TECHNISCHE UNIVERSITÄT MÜNCHEN

Lehrstuhl für Bodenökologie

Functional diversity of nitrogen transforming microbial communities during terrestrial ecosystem development

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Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

genehmigten Dissertation.

Vorsitzender: Univ.-Prof. Dr. J. C. Munch

Prüfer der Dissertation:

1. Hon.-Prof. Dr. M. Schloter
2. Univ.-Prof. Dr. I. Kögel-Knabner
3. Univ.-Prof. Dr. J. Zeyer
   (Eidgenössische Technische Hochschule Zürich, Schweiz)

Die Dissertation wurde am 08.08.2011 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 18.11.2011 angenommen.

„In den Wissenschaften ist viel Gewisses, sobald man sich

von den Ausnahmen nicht irre machen lässt

und die Probleme zu ehren weiß.“

(Johann Wolfgang von Goethe)

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List of publications and contributions

Publications

Research article:

I. **Töwe, S.**[*]; Wallisch, S.[*]; Bannert, A.; Fischer, D.; Hai, B.; Haesler, F.; Kleineidam, K.; Schloter, M. **(2011)** Improved protocol for the simultaneous extraction and column-based separation of DNA and RNA from different soils. Journal of Microbiological Methods **84:** 406-412

II. **Töwe, S**.; Kleineidam, K.; Schloter, M. **(2010)** Differences in amplification efficiency of standard curves in quantitative real-time PCR assays and consequences for gene quantification in environmental samples. Journal of Microbiological Methods **82:** 338-341

III. Brankatschk, R.[*]; **Töwe, S.**[*]; Kleineidam, K.; Schloter, M.[#]; Zeyer, J.[#] **(2011)** Abundances and potential activities of nitrogen cycling microbial communities along a chronosequence of a glacier forefield. The ISME Journal **5:** 1025-1037

IV. **Töwe, S.**; Albert, A.; Kleineidam, K.; Brankatschk, R.; Dümig, A.; Welzl, G.; Munch, J.C.; Zeyer, J.; Schloter, M. **(2010)** Abundance of microbes involved in nitrogen transformation in the rhizosphere of *Leucanthemopsis alpina* (L.) Heywood grown in soils from different sites of the Damma glacier forefield. Microbial Ecology **60**: 762–770

Review:

V. Ollivier, J.[*]; **Töwe, S**.[*]; Bannert, A.; Hai, B.; Kastl, E.; Meyer, A.; Su, M.; Kleineidam, K.; Schloter, M. **(2011)** Nitrogen turnover and global change. FEMS Microbiology Ecology **78**: 3-16

[*], [#] equally contributed

My contribution to the publications

I. I was mainly involved in evolving the simultaneous DNA/RNA extraction protocol, in coordinating the experiment and performing the qPCR analysis and the connected statistics. Regarding the manuscript, I was responsible to write the first draft of the material and methods part, the results and the discussion.

II. + IV. I was involved in planning and conducting the experiment and the sampling. The published data were ascertained by me. For publication IV the help of a statistician was consulted. The manuscripts are mainly based on my input.

III. I was involved in planning the experiment and the sampling. Regarding the subsequent analysis, I performed qPCR with the provided DNA extracts and potential enzyme activities of the nitrification and denitrification processes, respectively. Moreover, the determination of the C_{mic}, N_{mic}, DOC, DON, NO_3^- and NH_4^+ contents were done by me. The introduction and discussion part of the manuscript are mainly based on my input.

V. I was responsible for the chapter "Ecosystem development".

Summary

Nitrogen, as a macronutrient, is an important factor for ecosystem functioning and development. While the interplay of different nitrogen cycle processes and the respective functional communities is under permanent focus in agricultural ecosystems, in initial ecosystems only single processes like nitrogen fixation or denitrification have been investigated so far. In these ecosystems nitrogen is rare and thus a well balanced nitrogen turnover is needed to ensure the establishment of plants and microbial communities. To study ecosystem development glacier forefields are ideally suited, because until the end of the "Little Ice Age" around 1850 many alpine glaciers have been retreating and thereby exposed new material for soil formation. The emerged chronosequences enable the comparison of functional nitrogen cycle communities from initial to developed soils, originating from the same substrate and being located in narrow spatial boundaries. In this Ph.D. thesis, which was done in the frame of the Transregional Collaborative Research Centre 38, the Damma glacier forefield (canton Uri, Switzerland) was investigated as an example.

Besides the advantages of the chronosequence approach, one has to overcome some methodological challenges mainly attributed to the broad variety of samples ranging from low to high biomass content. Thus, in the first part of this thesis, methods for the nucleic acid extraction were compared and adapted. Moreover, an additional quality feature for quantitative real-time PCR measurements was established, to ensure that PCR efficiencies for samples with different template amounts are comparable.

In the second part, functional gene abundance pattern and potential enzyme activities were detected and correlated in bulk soil samples from sites being ice-free for 10, 50, 70, 120 and approximately 2000 years of the Damma glacier forefield. The processes and respective functional genes being under investigation were nitrogen fixation (*nifH*), mineralisation (*chiA, apr*), nitrification (*amoA* AOB, *amoA* AOA) and denitrification (*nirK, nirS, nosZ*). The obtained results suggest that initial soils (10 years) are dominated by mineralising microbes, which decompose deposited organic material, whereas highest abundance of nitrogen fixing communities was revealed in transient soils (50, 70 years), where first plant patches appeared. In the developed soils significantly highest potential nitrification and denitrification activities were detected, although high gene abundances were already found in the initial soils. This data, therefore, indicates that the genetic potential is not necessarily the driver for certain soil functions, but instead a well regulated gene expression.

In the third part, the microbial community in the rhizosphere of a pioneering plant was investigated, because plants are not able to fix nitrogen on their own and thus the microbially delivered nitrogen has a significant impact on plant growth. Therefore, a climate chamber experiment with *Leucanthemopsis alpina* (L.) Heywood was conducted. To detect changes in the plant-microbe interaction from initial to developed ecosystems, *L. alpina* was cultivated in a 10 and 120 year soil. Samples were taken after 7 and 13 weeks of incubation from the rhizosphere and the respective bulk soil. In this experiment the same functional communities were quantified as on the Damma glacier forefield. Data was related to the ammonium and nitrate contents as well as to the carbon and nitrogen status of the plant. The results showed a stronger rhizosphere effect in the 10 year soil. Moreover, *nifH* gene copy numbers were highest in the rhizosphere of the 10 year soil. Gene abundances of the remaining processes were higher in the 120 year soil. In particular the *nosZ* gene abundance strongly increased during the incubation.

Taken together, data indicates that the ecosystem development at the Damma glacier takes place in at least three phases. The bulk soil of initial sites of the Damma glacier forefield is dominated by mineralising microbes. Nitrogen fixing communities are favoured in connection with plants and low nitrogen contents in the transient soils, where the competition for nitrogen is more pronounced and the plant delivered energy-rich compounds. Contrarily, after 120 years of development, when the vegetation cover is closed, processes like denitrification and nitrification are enhanced. However, it has to be taken into account that this study is restricted to one glacier forefield only and the transferability to other initial ecosystems is still debatable.

Zusammenfassung

Stickstoff gehört zu den Makronährstoffen und spielt daher eine wichtige Rolle für die Funktionalität und Entwicklung von Böden. Die Wechselwirkung zwischen verschiedenen Prozessen des Stickstoffkreislaufs und der beteiligten funktionellen Gemeinschaften ist in landwirtschaftlichen Ökosystemen gut untersucht. Im Unterschied dazu beschränkt sich die Forschung von initialen Ökosystemen bisher auf ausgesuchte Prozesse wie die Stickstofffixierung und die Denitrifizierung. Besonders in jungen, stickstoffarmen Ökosystemen ist ein ausbalanciertes Zusammenspiel einzelner Stickstoffkreislaufprozesse wichtig, damit sich pflanzliche und mikrobielle Gemeinschaften etablieren können. Für die Erforschung der Ökosystementwicklung sind Gletschervorfelder besonders gut geeignet, da seit dem Ende der „Kleinen Eiszeit“ um 1850 viele Alpengletscher kontinuierlich abtauen, wodurch neues Ausgangsmaterial für die Bodenentwicklung freigelegt wurde. Die dadurch entstandenen Chronosequenzen ermöglichen die vergleichende Untersuchung von stickstofftransformierenden mikrobiellen Gemeinschaften in verschiedenen Bodenentwicklungsstadien. Der große Vorteil von Untersuchungen in Gletschervorfeldern ist die räumliche Nähe unterschiedlicher Entwicklungsstadien des Bodens, dessen Entwicklung vom selben Ausgangsmaterial beginnt. In dieser Doktorarbeit, die in die Forschungen des Sonderforschungsbereiches/Transregio 38 einzuordnen ist, wurde exemplarisch das Dammagletschervorfeld (Kanton Uri, Schweiz) untersucht.

Die Ökosystementwicklung ist unter anderem mit einem starken Anstieg der Biomasse verbunden. Die dadurch entstehende große Bandbreite von Proben mit geringer und hoher Biomasse, macht eine Anpassung der angewandten molekularen Methoden notwendig. Daher wurden im ersten Teil dieser Arbeit verschiedene DNA Extraktionsmethoden getestet, gegebenenfalls verbessert und auf ihre Reproduzierbarkeit und Ausbeute hin verglichen. Außerdem wurde sichergestellt, dass die PCR Effizienzen während der quantitativen real-time PCR vergleichbar waren, wenn Proben mit unterschiedlichem Ausgangsmaterial eingesetzt wurden. Zu diesem Zweck wurde ein zusätzliches Auswerteverfahren etabliert.

Im zweiten Teil der Doktorarbeit wurden funktionelle Genabundanzen und potenzielle Enzymaktivitäten gemessen und korreliert. Dafür wurden Bodenproben aus 10, 50, 70, 120 und ca. 2000 Jahre alten eisfreien Böden vom Dammagletschervorfeld untersucht. Die untersuchten Prozesse und funktionellen Gene waren: Stickstofffixierung mit *nifH*, Mineralisierung mit *chi*A und *apr*, Nitrifizierung durch Ammoniak oxidierende Archaeen und Bakterien mit *amoA* und Denitrifizierung mit *nirK*, *nirS* und *nosZ*. Die Untersuchungen haben

gezeigt, dass die initialen Böden (10 Jahre) von mineralisierenden Mikroorganismen dominiert werden, während Stickstofffixierer mit zunehmender Pflanzendichte ansteigen (50-70 Jahre). Obwohl die Abundanz von Nitrifizierern und Denitrifizierern bereits in initialen Böden relativ hoch ist, wurde die höchste potentielle Aktivität erst in den entwickelten Böden detektiert. Das lässt darauf schließen, dass nicht das genetische Potential, sondern eine gut regulierte Genexpression bestimmte Bodenfunktionen reguliert.
Pflanzen in initialen Ökosystemen sind abhängig von stickstofffixierenden Mikroorganismen, da sie nicht fähig sind selbstständig N_2 zu assimilieren. Aus diesem Grund wurde im dritten Teil der Doktorarbeit ein Klimakammerversuch mit der Pionierpflanze *Leucanthemopsis alpina* (L.) Heywood durchgeführt. Um Veränderungen in der Interaktion von Pflanzen und Mikroorganismen zu verfolgen, wurde die Pflanze in einem 10-jährigen und einem 120-jährigen Boden angezogen. Boden- und Rhizosphärenproben wurden nach einer Inkubationszeit von 7 und 13 Wochen untersucht. Es wurden dieselben funktionellen Gemeinschaften quantifiziert wie am Dammagletschervorfeld, die dann zu den Ammonium- und Nitratgehalten im Boden und der Rhizosphäre sowie dem Stickstoff- und Kohlenstoffgehalt der Pflanze in Bezug gesetzt wurden. Die Untersuchungen ergaben, dass der positive Einfluss der Rhizosphäre im 10-jährigen Boden am größten ist. Außerdem wurde in der Rhizosphäre des 10-jährigen Bodens die höchste Abundanz von Stickstoffixierern gemessen. Alle anderen funktionellen Gene resultierten in höheren Genabundanzen in dem 120-jährigen Boden. Besonders die *nosZ* Genkopienzahl in dem 120-jährigen Boden stieg während der Inkubation signifikant an.

Abschließend kann man sagen, dass die Ökosystementwicklung am Dammagletscher in mindestens drei Schritten abläuft. Die initialen Böden des Dammagletschervorfeldes werden von mineralisierenden Mikroorganismen dominiert. Die Bedeutung von Stickstofffixierern nimmt erst in Verbindung mit steigender Pflanzendichte zu, weil die Pflanze einerseits energiereiche Verbindungen bereitstellt aber andererseits mit den Mikroorganismen um die knappen Stickstoffresourcen konkurriert. Im Gegensatz dazu, nimmt die Bedeutung von Denitrifikation und Nitrifikation erst in den entwickelten Böden zu, wo die Pflanzendecke bereits geschlossen ist.
Einschränkend ist zu bemerken, dass diese Studie auf ein bestimmtes Gletschervorfeld und eine ausgewählte Pionierpflanze begrenzt ist und daher die Frage nach der Übertragbarkeit der Daten auf andere initiale Ökosysteme offen bleibt.

