 1159-1171.

Thompson, J. D., Plewniak, F., and Poch, O. (1999) A comprehensive comparison of multiple sequence alignment programs. Nucleic Acids Res. 27: 2682-2690.

Tiedje, J. M. (1988) Ecology of denitrification and dissimilatory nitrate reduction to ammonium. In: Biology of anaerobic microorganisms. A. J. B. Zehnder (ed.). John Wiley & Sons, New York, NY: pp. 179-244.

Tiunov, A. V., and Scheu, S. (2000) Microfungal communities in soil, litter and cast of *Lumbricus terrestris* L. (Lumbricidae): a laboratory experiment. Appl. Soil. Ecol. 14: 17-26.

Tscherko, D., Rustemeier, J., Richter, A., Wanek, W., and Kandeler, E. (2003) Functional diversity of the soil microflora in primary succession across two glacier forelands in the Central Alps. Eur. J. Soil. Sci. 54: 685-696.

Tscherko, D., Hammesfahr, U., Marx, M.-C., and Kandeler, E. (2004) Shifts in rhizosphere microbial communities and enzyme activity of *Poa alpina* across an alpine chronosequence. Soil Biol. Biochem. 36: 1685-1698.

Tscherko, D., Hammesfahr, U., Zeltner, G., Kandeler, E., and Böcker, R. (2005) Plant succession and rhizosphere microbial communities in a recently deglaciated alpine terrain. Basic Appl. Ecol. 6: 367-383.

Van Kessel, C., Horwath, W. R., Hartwig, U. A., Harris, D., and Lüscher, A. (2000) Net soil carbon input under ambient and elevated CO_2 concentrations: isotopic evidence after 4 years. Global Change Biol. 6: 435-444.

Van Kessel, C., Nitschelm, J., Horwarth, W. R., Harris, D., Walley, F., Lüscher, A., and Hartwig, U. A. (2000) Carbon-13 input and turnover in a pasture soil exposed to long-term elevated atmospheric CO_2. Global Change Biol. 6: 123-135.

Whitehead, D. C. (2000) Nutrient elements in grasslands – Soil-plant-animal relationships. CABI Publishing, New York: pp 369.

Wu, L., Thompson, D. K., Li, G., Hurt, R. A., Tiedje, J. M., and Zhou, J. (2001) Development and evaluation of functional gene arrays for detection of selected genes in the environment. Appl. Environ. Microbiol. 67: 5780-5790.

Yeates, G. W., Newton, P. C. D., and Ross, D. J. (2003) Significant changes in soil microfauna in grazed pasture under elevated carbon dioxide. Biol. Fert. Soils 38: 319-326.

Zak, D. R., Pregitzer, K. S., King, J. S., and Holmes, W. E. (2000) Elevated atmospheric CO_2, fine roots and the response of soil microorganisms: A review and hypothesis. New Phytol. 147: 201-222.

Zhou, J., Xia, B., Treves, D. S., Wu, L.-Y., Marsh, T. L., O'Neill, Palumbo, A. V., and Tiedje, J. M. (2002) Spatial and resource factors influencing high microbial diversity in soil. Appl. Environ. Microbiol. 68: 326-334.

Curriculum vitae

Name:	Kathrin Deiglmayr
Date of birth:	October 6th, 1974
Place of birth:	Munich, Germany
Parents:	Maria Helene Deiglmayr, née Ostertag Wilhelm Deiglmayr
Marital status:	married, one child

Education

09/1981 – 07/1985	Primary school in Augsburg
09/1985 – 07/1994	Grammar school at the "St. Anna-Gymnasium", Augsburg
07/1994	*Abitur* with A-levels in mathematics, french, geography and arts
10/1995 – 08/2001	Studies of agricultural sciences with focus on crop production at the Centre of Life Sciences in Weihenstephan/TU Munich

Occupational Activity

Since 4/2002	Scientific assistant at the Institute of Crop Production and Grassland Research, University of Hohenheim

International Research Experiences

10/2002 – 11/2002	Scientific sojourn at the INRA/Dijon with Dr. Philippot
05/2003 – 08/2003	Scientific sojourn in the USA at (i) the Oak Ridge National Laboratory, Tennessee with Dr. Zhou and (ii) the University of Minnesota, St. Paul with Prof. Sadowsky, supported by a DAAD grant
02/2005	Short-term scientific mission at the INRA/Dijon with Dr. Philippot supported by the COST Action 856

Other International Experiences

08/1994 – 06/1995	Volunteering in a Self-Help-Project in Mariannhill, KwaZulu Natal, South Africa
04/2000 – 05/2000	Placement at APCOP (Apoyo Para el Campesino Indígena del Oriente Boliviano) in the project "Implementation of agroforestry techniques"