1. Introduction

1.1 Outline of the Ph.D. thesis

In the last decades many Alpine glaciers have been retreating due to global warming and thereby exposed and released parent material for soil formation. Thus, chronosequences of differently developed soils emerged and display an ideal playground to study ecosystem development. Nitrogen, as an essential macronutrient, is especially necessary for ecosystem functioning and the establishment of higher plants. However, bedrock does not contain nitrogen and thus it is not surprising that in many studies it was stated that nitrogen is rare or even limited in initial ecosystems (Duc *et al.*, 2009; Kandeler *et al.*, 2006; Sigler and Zeyer, 2002). Only in the rhizosphere of pioneering plants local patches of enhanced nutrient availability were detected (Chapin *et al.*, 1994; Deiglmayr *et al.*, 2006; Tscherko *et al.*, 2005), but these are rather attributed to carbon than nitrogen. Thus, the colonisation with nitrogen fixing and decomposing microbes, which sustain the plant with low weight organic compounds (Lipson and Monson, 1998; Schimel and Bennett, 2004), ammonium (NH_4^+) and nitrate (NO_3^-) might display a selective advantage. In contrast, processes like nitrification and denitrification should be reduced, because they are connected with nitrogen lossess via leaching or the evolution of gaseous products. These examples illustrate that in soils of initial ecosystems an optimal internal nitrogen turnover is needed. But many studies focused only on single nitrogen cycle processes like nitrogen fixation (Crews *et al.*, 2001; Duc *et al.*, 2009; Yeager *et al.*, 2004) or denitrification (Deiglmayr *et al.*, 2006; Kandeler *et al.*, 2006), although the comprehension of a single process does not explain the interplay of different functional communities.

Therefore, it was the aim of this thesis: (i) To reconstruct the nitrogen cycle along the Damma glacier chronosequence by targeting marker genes for microbial biological nitrogen fixation (*nifH*), nitrogen mineralisation (*aprA*, *chiA*), nitrification (*amoA*) and denitrification (*nirK*, *nirS*, *nosZ*) and to correlate gene copy numbers to the respective potential enzyme activities. (ii) To analyse the impact of the rhizosphere of *Leucanthemopsis alpina* (L.) Heywood, a typical pioneering plant, on the abundance of functional nitrogen cycle communities in a less developed compared to a developed soil.

To be able to target these questions molecular methods were adapted to the challenging properties of the analysed samples.

1.2. The glacier forefield chronosequence

1.2.1. Global warming and the retreat of Alpine glaciers

During the "Little Ice Age" (~ 1200 to 1850 AD) the mean temperature dropped around 0.2°C and glaciers worldwide expanded (Lowell, 2000). Changes in climate, mainly increasing temperatures, caused the end of the "Little Ice Age".

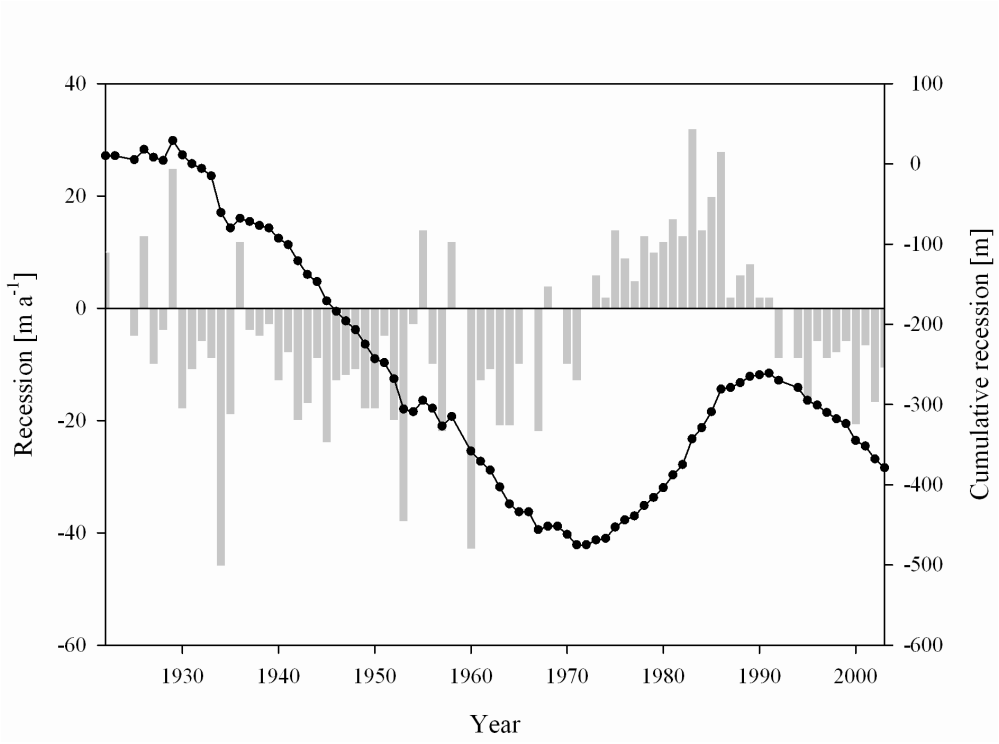

Figure 1. Recession (bars) and cumulative recession (dots) of the Damma glacier from 1922 to 2005 (Akademie der Naturwissenschaften Schweiz, 2010, http://glaciology.ethz.ch/messnetz/).

During the last century greenhouse gas emissions enhanced and resulted in an ongoing warming of 0.8°C, causing an imbalance of the accumulation and ablation phase of glaciers (Lowell, 2000). Especially heavy rainfalls during spring, which were followed by periods of drought, earlier snowmelt, higher UV-radiation and the deposition of aerosols, facilitated the retreat of glaciers (Horton *et al.*, 2006). Thus, between 1850 and 1970 more than 5000 Alpine glaciers had already lost 35% of their expanse and until 2000 they had lost 50%, which is an area of approximately 2237 km^2 (Zemp *et al.*, 2006).

The Damma glacier (canton Uri, Switzerland), for example, has retreated 10 m a^{-1} in average after the beginning of systematic measurements in 1921, except a short phase of expansion from 1972 to 1992, which is shown in Figure 1. Due to their constant retreat glaciers gradually released and exposed new material. This allows the study of the succession of plants, microbes and higher organisms as well as soil formation from a sandy textured deposit, in the following named as initial soils, to a differentiated, fertile soil with distinct horizons.

1.2.2. The chronosequence of the Damma glacier

A soil chronosequence is defined as a sequence of related soils that differ in their degree of profile development, because of differences in their age under comparable climatic conditions (Allaby, 2004). The Damma glacier forefield, as one example of a chronosequence, is located in canton Uri, Switzerland (8° 28′ E and 46° 38′ N). The forefield is bounded by two lateral moraines from 1850 and two terminal moraines from 1928 and 1992 (Göransson *et al.*, 2011; Noll and Wellinger, 2008). The parent material is composed of siliceous bedrock and builts up Regosols and Leptosols at the forefield and finally proceeds to a Cambisol outside the proglacial area (Dümig *et al.*, 2011; Lazzaro *et al.*, 2009).

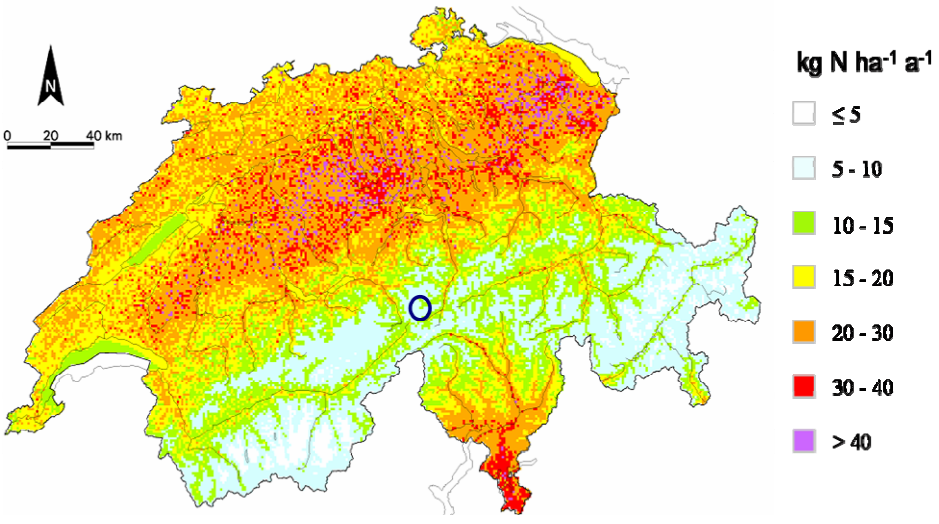

Figure 2. Map of nitrogen deposition in Switzerland (2000). The circle marked the approximate location of the Damma glacier (Federal Commission for Air Hygiene, 2005) (www.bafu.admin.ch/publikationen/publikation/00557).

The chronosequence has a NE exposition at an altitude of more than 2,000 m and a slope of 25%. At these altitudes the climate is extreme and highly variable. The mean annual rainfall amounted to 2,400 mm but is distributed irregularly during the year (Bernasconi, 2008; Duc *et al.*, 2009). The mean annual air temperature ranged between 0 and 5°C (Duc *et al.*, 2009), but due to the high solar radiation and light intensity at that high altitude the day surface temperature can reach up to 40°C in summer (Miniaci *et al.*, 2007). The high thermal radiation at night hampered the storage of the heat energy from the day (Landolt, 1992).
The initial sites of the chronosequence are characterized by Aar Granite as bedrock, scarce vegetation (< 20%) and nutrient shortage (Hämmerli *et al.*, 2007). Most carbon and nitrogen entered the forefield by wind and precipitation or via the glacial stream after snowmelt in spring (Hodkinson *et al.*, 2002; Saros *et al.*, 2010). As depicted in Figure 2 the northern agriculturally used areas of the Swiss Alps exhibit three to four times higher nitrogen deposition rates than being measured in the high alpine regions, where the Damma glacier is located. However, with ongoing ecosystem and soil development, plant coverage, carbon and nitrogen content as well as microbial biomass increased (Sigler *et al.*, 2002).

1.2.3. Weathering and ecosystem development

In most cases the initial bedrock is broken down by different weathering processes. Physical weathering is driven by the influence of heat, water, ice, pressure and abrasion, whereas chemical weathering results in dissolution by hydrolysis, mineral hydration or oxidation processes (Scheffer and Schachtschabel, 2002). A special case of chemical weathering is biological weathering, where dissolving chemicals originate from biological sources. Frey *et al.* (2010) revealed that especially *Arthrobacter* spp., *Janthinobacterium* spp., *Leifsonia* spp. and *Polaromonas* spp. as members of the phylum *β-Proteobacteria* were leading in the elemental release from granite of the Winterstock mountain near the Damma glacier. Microorganisms are known to accelerate the dissolution by excreting organic acids, siderophores or extracellular polysaccharides (Brantley *et al.*, 2001; Liermann *et al.*, 2000). In this regard, oxalate came out to be very effective in the dissolution of silicates mainly plagioclase feldspars (Welch *et al.*, 1999). Generally, oxalate can act as a ligand by complexing metal ions and thus facilitated the release of metals to solution or it accelerates a proton-promoted dissolution, because of the decreasing pH (Welch *et al.*, 1999). These examples illustrate that microbes give an important impulse for soil formation.

1.3. The role of nitrogen

Nitrogen is the fifth most abundant element in the solar system (Canfield *et al.*, 2010) and is beside carbon and other minerals like phosphate, sulphur or potassium one of the most important macronutrients for all living beings. It is an essential component of the most important polymers, namely proteins and nucleotides, and is further part of co-enzymes, photosynthetic pigments, secondary metabolites or other molecules. The most abundant nitrogen pool is N_2 (3.9 x 10^9 million tonnes) accounting for 78% of the atmosphere (Jenkinson, 2001). N_2 is an inert gas and thus not available for many organisms. It is introduced as plant available nitrogen by lightning, human activities like the Haber-Bosch process or special microorganisms, which are able to convert N_2 to NH_4^+ (Galloway *et al.*, 2004; Jenkinson, 2001; Maathuis, 2009). The preferred nitrogen compounds taken up by plants are NH_4^+, NO_3^- or low weight organic peptides smaller than 600 Da (Geisseler *et al.*, 2010). It depends on the soil type, climatic conditions and the plant species itself, which nitrogen compound will actually be assimilated. For example, in acidic reducing soils NH_4^+ is the dominant nitrogen form, whereas NO_3^- dominates alkaline and aerobic soils, where most NH_4^+ evaporates as ammonia (NH_3) (Maathuis, 2009).

1.4. The nitrogen cycle in soils

As plants are not able to take up all available forms of nitrogen, the transformation and degradation of nitrogen containing compounds is essential. Most of the relevant nitrogen cycle processes are exclusively performed by microorganisms. Consequently, plants depend on the microbial performance. As summarized in Figure 3, the nitrogen cycle is composed of different transformation steps, during which nitrogen undergoes oxidation states from +5 (NO_3^-) to -3 (NH_3). This includes the biological nitrogen fixation (BNF), nitrogen mineralisation, the aerobic oxidation of NH_3 to NO_3^- and the anaerobic oxidation of ammonium (Anammox) to N_2, as well as denitrification of NO_3^- to gaseous end products and the dissimilatory reduction of nitrate to ammonium (DNRA). As the dominant processes of the nitrogen cycle in aerated soils are instead BNF, mineralisation, nitrification and denitrification, this Ph.D. thesis focussed on these processes.

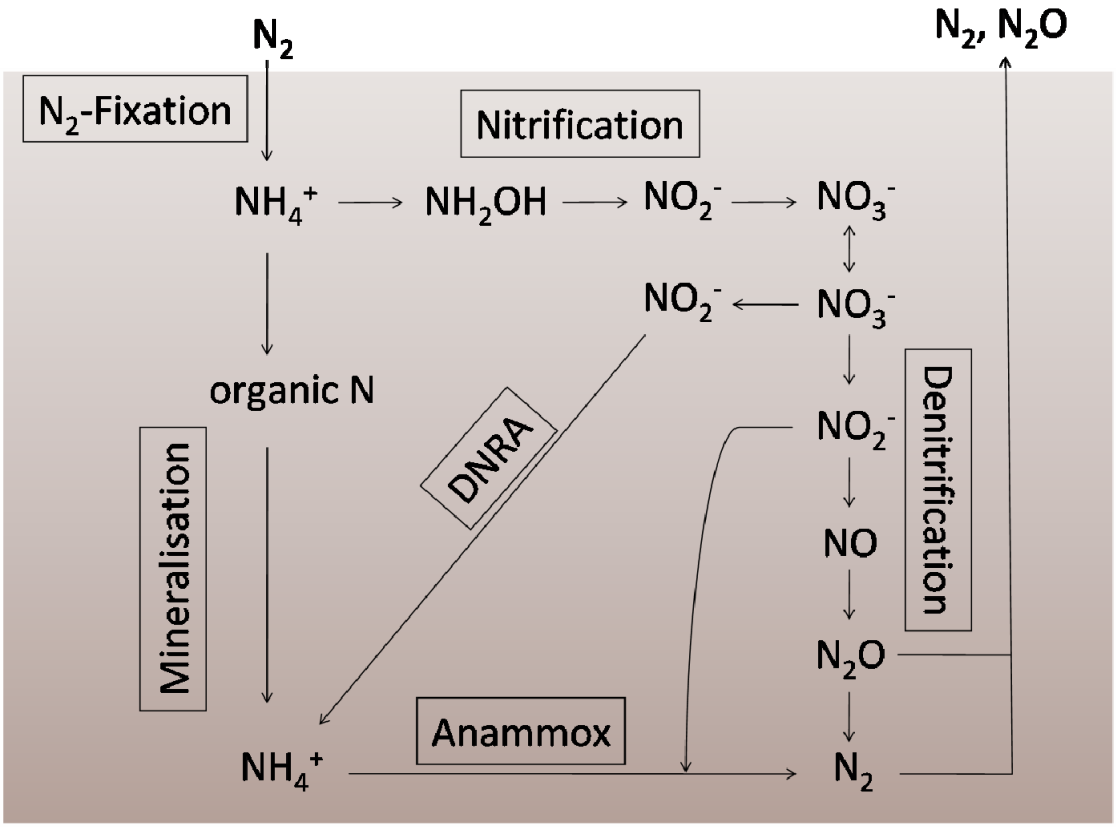

Figure 3. A simplified scheme of the nitrogen cycle including N_2-fixation, nitrification, denitrification, mineralisation, dissimilatory nitrate reduction to ammonium (DNRA) and anaerobic ammonium oxidation (Anammox) (modified after Arrigo (2005) and Francis *et al.* (2007)).

1.4.1. Biological nitrogen fixation

Inorganic nitrogen can enter the soil via three different ways: (i) by BNF, (ii) by the mineralisation of biomass or (iii) by deposition. The conversion of N_2 to NH_4^+, as shown in Figure 4, can be performed by free-living, symbiotic or plant associated nitrogen fixing bacteria or archaea, which are summarized as diazotrophs. Although, the ability to fix nitrogen might be an advantage in nitrogen limited habitats, it is also connected with a high demand of energy. At least 16 molecules of ATP are needed to fix one molecule N_2 (Zehr *et al.*, 2003).

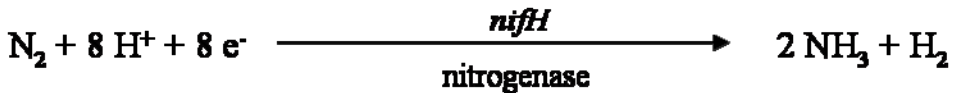

Figure 4. Stoichiometry of the biological nitrogen fixation (BNF). The functional gene *nifH*, which encodes the dinitrogenase reductase subunit of the nitrogenase, is frequently used as marker gene to analyse BNF (adapted from Hai (2010)).

The reduction of N_2 is catalysed by a metallo-enzyme called nitrogenase (EC 1.18.6.1). There are four distinct groups of nitrogenases, from which three originate from a common ancestor, while the fourth type developed independently, but was only found in the thermophilic streptomycete *Streptomyces thermoautotrophicus* (Ribbe *et al.*, 1997). The three closely related nitrogenases are the molybdenum-based (Mo-), the vanadium-based (V-) and the iron-based (Fe-) nitrogenase, from which the first one is found in all nitrogen fixing microbes, while the others occur randomly as additional enzyme (Dean *et al.*, 1993). From the ecological point of view that is a reasonable distribution, as molybdenum is the most efficient catalyst for the reduction of N_2 followed by vanadium and iron (Newton, 2007). The Mo-nitrogenase consists of two metallo-proteins (Burris, 1991). The MoFe-protein, called dinitrogenase, is a heterodimer with a molecular mass of 230 kDa, which is responsible for the substrate binding and encoded by *nifD* and *nifK*. The Fe-protein, termed dinitrogenase reductase, is encoded by *nifH*, which builds up a 64 kDa homodimer and performed the ATP hydrolysis and the electron transport to the MoFe-protein (Newton, 2007). The nitrogenase is sensitive against O_2, because it irreversibly inhibits the enzyme by oxidizing the Fe-S cofactor. Nevertheless, not only obligate anaerobic bacteria (e. g. *Clostridium, Desulfovibrio*) are able to perform BNF but also a variety of facultative anaerobic (e. g. *Klebsiella, Bacillus*) and aerobic bacteria (e. g. *Azotobacter, Cyanobacteria, Herbaspirillum*) (Zehr *et al.*, 2003). However, all of them need necessarily anaerobic or microaerophilic conditions to perform BNF (Burris, 1991). Therefore, different mechanisms exist to shield the nitrogenase from oxygen. For example, *Cyanobacteria* develop special cells, the heterocysts, where no O_2 is produced photosynthetically (Fay, 1992). Aerobic bacteria keep the internal O_2 concentration low by very high respiration rates (Marchal and Vanderleyden, 2000).

1.4.2. Nitrogen mineralisation

Beside the enormous source of nitrogen in the atmosphere a vast pool of organic nitrogen is present in soils comprising a broad spectrum of substrates. Thereby proteins perceive a proportion of 40% of the total nitrogen in soil (Schulten and Schnitzer, 1998) and chitin is the most abundant polymer in nature serving as carbon and nitrogen source (Cohen-Kupiec and Chet, 1998). The degradation of proteins or chitin is performed by extracellular proteases and chitinases, respectively. Both enzymes are ubiquitously distributed, for example, in plants, bacteria and fungi. The expression of chitinolytic and proteolytic functional genes is regulated by substrate induction (proteins, chitin or low amounts of poly-β-1,4-acetylglucosamine) and product repression (amino acids, NH_4^+, glucose or high levels of poly-β-1,4-acetylglucosamine), respectively (Felse and Panda, 1999; Geisseler *et al.*, 2010).

Regarding extracellular proteases, microbes are supposed to have higher substrate affinities compared to plants (Ji and Brune, 2005). Generally, two major groups of proteolytic enzymes exist: on one hand, endoproteases cleave the interior linkages of the substrate and on the other hand exoproteases attack the free ends of proteins. As a direct uptake of proteins does not occur, proteolytic enzymes are predominantly extracellular (Loll and Bollag, 1983). Proteolytic enzymes can be classified after the catalysed reaction, the chemical nature of the catalytic site or the evolutionary relationship (Rao *et al.*, 1998). Addressing the functional group in the active site proteases are classified in four groups: the serine- (EC 3.4.21), the aspartic- (EC 3.4.23), the cysteine- (EC 3.4.22) and the metalloproteases (EC 3.4.24). These groups are again subdivided after their optimal proteolysis pH into acidic, neutral and alkaline proteases. In bacteria the alkaline metalloproteases (Apr), neutral metalloproteases (Npr) and serine proteases (Ser) are prevalent (Kalisz, 1988). Metalloproteases require divalent ions for their activity, while serine proteases exhibit a serine group at their active site. The substrate spectrum of neutral metalloproteases is limited to hydrophobic amino acids, while the alkaline metalloproteases and the serine proteases cover a broad range of substrates (Rao *et al.*, 1998). A study from Bach and Munch (2000) revealed that protein degradation is mainly catalysed by neutral and alkaline metalloproteases in arable soils, grasslands and a beech forest. Moreover, it seems that proteolytic communities in soil are dominated by *Pseudomonas* spp., which are known to comprise primarily alkaline metalloproteases (Bach and Munch, 2000; Sakurai *et al.*, 2007) and are widely distributed in terrestrial ecosystems (Janssen, 2006). Hence, *apr* as marker gene for alkaline proteases is particularly suitable to follow dynamics of proteolytic communities.

In contrast to the ubiquitous distribution of proteins, chitin, a poly-β-1,4-acetylglucosamine (GlcNAc), is mainly present in insects, crustaceans and most fungi. The degradation of chitin is catalysed by the chitinase (EC 3.2.1.14). In contrast to plants or fungi, where the chitinase is used as defence mechanism against chitin-containing organisms or for cell wall development, respectively, bacteria meet their nutritional demands by the degradation of chitin (Cohen-Kupiec and Chet, 1998). The degradation can be performed by many gram-positive and gram-negative bacteria, where the majority belongs to the genera *Pseudomonas, Aeromonas, Cytophaga johnsonae, Lysobacter, Arthrobacter, Bacillus* and actinomycetes (Gooday, 1990). Based on amino acid sequence similarities, chitinases are divided into five classes (Hamel *et al.*, 1997), which can be grouped in family 18 (class III and V) and 19 (class I, II and IV) of the glycosyl hydrolase classification system (Henrissat, 1991). However, both families are structurally not related. While family 19 glycosyl hydrolases are exclusively represented by plant chitinases, class III chitinases of the glycosyl hydrolase family 18 are composed of plant and fungi derived chitinases and class V is comprised of bacterial chitinases. Bacterial chitinases can be clustered in three subfamilies namely chitinase A, B and C (Metcalfe *et al.*, 2002; Suzuki *et al.*, 1999). They all have in common, that conserved glutamic acid and aspartic acid residues are essential for the hydrolysis of chitin (Watanabe *et al.*, 1993). Bacteria can inhabit different chitinase subfamilies to utilize a broader spectrum of the diverse forms of chitin. Specifically chitinase A was detected in many different environments and is thus particularly suitable to analyse the diversity and distribution of chitinolytic bacteria (Xiao *et al.*, 2005).

1.4.3. Nitrification

The NH_3 produced during nitrogen fixation and mineralisation can be used as a substrate for nitrification. Thus, nitrification has an important influence on the amount of plant available nitrogen, because NH_3 is transferred to the mobile nitrate, which can also serve as electron acceptor for denitrification. The stepwise oxidation of NH_3 to NO_3^-, as shown in Figure 5, is carried out by two different functional groups: First, the oxidation of NH_3 to NO_2^- is performed by ammonia oxidising bacteria (AOB) or ammonia oxidising archaea (AOA), followed by the oxidation of NO_2^- to NO_3^- by nitrite oxidising bacteria (NOB).
All nitrifying bacteria are known to be chemolithoautotrophic, meaning that NH_3 serves as sole energy source and reductant and that CO_2 displayed the main carbon source (Kowalchuk and Stephen, 2001).

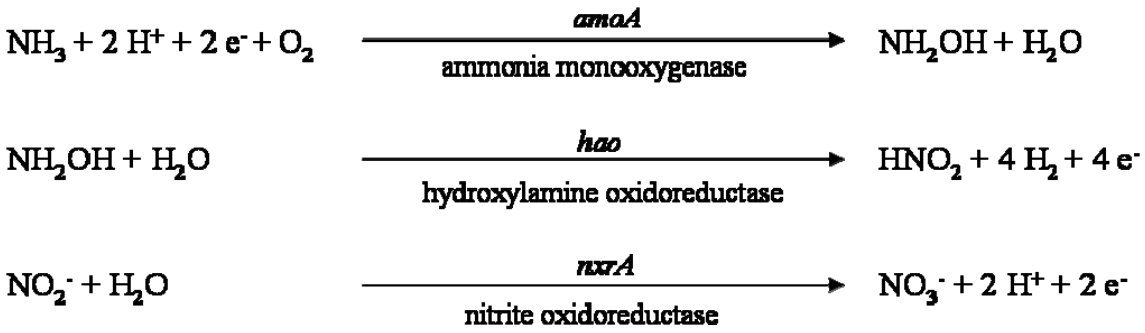

Figure 5. Stoichiometry of the nitrification. Below the arrows the responsible enzymes are displayed and above the targeted marker genes (adapted from Hai (2010)).

The majority of AOB is restricted to the *β-Proteobacteria* (e.g. *Nitrosomonas, Nitrosococcus, Nitrosospira*), whereas NOB were found to belong to *α-* (*Nitrobacter*), *β-* ('*Candidatus* Nitrotoga arctica'), *γ-* (*Nitrococcus*) and *δ-Proteobacteria* (*Nitrospina)* and to *Nitrospira* (phylum *Nitrospira*) (Daims *et al.*, 2001; Jetten *et al.*, 1997).

As shown in Figure 6, the complete aerobic oxidation of ammonia to nitrite requires four different proteins. The rate limiting step, the oxidation of NH_3 to hydroxylamine, is catalysed by the ammonia monooxygenase (Amo, EC 1.14.99.39). Afterwards hydroxylamine will be dehydrogenated to NO_2^- by the hydroxylamine oxidoreductase (Hao, EC 1.7.3.4). During that process four electrons are released and relayed to the ubiquinone pool via two interacting tetraheme cytochromes (cyt), the cyt c554 and the cyt c_M552 encoded by the respective genes *cycA* and *cycB* (Hooper *et al.*, 1997). Two of the electrons are recycled for the initial ammonia oxidation and the remaining ones are used for the generation of energy (Jetten *et al.*, 1997). The Hao is well characterized on the DNA and protein level. It is a multimeric protein consisting of 2-3 subunits with a size of 62 kDa. It contains at least seven c-type hemes and one heme P_{460} (Arciero *et al.*, 1993; Hendrich *et al.*, 1994). In contrast, information about the Amo is based on sequence homologies to a copper-dependent particulate methane monooxygenase (pMmo). Thus, Amo is described as multimeric transmembrane copper-enzyme (Holmes *et al.*, 1995). It is composed of three subunits, which are encoded by the respective genes of the *amoCAB* operon (Arp *et al.*, 2007; Klotz *et al.*, 1997), where *amoA* is used as functional marker gene for molecular analysis (Rotthauwe *et al.*, 1997).

In the last years the knowledge of the ammonia oxidation reached a new level due to the detection of new key players and processes like AOA and Anammox, respectively. In this respect, AOA came out to play an important role in aquatic and terrestrial ecosystems.

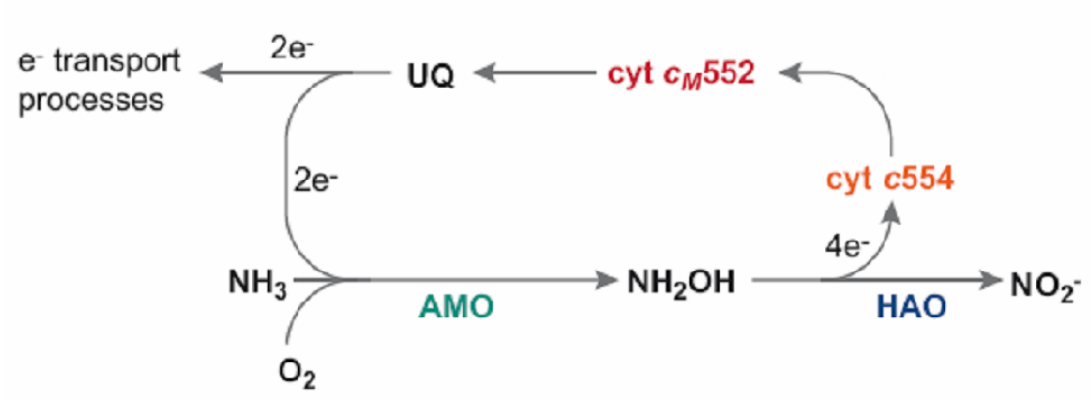

Figure 6. A schematic illustration of the electron flow during the bacterial oxidation of ammonia to nitrite (modified after Arp *et al*. (2007)).

Studies on the abundance of ammonia oxidisers mostly revealed a dominance of AOA over AOB (Prosser and Nicol, 2008). Targeting *amoA* transcript abundances and ammonia oxidation activities the dominance of the AOA is not as clear as for the gene abundance data. Instead, data indicates a niche separation of AOA and AOB in terms of their performance. For example, Di *et al.* (2009) figured out that AOB drive nitrification activity in nitrogen-rich grassland soils. In contrast, a positive correlation between nitrification activity and AOA abundance was observed in acidic agricultural soils (Gubry-Rangin *et al.*, 2010). Although, activity of the Amo of AOA was proven on the mRNA (Hatzenpichler *et al.*, 2008; Treusch *et al.*, 2005) and cultivation level (Könneke *et al.*, 2005), remarkable differences to the bacterial Amo have been revealed during metagenomic studies of a fosmid clone (54d9), for example differences in the DNA sequence, the absence of the Hao and a generally different operon-structure (Treusch *et al.*, 2005). However, bacterial and archaeal Amo exhibit conserved AA residues in their metal centre (Nicol and Schleper, 2006). Based on 16S rRNA studies AOA were firstly grouped to the 1.1a (marine) (Könneke *et al.*, 2005) and 1.1b (terrestrial) lineage (Treusch *et al.*, 2005) of the *Crenarchaeota*. Recent studies, based on the genomes of *Nitrososphaera gargensis*, *Cenarchaeum symbiosum* A and *Nitrosopumilus maritimus* SCM1, revealed that AOA form a distinct phylum within the archaea named *Thaumarchaeota* (Brochier-Armanet *et al.*, 2008; Spang *et al.*, 2010).

Anammox was first discovered in anoxic bioreactors of waste water treatment plants (Mulder *et al.*, 1995). The oxidation of NH_4^+ to N_2 via hydrazine (N_2H_4) as intermediate was performed by novel organisms belonging to the *Planctomycetales*. Further studies revealed that anaerobic ammonium oxidising bacteria are distributed ubiquitously in aquatic

environments (Engström *et al.*, 2005; Francis *et al.*, 2007; Kuypers *et al.*, 2003; Meyer *et al.*, 2005). Recent studies also confirmed the presence of anaerobic ammonium oxidising bacteria in terrestrial habitats (Hu *et al.*, 2011; Humbert *et al.*, 2010).

The second step of the nitrification pathway, the oxidation of NO_2^- to NO_3^-, is catalysed by the nitrite oxidoreductase (Nxr). As NOB rely on the nitrite produced during initial ammonia oxidation, these organisms have been studied less frequently and many studies are restricted to *Nitrobacter* (Meincke *et al.*, 1992; Starkenburg *et al.*, 2006). The Nxr of *Nitrobacter* is an iron-sulfur molybdoprotein and is located at the inner cell membrane and at intracytoplasmic membranes (Meincke *et al.*, 1992). Depending on the isolation method 2-3 subunits were ascertained with a $\alpha_2\beta_2\gamma_1$ stoichiometry (Meincke *et al.*, 1992; Sundermeyer-Klinger *et al.*, 1984). The α-subunit, encoded by *nxrA*, contains the substrate binding site with the molybdopterin cofactor (Meincke *et al.*, 1992; Sundermeyer-Klinger *et al.*, 1984). The β-subunit, encoded by *nxrB*, is with its [Fe-S] cluster responsible for the electron transport from either the α- to the γ-subunit or to the electron transport chain (Kirstein and Bock, 1993). However, recent studies based on molecular tools rather than cultivation revealed that *Nitrospira* is the most diverse and abundant NOB in most environments (Daims *et al.*, 2001). The characterization of the Nxr of "*Candidatus* Nitrospira defluvii" based on a complete genome reconstruction uncovered remarkable differences between the Nxr of *Nitrobacter* and *Nitrospira*, which are mainly attributed to the lacking transmembrane helices of the Nxr of *Nitrospira* (Lücker *et al.*, 2010). As to date less data is available on other NOB genomes, a generalisation of the molecular Nxr mechanism is awkward.

1.4.4. Denitrification

The emerging NO_3^- during nitrification can have several destinations: (i) it can be assimilated by plants; (ii) it can be leached out; (iii) it is used as substrate for the dissimilatory nitrate reduction to ammonium (DNRA); or (iv) it can be used as alternative terminal electron acceptor under anaerobic conditions. During this process, called denitrification, NO_3^- is stepwise reduced to gaseous nitrogen compounds like N_2O or N_2, which is depicted in Figure 7. The ability to denitrify is widespread among bacteria, archaea and fungi (Hayatsu *et al.*, 2008; Philippot *et al.*, 2007).

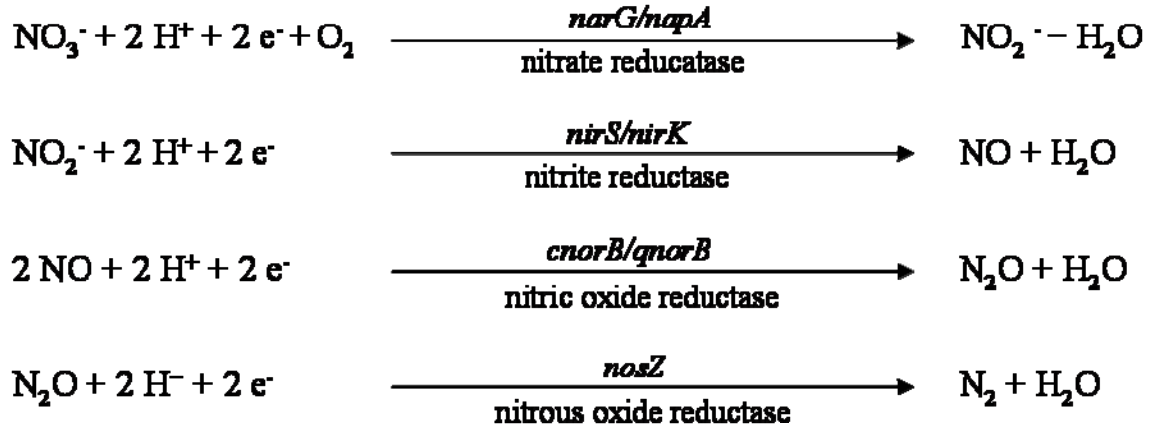

Figure 7. Stoichiometry of the reduction of nitrate to N_2. Below the arrows the responsible enzymes are displayed and above the targeted marker genes (adapted from Hai (2010)).

Two enzymes are known to be responsible for the initial reduction of NO_3^- to NO_2^-, the membrane-bound (Nar, EC 1.7.99.4) and the periplasmic located nitrate reductase (Nap, EC 1.9.6.1). Both enzymes can occur in parallel in one organism (Roussel-Delif *et al.*, 2005). Nap is a heterodimer consisting of a c cytochrome and a catalytic subunit with a molybdopterin cofactor and a [4Fe-4S] cluster encoded by the genes *napB* and *napA,* respectively (Philippot, 2002). The membrane-bound Nar consists of three subunits. The α-, β-, and γ-subunits represent the catalytic subunit with the molybdopterin cofactor, four [4Fe-4S] cluster and 2 *b*-type hemes, respectively, which are encoded by the respective genes *narG*, *narH*, and *narI* (Zumft, 1997). For both enzymes it is necessary that NO_3^- is imported, because Nap is located periplasmatic and the α-subunit of Nar is facing the periplasm as well (Philippot, 2002). However, the first step in the denitrification cascade often led to misinterpretations of data, as organisms performing DNRA contain the same nitrate reductases as denitrifying ones. Therefore, Mahne and Tiedje (1995) proposed criteria to identify real denitrifying microorganisms. First, the reduction of NO_3^- must be coupled to an increase in growth yield and second N_2O or N_2 have to be the end products.

For the nitrite reduction two evolutionary unrelated proteins are described, which differ in structure and prosthetic metal but are equal concerning functionality (Glockner *et al.*, 1993). On one hand a cytochrome cd_1-nitrite reductase (NirS, EC 1.7.2.1) and on the other hand a copper-nitrite reductase (NirK, EC 1.7.2.1) catalyse the reduction of nitrite to nitric oxide (NO), the first gaseous product (Philippot, 2002). While NirK is a homotrimer with 36 kDa

and two copper atoms per monomer, NirS is a homodimer with 64 kDa and one heme cd_1 per monomer (Jetten *et al.*, 1997; Zumft, 1997). Although being functionally redundant recent studies revealed (Hai *et al.*, 2009; Kandeler *et al.*, 2006; Sharma *et al.*, 2005) a niche differentiation of *nirK* and *nirS* harbouring bacteria, with *nirK* being dominant in the rhizosphere and *nirS* in the bulk soil. Moreover, studies from Heylen *et al.* (2006) underlined the hypothesis that *nirK* tends to be distributed by horizontal gene transfer, reflected by the finding that *nirK* rather showed sequence similarities to bacteria from the same habitat than to related taxa, as it is for *nirS*.

The NO-reductase is regulatory coupled to the nitrite reductase (Jüngst and Zumft, 1992), for that reason it is often omitted from molecular studies. Anyhow, it fulfils a key step during denitrification, as it introduced the N-N bond in the course of reducing NO to N_2O, which moreover displayed the first "true" denitrification product. The NO-reductase (EC 1.7.99.7) is bound to the membrane via 12 membranehelices (Philippot, 2002). It consists of two subunits, one with 16 kDa comprising the heme *c* and one with 33 kDa displaying the heme *b* (Zumft *et al.*, 1994). Moreover, a distinction is made between NO-reductases, because one received their electrons from a cytochrome *c* or a pseudoazurin (cNor) and the other gets its electrons from the quinol pool (qNor) (Philippot *et al.*, 2007).

The final reduction of N_2O to N_2 is performed by the N_2O-reductase (Nos, EC 1.7.99.6), which is a homodimer of two 70 kDa subunits, where each subunit incorporates four copper atoms (SooHoo and Hollocher, 1991). For molecular studies the *nosZ* gene is predominantly used, which encodes the catalytic subunit (Henry *et al.*, 2006). Two conserved domains have been identified in NosZ. On one hand the CuA-domain, which is responsible for the electron entry and on the other hand the CuZ-domain, which displayed the catalytic site (Zumft *et al.*, 1990). The N_2O-reductase is most sensitive to oxygen (Morley *et al.*, 2008), consequently oxygen has a strong influence on the N_2O:N_2 ratio.

Interestingly, NO_3^- availability only influenced the actual denitrification activity, whereas the denitrifying community in soils is shaped by environmental parameters like organic carbon content, pH, moisture and temperature (Wallenstein *et al.*, 2006).

Due to the different lifestyles of microorganisms involved in the nitrogen cycle and different requirements for the performance of the respective enzymes, the environment has a big impact on the respective relevance of the single processes and favoures one or another group involved in the nitrogen cycle.

1.5. Ecology of the terrestrial nitrogen cycle

1.5.1. Initial ecosystems

Different natural and anthropogenic activities led to the release and exposition of new terrain for soil formation. Therefore, glacier forefields, sand dunes, volcanoes or restoration sites are admired research objects to investigate initial ecosystem development and colonisation. These ecosystems are often characterized by nutrient shortage, barren substrate and scarce vegetation (Crews *et al.*, 2001; Lazzaro *et al.*, 2009; Nemergut *et al.*, 2007; Smith and Ogram, 2008). To survive under these harsh conditions a quick response to changing environmental conditions is mandatory. Hence, it is not surprising that Sigler *et al.* (2002) revealed a dominance of *r*-strategists at initial sites of the Damma glacier.

The establishment of plants is an important step in ecosystem development, because the penetration of roots stabilises the slope, and exudates and decaying litter material are main sources of organic matter (Duc *et al.*, 2009; Grayston *et al.*, 1997; Körner, 2004). However, plants rely on the availability of nitrogen, which is rare in initial soils of glacier forefields, as only traces are released from bedrock material (Frey *et al.*, 2010). Thus, nitrogen must be introduced from external sources. Two possibilities are most likely: first, the input via nitrogen deposition and second, the BNF by diazotrophs. Regarding BNF, Crews *et al.* (2001) demonstrated that the total nitrogen input in young lava flows was mainly driven by nitrogen fixation. However, contradictory data exists, dealing with *nifH* diversity, abundance and potential nitrogen fixation activity. The highest diversity was detected in young development stages of biological soil crusts and glacier forefields but similarly lowest *nifH* gene abundances and potential enzyme activities were determined (Duc *et al.*, 2009; Yeager *et al.*, 2004).

At a first glance nutrients are limited in initial ecosystems, thus researchers assumed that the first phase is dominated by autotrophic organisms like cyanobacteria, algae, mosses and lichens (Schmidt *et al.*, 2008; Walker and del Moral, 2003). However, this theory is under discussion, because recent studies found an initial heterotrophic phase (Bardgett *et al.*, 2007; Hodkinson *et al.*, 2002) where microbes feed on ancient and recalcitrant carbon. These microorganisms were randomly transported through air, animals or water, especially the glacial stream transports small microbial communities, which originate from cryoconite holes of the glacier ablation zone (Xiang *et al.*, 2009). Beside these carbon pools, the rhizosphere of plants also displays a nutrient hotspot. Plants can release between 10 and 40% of their fixed

carbon to the rhizosphere (Hutsch *et al.*, 2002; Merbach *et al.*, 1999; Paterson and Sim, 2000) and thereby enhance microbial abundance and activity, which is known as the "rhizosphere effect" (Butler *et al.*, 2003; Hartmann *et al.*, 2008; Raaijmakers *et al.*, 2009). It was shown by Miniaci *et al.* (2007) at initial sites of the Damma glacier forefield that this effect still has an influence at 20 cm distance to the plant. The advantage for microbes regarding carbon availability in the rhizosphere is undisputed, but debatable in terms of nitrogen availability, because plants are not able to fix nitrogen themselves. Thus, the question rose whether plants and microbes compete for the limited nitrogen resources (Kaye and Hart, 1997; Schimel and Bennett, 2004).

In initial ecosystems generally and in the rhizosphere of sparsely distributed plants particularly, processes like nitrification and denitrification should be reduced, as they are connected with nitrogen losses by leaching of highly mobile NO_3^- or the formation of gaseous products, respectively. Plants are known to be able to create beneficial conditions within their direct vicinity, for example they exude nitrifier-inhibiting secondary metabolites like tannins, polyphenolic substances or monoterpenes (Briones *et al.*, 2003; Kowalchuk and Stephen, 2001; Ward *et al.*, 1997). Nevertheless, specific studies targeting ammonia oxidising microbes in initial ecosystems are rare, but investigations at the Rotmoosferner and Ödenwinkelkees glaciers (Austria) (Nicol *et al.*, 2005; Nicol *et al.*, 2006) gave hints about an archaeal nitrifying potential by revealing a dominance of the *Crenarchaeota* lineage 1.1.b in the bulk soil of initial sites. However, these studies addressed the whole *Crenarchaeota* phylum and did not also consider AOB.

In contrast to the small group of ammonia oxidising microbes, the ability to denitrify is widely distributed among several groups of microorganisms. Moreover, it is well known that, for example, *nirK* is distributed via horizontal gene transfer (Heylen *et al.*, 2006). Thus, it is not surprising that high gene copy numbers and diversity of functional denitrification genes were detected at the Rotmoosferner glacier (Deiglmayr *et al.*, 2006; Kandeler *et al.*, 2006). However, as also shown for potential nitrogen fixation activity, measured nitrate reductase activities were low at initial sites of glacier forefields (Deiglmayr *et al.*, 2006).

While, several studies exist targeting a single process of the nitrogen cycle, mainly nitrogen fixation or denitrification, studies comparing different processes are lacking. Thus, making statements about different behaviours of the functional communities is difficult.

1.5.2. Changes during ecosystem development

In contrast to initial ecosystems, developed ones like natural grasslands are characterized by the formation of distinct soil horizons, dense and diverse plant coverage and hence a broader spectrum of available nutrients. Plants do not only differ in density but also in their properties in developed ecosystems. While fast-growing plants from initial sites produce nitrogen-rich high quality litter and thus promote bacterial-dominated food webs, slow-growing plants from developed ecosystems produce low-quality, phenol-rich litter, which favour fungi-dominated food webs (Bardgett and Walker, 2004). Due to the high plant coverage connected with a strong root penetration of the soil, the plant has a major influence on microbial communities. For example, higher amounts of carbon are available due to the higher exudation of organic substances like malate, citrate and oxalate (Jones, 1998), which generally enhance microbial abundance and activity in the rhizosphere. Moreover, root debris and plant litter, which are primarily water-insoluble and of high-molecular weight, provide a broad substrate spectrum for extracellular enzymes, which make these substances accessible for microbes. Thus, it is not surprising that much higher enzyme activities and thus also higher mineralisation potentials were detected at developed sites compared to initial sites of glacier forefields (Tscherko *et al.*, 2003). Because of the steady supply with nutrients, *k*-strategists, pursuing a strategy of maintenance, rather than *r*-strategists have been detected (Sigler *et al.*, 2002).

With the higher diversity of plants in developed ecosystems legumes also establish and play a main role for the nitrogen budget of ecosystems. In this regard, Jacot *et al.* (2000) showed that 70 to 95% of the nitrogen requirements of legumes in the Swiss Alps were provided by symbiotic nitrogen fixation. Contrarily, initial ecosystems are dominated by free-living diazotrophs (Duc *et al.*, 2009), because legumes are not yet well established (Tscherko *et al.*, 2003). In addition to the symbiotic and plant-associated diazotrophs, free-living heterotrophic nitrogen fixing microbes also have advantages in developed ecosystems, because enough carbon is available serving as energy source even in the bulk soil.

The high plant coverage is further connected with a strong root penetration of the soil, which caused lower partial oxygen pressures in the rhizosphere, attributed to the respiration activity of plants and microbes. Both, the availability of electron donors and the reduced oxygen favour denitrification. In contrast, the water consumption of the plants creates better conditions for gas exchanges, which are in turn connected with increasing oxygen concentrations and thus reduced denitrification (Philippot *et al.*, 2007). However, microhabitats with low redox potentials and optimal denitrifying conditions may be retained.

Another important factor is the genesis of functionally connected conglomerations, because the diffusion coefficient in soil is much lower than in aqueous solutions. This is mainly attributed to interactions between the ions in the soil solution and the solid phase and the different length and thickness of water films in the soil (Scheffer and Schachtschabel, 2002). Hence, it is important for microbes to be in the direct vicinity of nutrients. In this respect, Schimel and Bennett (2004) claimed that in nitrogen-poor habitats, nitrifying organisms only establish in the direct vicinity of mineralisers, which provide enough NH_3 and Frank *et al.* (2000) revealed a positive and linear correlation of nitrogen mineralisation, nitrification and denitrification activity in a Yellowstone Park grassland.

1.6. From the gene to the enzyme: Methods to quantify functional communities in soil

Functional communities in soil can be described on different levels as illustrated in Figure 8. Many studies have been performed to assess the microbial potential by quantifying the particular functional genes. Analyses on the DNA level have the advantage that DNA is relatively stable and thus easy to handle. Moreover, it is possible to track long term effects on the functional community. However, gene abundance data only reflects the numerical dominance of a certain functional group, but include no information about their actual activity. Moreover, a direct inference to bacterial cell numbers is often not possible as gene copy numbers per genome can vary depending on the community composition (Klappenbach *et al.*, 2000). A first hint about the activity of functional groups is given by the quantification of mRNA transcripts, but RNA compared to DNA has a short half-life period. For example, *nirS* mRNA is only stable for 13 minutes in pure culture (Hartig and Zumft, 1999). Hence, obtained data reflects only a snapshot, which is strongly influenced by spatial heterogeneity or environmental factors. Thus, for the analysis of RNA high numbers of replicates and controlled conditions might be required. Although, it is possible to detect gene expression by addressing mRNAs, a definite statement about the actual activity is not possible, because transcripts are still need to be processed. Therefore, proteins display a better alternative as they are at the end of the regulatory cascade and beyond any transcriptional, translational or post-translational modification (Philippot and Hallin, 2005).

Regardless, which step of the regulatory cascade will be analysed, the work with environmental samples brings along some challenges, which are attributed to the respective properties of the sample.

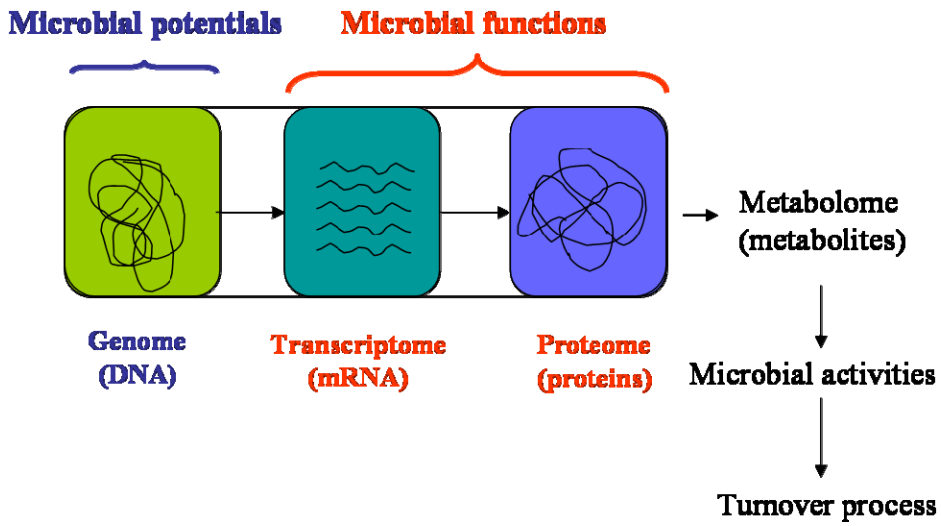

Figure 8. Scheme of the different levels, which can be addressed during the characterisation of functional communities (Schloter *et al.*, 2008).

Concerning the nucleic acid extraction that includes the removal of humic acids, the reproducibility and sufficient extraction of nucleic acids, which is especially problematic in ecosystems with low biomass. Thus, the choice of the most applicable extraction procedure is a crucial point. For example, an approach where simultaneous analyses of DNA and RNA will be done requires a co-extraction of both to reduce the introduced biases caused by different extraction protocols. Basically, two approaches exist to extract nucleic acids (Herrera and Cockell, 2007). Either cells were first separated from the soil matrix (Delmotte *et al.*, 2009; Holben *et al.*, 1988), which is followed by cell lysis and nucleic acid extraction or cells were directly lysed in the soil before the subsequent nucleic acid extraction (Roose-Amsaleg *et al.*, 2001). Nowadays, the second approach is used more frequently. Therefore, a nucleic acid extraction based on a subsequent phenol-chloroform isolation followed by a nucleic acid precipitation is applied or column based commercial kits are used. After the isolation and purification of the nucleic acids and, if needed, a reverse transcription of the mRNA to cDNA, samples can be used for follow up analyses.

To describe changes in the abundance of different functional communities PCR based approaches targeting functional genes or 16S rRNA genes of specific functional groups like ammonia or nitrite oxidisers are often used. In the last decade quantitative real-time PCR (qPCR) displaced the traditional “end-point” PCR, where an evaluation of the PCR products was only possible after the reaction was completed (Sharma *et al.*, 2007). In contrast, the

qPCR method allows a real-time detection of the amplicons after each PCR cycle (Rasmussen *et al.*, 1998; Wittwer *et al.*, 1997). Therefore, various fluorescent reporter molecules can be used. Generally, three different chemistries can be applied to detect the increase of the amplicon amount (Zhang and Fang, 2006): (i) dsDNA binding dyes like SybrGreen®, which have the disadvantage that they do not distinguish between PCR amplicons, primer-dimers and unspecific products, (ii) sequence-specific labelled probes, and (iii) fluorescent labelled primers. In order to estimate the initial amounts of the template serially diluted plasmids containing the functional gene of interest can be used. A proportional relationship between initial template amounts and amplified gene copy numbers is given at the threshold cycle (Ct), where the fluorescence exceeds the background and amplification is in a linear phase (Heid *et al.*, 1996). Although, qPCR is a frequently used method in microbial ecology, it suffers from some PCR related limitations. First of all environmental samples comprise a huge heterogeneity of templates within a single sample and thus a mixture of different primer sequences is needed to target most of the bacteria during the qPCR run. The use of degenerated primer might lead to reduced primer specificities. Moreover, the templates might develop different secondary structures and the polymerase itself could make mistakes in incorporating nucleotides (Eckert and Kunkel, 1991; Pallansch *et al.*, 1990; von Wintzingerode *et al.*, 1997). Moreover, the qPCR efficiency is influenced by the amplicon length, which should not exceed 500 bp (Pfaffl, 2001), and the degree of the contamination with humic acids or other inhibiting substances. Thus, it is indispensable to submit the raw data to a strict quality check before it is possible to make comparisons between various functional communities or different experiments.

However, if it is the aim of a study to target the active community or to measure turnover rates, analyses on the level of proteins or enzyme activities are more sensible. Proteins can be detected *in situ* or after the separation of cells from the soil matrix. For example Metz *et al.* (2003) used phylogenetic oligonucleotides and a monoclonal antibody against NirK combined with flow cytometry to quantify the denitrifying community in the rhizosphere of wheat roots and wastewater sewage treatment plants. However, the extraction of proteins from soil is difficult and it is unclear how stable extracted enzymes are if the substrate is not available (Dick, 1992; Philippot and Hallin, 2005). Thus, in microbial ecology it is common practice to measure potential enzyme activities in the laboratory or gas fluxes, which are indirectly linked to enzyme activities (Baldrian *et al.*, 2008; Duc *et al.*, 2009; Hoffmann *et al.*, 2007; Muhr *et al.*, 2008; Murray and Knowles, 1999; Tscherko *et al.*, 2004). To compare the enzymatic potentials of differently treated soils standardised *ex situ* protocols are used. The use of stable

conditions determining the optimal pH, temperature and nutrient concentrations enhanced the comparability of data. Potential enzyme activities can be detected by following the decrease of the substrate or the increase of the product, where fluorescent labelled substrates or special dyes were used, respectively. Regarding the nitrogen cycle, methods measuring potential nitrogen fixation (Tsunogai *et al.*, 2008), chitin and protein degradation (Hendel and Marxsen, 2005), nitrification (Hoffmann *et al.*, 2007) and denitrification (Luo *et al.*, 1996; Smith and Tiedje, 1979) are well established.

1.7. Aims and hypotheses of the Ph.D. thesis

It is unquestioned that nitrogen, as an essential macronutrient, plays a major role in ecosystem development. As many studies only focused on the role of single nitrogen cycle processes in ecosystem development, contradictory statements about the relevance of one or another process and the interaction of plants and microbes exist. In this regard, the question rises whether mineralisation or nitrogen fixation dominate in initial ecosystems or even a completely different process and, moreover, if plants display a nutrient hotspot or if they instead compete with microbes for the less abundant nitrogen.

Therefore, it was the aim of this thesis to reveal the succession of the terrestrial nitrogen cycle along a chronosequence of differently developed soils and to uncover the role of plants in this respect.

The following hypotheses have been tested in the frame of this thesis:

(i) Although, high gene copy numbers might occur at initial sites, nitrification and denitrification activities parallel the succession of plants, whereas the mineralisation of the rare organic compounds and the additional nitrogen input by BNF are already important at the beginning of the ecosystem development.

(ii) Moreover, plants from less developed soils strongly depend on the nitrogen delivered by microbes via BNF.

(iii) In contrast, a balanced relationship concerning nitrogen exchange is expected in more developed soils with higher abundance of microbes involved in nitrification and denitrification.

To test these hypotheses a field study and a climate chamber experiment were conducted. In the course of the field study, bulk soil samples from sites being ice-free for approximately 10-, 50-, 70- and 120-years (a) and from a reference site of around 2000a were analysed. Functional gene abundance patterns were achieved by qPCR of subunits of enzymes involved

in BNF (*nifH*), proteolysis and chitinolysis (*aprA* and *chiA*), nitrification (*amoA* AOA, *amoA* AOB) and denitrification (*nirS*, *nirK* and *nosZ*). Gene abundance data were correlated with the respective potential enzyme activities to reveal the actual relevance of the different functional communities and were further linked to different soil chemical parameters like dissolved organic carbon (DOC) and nitrogen (DON), NH_4^+, NO_3^- and pH (**Publication III**).

The climate chamber experiment aimed to analyse the plant-microbe interactions in the rhizosphere of *Leucanthemopsis alpina* (L.) Heywood, which is distributed along the whole glacier forefield even on the scarcely vegetated initial sites (Hämmerli *et al.*, 2007). The plant was cultivated in a 10a and 120a old soil for 7 and 13 weeks under controlled glacier mimicking conditions. Afterwards functional gene abundance patterns were compared in the rhizosphere and the respective bulk soil and linked to the NH_4^+, NO_3^-, plant carbon and nitrogen values (**Publication IV**).

As microbial biomass increased dramatically during ecosystem development molecular methods must be adapted to low biomass as well as high biomass samples. Therefore, pre-experiments targeting the reproducible extraction of nucleic acids from especially low biomass samples (**Publication I)** and the comparability of qPCR efficiencies in samples with low and high template amounts (**Publication II**) were performed.

2. Discussion

The present study was part of the Transregional Collaborative Research Centre 38, which investigated structures and processes of initial ecosystem development and was funded by the Deutsche Forschungsgemeinschaft and the Brandenburg Ministry of Science, Research and Culture (MWFK, Potsdam).

Chronosequences of soils with different development stages, which resulted among others from the ongoing retreat of glaciers, are ideally suited to investigate primary succession of microbial communities. In this study the Damma glacier forefield was chosen as an example, because this site has the advantage that it was extensively characterized in the course of the BigLink project (Biosphere-Geosphere interactions: Linking climate change, weathering, soil formation and ecosystem evolution, http://www.cces.ethz.ch/projects/clench/BigLink) and thus diverse background data and an already established sampling grid were available.

The focus of this Ph.D. thesis was to study prokaryotes involved in nitrogen turnover, because nitrogen as a macronutrient is essential for the establishment of plants and thus ecosystem development, but is not provided by the weathering of the bedrock material. Thus, microbes and plants being not able to fix N_2, strongly rely on the external input of nitrogen or the nitrogen fixing activity of other microbes. However, as nitrogen availability changed during ecosystem development, the relevance of one or another functional nitrogen cycle community might change as well. To cover the interplay of the different functional communities an extended nitrogen cycle approach was performed including nitrogen fixation, mineralisation, nitrification and denitrification.

2.1. The chronosequence approach

Although chronosequences facilitate the study of ecosystem development within relatively narrow spatial boundaries, this approach confronts researchers with different problems. Ecosystem development is not a linear process, but instead progresses in different phases (Deiglmayr *et al.*, 2006; Nicol *et al.*, 2005; Tscherko *et al.*, 2004). Thus, choosing the right sampling points along a chronosequence is essential. Therefore, the established and well characterised sampling grid at the Damma glacier constitutes a good basis. Moreover, glacier forefields are characterised by a large spatial heterogeneity attributed to the slope, the alteration of the glacial stream, the irregular distribution of vegetation patches and the uneven

surface. These properties create different microclimatic conditions along the chronosequence in terms of solar radiation, humidity, wind exposition and erosion. Despite these variations in environmental conditions soil age came out to be the main driver of ecosystem development (Duc *et al.*, 2009; Nemergut *et al.*, 2007), whereas other site characteristics have only a minor influence.

2.2. Methodological challenges

Beside the visible site specific heterogeneity at the soil surface, parameters like microbial biomass and organic matter content, which remarkably varied, had a direct impact on the applied molecular methods. The extractable DNA content increased from 2.3 µg DNA g^{-1} dry weight (dw) at site 10a to 28 µg DNA g^{-1} dw at the reference site outside the glacier forefield. Moreover, total organic carbon (TOC) and DOC contents increased 57- and 11-fold, respectively, and consequently also humic substances (**Publication III**). These conditions resulted in three main challenges for molecular downstream analyses: (i) the reproducible extraction of sufficient amounts of DNA from low biomass samples; (ii) the high variation of the DNA and humic substance content along the chronosequence might influence the qPCR efficiency and reliability and, (iii) the question about how to relate or normalise data obtained from that environment.

The choice of the nucleic acid extraction protocol influenced both the achievable DNA concentration and the DNA purity. The generally low yield of extractable genetic material, being below 10 μg^{-1} g^{-1} soil at initial sites of glacier forefields (Kandeler *et al.*, 2006; Noll and Wellinger, 2008; Sigler and Zeyer, 2002), especially, displayed a challenge for a reproducible DNA extraction. Nowadays, the most frequently used procedure to isolate nucleic acids from soil material is the direct extraction method, where cells are directly lysed from the soil matrix (Roose-Amsaleg *et al.*, 2001). For quantitative downstream analyses it has to be steered to a middle course between nucleic acid purity and quantity. In this regard, **Publication I** revealed the highest and most reproducible DNA contents, when the soils were extracted with the FastDNA® SPIN Kit for Soil (MP Biomedical, France). However, that was also connected with the highest contamination of humic substances, mostly attributed to the similar physicochemical properties of nucleic and humic acids, which in turn leads to competitively binding to absorption sites of the applied minicolumns (Harry *et al.*, 1999; Lear *et al.*, 2010). A common method for qPCR measurements to overcome this problem is to dilute the nucleic acid extracts until inhibitory substances are below an effective threshold. In contrast, this

strategy is not advisable for diversity analysis, because less abundant species can be thinned out and consequently the detected diversity is underestimated. This example showed that requirements for the nucleic acid extraction change with the subsequent application.

While, the challenges for the DNA extraction are mainly attributed to low biomass samples, the qPCR approach is affected by diverse parameters. On the one hand, PCRs performed with less abundant templates are more prone to PCR biases and unspecific amplifications, which might be problematic for samples from the initial sites. On the other hand, the abundance of the template can oscillate within two to three orders of magnitude along the chronosequence or between bulk soil and rhizosphere as shown for *nirS* at the Rotmoosferner glacier (Kandeler *et al.*, 2006), for *nifH* and *amoA* AOA in **Publication III** and *chiA* in **Publication IV**. As it is known that the performance of the polymerase is best at a certain ratio of the PCR components (Zhang and Fang, 2006), different amounts of the template might have an effect on the PCR reaction. Hence, it is absolutely necessary to validate the quality of each qPCR run. Therefore, a guideline about the "Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE)" has been published (Bustin *et al.*, 2009). However, the recommended validation of the qPCR is mostly restricted to the quality of the standard curve (R^2 of the standard curve, qPCR efficiency calculated from the slope of the standard curve, y-intercept values) and the specificity of the PCR product (melting curve analysis, agarose gel analysis). Although the calculated qPCR efficiency using the equation

$$Eff_{slope} = [10^{(-1/slope)} - 1]$$

might result in high PCR efficiencies, this is only an expression about the accuracy of the serial dilution of the standard. In order to proof the reliability and comparability of data, a comparison of the individual PCR efficiency of samples with different characteristics like template amount or inhibitor concentrations is needed. Therefore, an algorithm was developed, where at the log-linear part of each amplification curve a linear regression was performed to calculate the individual PCR efficiencies (Ramakers *et al.*, 2003; Ruijter *et al.*, 2009). By using the corresponding analysis tool, the LinRegPCR program, it was possible to check if the qPCR reactions were comparable in soils with different template amounts and chemical properties. The analysis of gene abundance data from the 10a and 120a soil revealed (**Publication II**) that the qPCR performance was similar for both soils, although they are most different in terms of biomass and chemistry. Subsequently, that procedure was applied for all qPCR data sets as an additional quality parameter to the MIQE guideline.

The increasing biomass has not only a direct influence on the application of the different molecular methods but also on the data processing and evaluation. Thus, the decision of how to relate the data must be made carefully. Basically there are three possibilities to display the obtained data: (i) one can relate the data to gram of soil, (ii) to ng extracted DNA or (iii) to 16S rRNA gene copies. Regarding the first alternative the interpretation of data might be trivial, because the increasing biomass caused an increase in gene abundances and potential enzyme activities. In contrast, the remaining opportunities exclude the biomass factor, so that it is possible to compare the relative proportions of different functional groups. However, it is common knowledge that the number of 16S rRNA operons can vary between one and 15 copies in different bacterial taxa (Klappenbach *et al.*, 2000). As the bacterial diversity changed along glacier forefields (Sigler and Zeyer, 2002), it is difficult to carry out calculations with a mean operon number for that ecosystem. Hence, for the interpretation of the chronosequence results, data was related to DNA contents.

2.3. Succession of the nitrogen cycle

Succession is a reasonably directional process of community development, which ends up in a stabilised ecosystem. The process itself is driven by the modifications of the inhabiting community but the pattern, the rate of change and the finally reached system is regulated by the physical environment (Odum, 1969). Basically succession can be divided into two groups: primary and secondary succession. Succession is called primary when an environment is colonised by plants or other organisms for the first time. This includes deglaciated regions, volcanically active areas and bare rocks. In contrast, secondary succession occurred when previously colonized sites will be disturbed by heavy grazing, firestorms or ploughed fields, which afterwards have been laid fallow or other disturbing events (Wali, 1999).
Regarding primary succession, it is argued that initial organisms have the task to pave the way for the establishment of others by creating a more hospitable environment. This mechanism is known under the term “facilitation pathway” (Connell and Slatyer, 1977).

2.3.1. Which functional communities dominate the initial sites?

At initial sites of the Damma glacier plant coverage is below 20% (Hämmerli *et al.*, 2007) and nitrogen compounds like DON, NO_3^- and NH_4^+ were lowest, for example NH_4^+ revealed 400 times lower contents at the 10a compared to the reference site. Thus, at a first glance, it is

most likely that BNF might be the dominating process, which would be in agreement with results from recently deglaciated sites of the Peruvian Puca Glacier (Schmidt *et al.*, 2008) and young lava flows from the Hawaiian Mauna Loa (Crews *et al.*, 2001). Against this assumption, gene abundance data revealed significantly highest *nifH* gene abundances at the 50a site, where first plant patches appear, regardless of whether being related to gram of soil or ng DNA. On the contrary, *apr* genes per ng DNA were most abundant at the 10a site and additionally relative potential protease and chitinase activities were highest. The potential enzyme activities of the remaining processes were below the detection limit at the 10a site or even much lower compared to the reference site. There are three possibilities explaining the dominance of the mineralising community and the under-representation of the nitrogen fixing community: (i) As shown by Göransson *et al.* (2011) carbon is the primary limiting resource at initial sites of the Damma glacier. Thus, the needed energy (16 ATP per molecule N_2) to fix nitrogen might be lacking. (ii) Due to the sparse vegetation, the competition between plants and microbes for nitrogen resources in the bulk soil might be low. (iii) In this regard, the deposited nitrogen, which is in total between 10-15 kg N ha^{-1} a^{-1} in this area (Federal Commission for Air Hygiene, 2005) and where the particulate proportion perceived at least 0.63 kg N ha^{-1} a^{-1}, might be high enough to meet the demands of the microbes. In addition to the detected allochthonous organic material, microbes might also feed on ancient recalcitrant carbon (Bardgett *et al.*, 2007; Hodkinson *et al.*, 2002).

As it was demonstrated that the rhizosphere of pioneering plants displayed a nutrient hotspot (Duc *et al.*, 2009; Miniaci *et al.*, 2007), at least in terms of carbon, a climate chamber experiment with *Leucanthemopsis alpina* (L.) Heywood as model plant and carbon hotspot was conducted (**Publication IV**). *L. alpina* was cultivated for 7 and 13 weeks in a 10a and 120a soil exhibiting different total carbon contents of 0.7 g kg^{-1} and 12 g kg^{-1} bulk soil, respectively. Gene abundance data revealed that the *nifH* gene copy numbers were the only one displaying highest values in the rhizosphere grown in the 10a soil after 7 weeks of incubation. Additionally, the percentage of nitrogen in the plant biomass significantly increased and consequently the C/N ratio decreased from 33 to 10 in the green biomass of *L. alpina* from the 10a soil. On the contrary, the C/N ratio of the plants from the 120a soil remained constant. Therefore, two scenarios might explain the increased nitrogen content of *L. alpina* and the enhanced abundance of nitrogen fixers. First the plant assimilated the microbially derived NH_4^+ as predicted by the "classical" paradigm of the nitrogen cycle (Black, 1993; Chen *et al.*, 2003; Schimel and Bennett, 2004) and thus nitrogen fixing microbes were enriched. Or secondly, microbes and plants compete for the organic nitrogen

compounds as demonstrated for nitrogen poor environments in different studies (Chapin *et al.*, 1993; Jingguo and Bakken, 1997; Kaye and Hart, 1997; Schimel and Chapin, 1996), with the plant being able to win the competition over time. Consequently, nitrogen fixers have an advantage compared to other functional communities in this situation.
All data together indicate that the initial sites of the Damma glacier are mainly dominated by mineralising microbes, whereas nitrogen fixing microbes instead appear when plant coverage increased and a competitive situation for limited nutrients like nitrogen emerged.

2.3.2. Does gene abundance reflect the potential activity?

Functional gene abundance reflects the potential number of microbes performing a certain process. However, the quantity of microbes producing active enzymes is usually much lower (Philippot and Hallin, 2005), because enzymes do not pass all regulatory levels during the maturation. This includes, that genes are not transcribed, mRNA is not translated, proteins undergo posttranslational modifications or mRNA and proteins are degraded (Röling, 2007). Moreover, it is possible that functional enzymes cannot perform their assigned reaction, because for various reasons the substrate is not available, for example: (i) the substrate is simply less abundant; (ii) it is not accessible, because it is physically associated with other substrates; (iii) it might be trapped inside of pores or microaggregates; (iv) or it might form complexes with recalcitrant humic matter (Burns, 1982). Due to the number of factors influencing the expression of genes or the accessibility of substrates, the question rises to which extent gene abundance is a good proxy for the actual activity of a certain functional community. Hence, we correlated gene abundance data and potential enzyme activities of the different nitrogen cycle processes along the Damma glacier chronosequence (**Publication III**). The different nature of the processes might lead to variable correlations of abundance and activity. For example denitrification is facultative under aerobic conditions, whereas the oxidation of NH_3 is obligatory for the energy budget of ammonia oxidising bacteria. Thus, a better correlation for nitrification can be expected compared to denitrification. Against that theory, correlations were only found for chitinolytic ($R^2 = 0.46$; $p < 0.001$) and proteolytic ($R^2 = 0.50$; $p < 0.001$) microbes and for N_2O reducers ($R^2 = 0.64$; $p < 0.001$).
The ability to degrade proteins and chitin is widespread among microorganisms (Cohen-Kupiec and Chet, 1998; Kumar and Takagi, 1999). Therefore, it is not surprising that protease and chitinase activities follow the increase in microbial biomass. This trend is corroborated by studies from Tscherko *et al.* (2004; 2003), who also detected increasing protease and chitinase

activities along the Rotmoosferner glacier forefield. As it is assumed for chitinases and extracellular proteases that they might be constitutively excreted (Burns, 1982; Felse and Panda, 1999), a strong correlation between functional gene abundance and enzyme activity is very likely. Interestingly, Olander *et al.* (2000) proposed a negative feedback of chitinase activity and nitrogen supply, which is probably attributed to the repression of the activity by high levels of N-acetyl-glucosamine. Obviously, at the Damma glacier forefield and even at the reference site the critical level of nitrogen is not reached. Lowest nitrogen contents in the mentioned study were around 0.9 % total nitrogen, while at the Damma glacier values did not exceed 0.3 % total nitrogen and thus were far away from a negative feedback situation. The good correlation of activity and abundance of mineralising or instead decomposing microbes might also be attributed to the unique regulation of enzyme induction mainly based on substrate induction and/or product repression. For example, chitinase activity is triggered by chitin availability (Felse and Panda, 1999). As fungi, as a source of chitin, strongly increased along glacier forefields (Bardgett and Walker, 2004; Ohtonen *et al.*, 1999), it is not surprising that chitinase activity did as well. The same was found for proteases, which are mainly induced by protein availability and additionally exhibit a broad substrate spectrum (Geisseler and Horwath, 2008).

For BNF, abundance and activity were uncoupled with highest gene copy numbers observed in the intermediate stages, whereas potential activity peaked in the 120a and 2000a sites. The low nitrogen fixation activity in the initial and intermediate soils might be attributed to: (i) low carbon contents and thus low energy availability, (ii) a shift from a cyanobacteria dominated community (Duc *et al.*, 2009; Nemergut *et al.*, 2007), with low turnover rates, to a symbiotic or plant associated community, exhibiting much higher turnover rates (Cocking, 2003), and (iii) the possibility that not all microbes able to fix nitrogen, are actively participating in BNF as shown by Bürgmann *et al.* (2005). The first theory is corroborated by findings from Duc *et al.* (2009) and the pot experiment with *L. alpina,* where the input of carbon in the rhizosphere accomplished an increase of nitrogen fixation activity or *nifH* gene copy numbers, respectively. The second theory is underlined by the potential nitrogen fixation assay applied. As samples were incubated in the dark for two weeks the amount of phototrophic fixed nitrogen should be low compared to heterotrophic nitrogen fixation (Yeager *et al.*, 2004). Thus, the measurement confirms an increase of the heterotrophic nitrogen fixation. As shown in many other studies from initial ecosystems, (Garcia-Pichel *et al.*, 2001; Schmidt *et al.*, 2008; Yeager *et al.*, 2004) cyanobacteria might rather play a role in ecosystem engineering by secreting polysaccharide sheath and thus stabilising the slope and

increasing the water holding capacity of the substrate, than introducing remarkably amounts of nitrogen to the ecosystem.

Regarding nitrification, it might be assumed that nitrification plays a minor role along the Damma glacier chronosequence due to the low pH dropping from 5.1 to 4.1. Consequently, the equilibrium of NH_3 and NH_4^+ is shifted to NH_4^+, which is not used as substrate by the ammonia monooxygenase (Kowalchuk and Stephen, 2001; Suzuki *et al.*, 1974). However, mechanisms exist to overcome the problems being connected with acidic environments, for example the intracellular hydrolysis of urea, the growth in alkaline microsites or the formation of biofilms (De Boer and Kowalchuk, 2001; Tourna *et al.*, 2011). Therefore, it might be that at the less acidic initial sites of the glacier forefield these mechanisms allow the AOB to outcompete AOA because of their higher turnover rates per cell (de la Torre *et al.*, 2008; Könneke *et al.*, 2005; Prosser, 1989; Ward, 1987). However, with ongoing succession the pH steadily decreased and AOA are assumed to be more successful under these conditions (Nicol *et al.*, 2008), which are further favoured by the low NH_4^+ concentrations (Di *et al.*, 2010; Di *et al.*, 2009). Consequently, the potential nitrification activity increased with ongoing succession and rather correlates with the abundance of AOA ($R^2 = 0.49$, $p = 0.07$) than AOB ($R^2 < 0.01$, $p = 0.95$). A further advantage of AOA compared to AOB is the recently discovered property of soilborne AOA to pursue a mixotrophic lifestyle (Jia and Conrad, 2009; Tourna *et al.*, 2011), meaning that they are also able to use organic energy sources. Therefore, it is not surprising that the relative abundance of AOA increased with increasing DOC contents, whereas the abundance of AOB decreased. Thus, it seems that a shift from AOB to AOA driven nitrification occurred along the Damma glacier forefield.

In contrast to ammonia oxidation, denitrification is only performed under anoxic conditions. Otherwise oxygen is preferred as terminal electron acceptor. Interestingly, only *nosZ* gene copy numbers increased with ongoing succession and hence correlated with the potential denitrification activity, while *nirS* and *nirK* containing microbes decreased. The correlation of *nosZ* gene abundance and potential denitrification activity was also observed in studies of Hallin *et al*. (2009) and Phillipot *et al.* (2009). Generally, denitrification is strongly influenced by NO_3^-, O_2 and organic carbon availability. Thus, it is not surprising that potential denitrification activity increased along the chronosequence, as carbon and nitrogen contents also had and the increasing plant coverage connected with elevated root penetration lowered the partial oxygen pressure in the surrounding soil. That only *nosZ* correlates with the activity might be attributed to the low NO_3^- content in the habitat, so that it is important to extract optimal amounts of energy during the reduction of one molecule of NO_3^-. In that respect it

was shown that the N_2O/N_2 ratio is positively related to the NO_3^- concentration (Dendooven *et al.*, 1994). Moreover, the N_2O reductase is most sensitive against O_2 (Morley *et al.*, 2008; Otte *et al.*, 1996) and therefore N_2O reducers might have an advantage with increasing plant coverage. This assumption is corroborated by the data obtained from the pot experiment with *L. alpina*, where only the gene copy number of *nosZ* significantly increased in the rhizosphere with ongoing plant and root development, while *nirK* and *nirS* harbouring microbes did not respond. In contrast to our data, Cuhel *et al.* (2010) found a correlation of *nirS* and the potential denitrification activity. Regardless, the NO_3^- contents of that soil were five times higher than values from the reference site of the Damma glacier chronosequence, while the microbial biomass only doubled. Hence, much more NO_3^- is available per microbe in the investigated pasture and therefore the relatively high nitrate concentrations might favour a truncated denitrification pathway.

2.4. The role of plants: competitors or drivers?

The competition between microbes colonising the barren initial substrate is assumed to be relatively low. That is on the one hand, because the density of the microbes compared to the nutrient content is high enough to meet their demands (Deiglmayr *et al.*, 2006). And on the other hand, a spatial isolation of soil particles might also lead to a spatial isolation of microbes, which in turn reduced directly competitive events (Zhou *et al.*, 2002). Nevertheless, one can already find sparsely distributed pioneering plants at the very young sites of glacier forefields such as *L. alpina*, *Agrostis gigantea* or *Cerastium uniflorum* (Göransson *et al.*, 2011; Miniaci *et al.*, 2007; Tscherko *et al.*, 2003). Interestingly, legumes did not belong to the dominant plant species at the initial sites of alpine glacier forefields. There are two reasons explaining the absence of legumes from the initial sites: First many legumes like *Lotus alpinus* form heavier seeds than *L. alpina* or *C. uniflorum* (Pluess *et al.*, 2005; Tackenberg and Stöcklin, 2008) and thus the dispersal via wind is more difficult. Second the establishment and maintenance of a rhizobia-legume symbiosis is a very energy consuming process (Merbach *et al.*, 1999) and therefore may be too energy costly under these harsh conditions. Regarding other pioneering plants, researchers revealed that at initial sites especially, microbes are much more abundant and active in the direct vicinity of the plant compared to the bulk soil (Bardgett and Walker, 2004; Deiglmayr *et al.*, 2006; Duc *et al.*, 2009; Tscherko *et al.*, 2004). Similar results were obtained during the pot experiment with *L. alpina,* where all functional gene abundances were significantly higher in the rhizosphere

compared to the bulk soil in the 10a soil. Whereas the impact of the plant decreased with soil age, when parameters like organic carbon increased in the bulk soil. The positive effect of the plant on the microbial abundance and activity is known as the "rhizosphere effect". The term "rhizosphere" was already in 1904 defined by Lorenz Hiltner as "the soil influenced by the root" (Hartmann *et al.*, 2008). That effect is mainly attributed to rhizodeposition and litter input by the plant, which delivered carbon and thus also energy to the root associated microbes. Therefore in terms of carbon, the pivotal role of the plant is unquestionable, as CO_2 is fixed by the plant itself during photosynthesis and the plant allocated up to 40% of the fixed CO_2 to the microbes (Paterson and Sim, 2000). However, a competition between plants and microbes about nitrogen might occur in initial ecosystems. Therefore, it is particularly interesting to investigate the impact of pioneering plants on different functional communities of the nitrogen cycle (**Publication IV**). For example, processes like nitrification and denitrification might be reduced in the rhizosphere of pioneering plants, because they are connected with a loss of nitrogen via leaching of NO_3^- or gaseous products, respectively. In contrast, the enrichment of free-living diazotrophs and mineralising microbes in the rhizosphere might display an advantage for both parties.

In this respect, only *nifH* harbouring microbes revealed highest abundance in the rhizosphere of the 10a soil after 7 weeks of incubation, whereas all other functional groups were higher in the 120a soil. Similarly, the C/N ratio of the plant biomass was highest, which was attributed to low nitrogen contents in the plant. However, the percentage nitrogen content of the plant significantly increased after 13 weeks of incubation in the 10a soil from 1.28% to 3.94%, while the carbon content remained constant. This observation fits nicely with the assumption that microbes are able to win the competition over a short timescale, because of their higher volume-surface ratio, higher growth rate and substrate affinity (Hodge *et al.*, 2000). On the contrary, plants are more effective over a long time period, because of their longer lifespan, whereby they compete different times for the same nitrogen source and additionally they are able to retain assimilated nitrogen over a longer time period (Hodge *et al.*, 2000; Nordin *et al.*, 2004). This theory is further corroborated by the observation that the abundance of the different functional groups generally decreased during the incubation. The only functional group being less affected by incubation time and plant development stage is the *chiA* harbouring community, which is underlined by studies where a selection of heterotrophic organisms in the rhizosphere was found (Miethling *et al.*, 2000; Tscherko *et al.*, 2004). It was not surprising that mineralising microbes in general are highly abundant. The relatively high abundance of *chiA*, in particular, which is with 5×10^7 copies g^{-1} dw even higher than *nifH,*

might be attributed to higher amounts of fungal biomass in the rhizosphere compared to the bulk soil (Tscherko *et al.*, 2004). The degradation of chitin has the advantage that low weight organic compounds consisting of carbon and nitrogen are released. The ability of plants to assimilate amino acids, amino sugar or small peptides seems to be ubiquitously distributed among different ecosystems and plays a particular role in cold and wet habitats (Lipson and Monson, 1998; Näsholm *et al.*, 2009; Schimel and Bennett, 2004). These findings are underlined by the discovery of three transporters for the amino acid uptake in *Arabidopsis thaliana* (Hirner *et al.*, 2006; Lee *et al.*, 2007; Svennerstam *et al.*, 2008).

Regarding ammonia oxidising microbes, similar gene copy numbers of *amoA* AOB were found in the bulk soil of *L. alpina* and the Damma glacier forefield with values of 3.2×10^6 and 1.4×10^6 copies g^{-1} dw, respectively. In contrast, *amoA* AOA were below the detection limit in both, the bulk soil and the rhizosphere of *L. alpina* in the pot experiment. These results are in line with studies from Di *et al.* (2010; 2009), who revealed that AOB can cope much better with high concentrations of NH_3 than AOA, which is the case of the rhizosphere. Moreover, Fan *et al.* (2010) showed that only the composition of the AOB community correlates with the potential nitrification activity in the rhizosphere of faba bean and maize, whereas AOA were unaffected. Data indicates that a niche separation between AOA and AOB occurred on one hand along the Damma glacier chronosequence as discussed in chapter 2.3.2 and on the other hand in the rhizosphere.

A similar observation was made for the functional genes of the nitrite reductases *nirS* and *nirK*. While *nirK* was found in constantly high gene copy numbers in the rhizosphere of *L. alpina* and to a lower extent in the bulk soil, *nirS* was only detected in the bulk soil of the 120a soil. This niche differentiation of *nirS* and *nirK* communities was also observed in the rhizosphere of grain legumes (Sharma *et al.*, 2005). The more interesting aspect about the functional denitrifying community is the constant abundance of *nirK* and the strongly increasing abundance of *nosZ* in the rhizosphere of the 120a soil. This hints towards a shift within the community from microbes with a truncated denitrification pathway to one with a fully developed one. Obviously, the conditions for denitrification become more favourable with ongoing plant development, mainly attributed to the elevated root system, which leads to a reduced partial oxygen pressure in the rhizosphere, because of the root respiration (Rheinbaben and Trolldenier, 1984).

Finally, it might be concluded that the proposed positive feedback (Reynolds *et al.*, 2003) between plants and microbes in developing ecosystems could be confirmed by the revealed data. However, the significance of the rhizosphere effect decreased with ecosystem

development, due to the increasing organic carbon content in the bulk soil. But nevertheless, it seems that plants are able to create their optimal rhizosphere community, maybe by actively changing their exudation pattern in order to influence the establishment of one or another microbial community as shown for *L. alpina* (Edwards *et al.*, 2006), or exuding secondary metabolites like tannins, polyphenolic substances or monoterpenes, which can inhibit certain functional groups (Briones *et al.*, 2003; Cocking, 2003; Kowalchuk and Stephen, 2001; Ward *et al.*, 1997). Consequently, the plant selects for special functional communities rather than taxonomic groups (Singh *et al.*, 2004).

3. Conclusions

The investigation of unaffected chronosequences of ecosystem development gives insights into the natural evolution of certain ecosystem functions. Nitrogen, which is on one hand turned over in a relatively closed cycle compared to carbon and on the other hand rare at many initial sites of ecosystem development, represents a good model to study the succession of different functional microbial communities.

Using molecular techniques like qPCR and potential enzyme activity measurements, it was possible to follow changes in the abundance and potential activity of functional communities. However, due to the broad range of biomass, pre-experiments about the reliability of nucleic acid extraction protocols (**Publication I**) and qPCR standard curves (**Publication II**) were performed. Based on that, our data indicates that the succession of nitrogen cycle processes along the Damma glacier forefield proceeded in at least three stages, which are depicted in Figure 9 (**Publication III**). The initial phase (10a) is characterized by the high abundance and potential activity of mineralizing microbes.

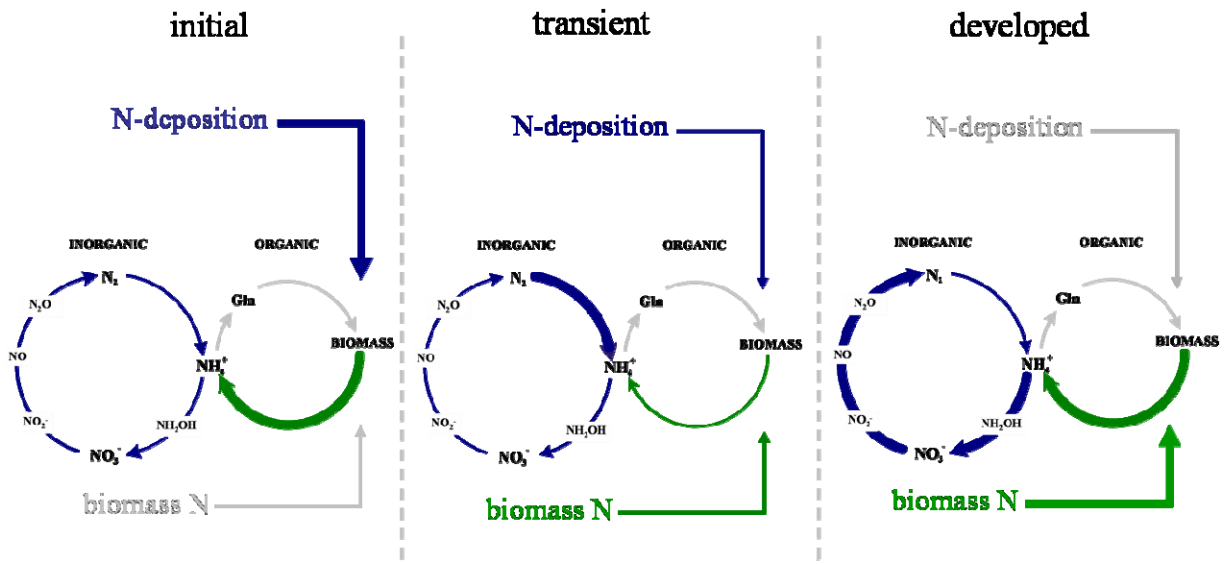

Figure 9. Scheme of the succession of the nitrogen cycle. The initial phase comprises the 10a soils, the transient phase the 50a and 70a soils and the developed phase includes the 120a and 2000a soils. The size of the arrows indicated the relevance of the respective process including nitrogen deposition and biomass derived nitrogen.

Moreover, the deposited nitrogen and other external nitrogen inputs seemed to be an important source of nitrogen, which allows the microbes to meet their demands. In contrast, in the transient phase (50a, 70a), where first plant patches appear, the competition between plants and microbes increased and therefore the importance of nitrogen fixation did as well. That observation was corroborated by the pot experiment with *L. alpina* (**Publication IV**), which on one hand served as carbon hotspot in the 10a soil and on the other hand competes with the microbes for nitrogen. In this regard, data revealed highest *nifH* gene copy numbers in the rhizosphere of *L. alpina* cultivated in the 10a soil and moreover, the plant was able to accumulate higher percentages of nitrogen in the 10a compared to the 120a soil during the incubation. While the initial and transient development stages are dominated by processes being connected with nitrogen input or release from biomass, nitrification and denitrification processes are already facilitated after 120a of soil development. At that point, it is very likely that most of the requirements are covered by biomass derived nitrogen. Moreover, correlations of gene abundance data and potential enzyme activities revealed that most of the potential enzyme activities depend on environmental conditions (e.g. denitrification) or a shift within the functional community (e.g. nitrification and nitrogen fixation) rather than on gene copy numbers (**Publication III**).

Although our results revealed significant changes in the abundance and potential activity of different functional communities along the Damma glacier forefield and differences in the abundance of functional communities in the rhizosphere of *L. alpina*, one has to keep in mind that we only analysed one glacier forefield and one pioneering plant, respectively. Especially, differences in climatic conditions, bedrock material (silicoues or calcerous), deposition rates or exposition of the glacier forefield might significantly influence ecosystem development and therefore, a generalisation of the data is at the moment not possible. For future studies, it would be interesting to check if our data is transferable to other initial chronosequences like inland dunes or other biological hotspots like biological soil crusts. Moreover, the establishment of high-throughput sequencing methods in recent years would further allow the investigation of food web dynamics by meta-genome or meta-transcriptome studies.

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List of abbreviation

a	year
AD	anno domini
amoA	gene encoding the α-subunit of the ammonia monooxygenase
amoCAB	operon encoding the ammonia monooxygenase
Anammox	anaerobic ammonium oxidation
AOA	ammonia oxidising archaea
AOB	ammonia oxidising bacteria
Apr	bacterial alkaline metalloprotease
apr	gene encoding the alkaline metalloprotease
ATP	adenosine triphosphate
BNF	biological nitrogen fixation
chiA	gene encoding the bacterial chitinase group A
C_{mic}	microbial carbon
cNor	NO-reductase receiving electrons from cytochrome *c* or a pseudoazurin
Ct	threshold cycle
CuA-domain	domain of the electron entry of the nitrous oxide reductase
CuZ-domain	catalytic site of the nitrous oxide reductase
cycA	gene encoding the cytochrome c554
cycB	gene encoding the membrane bound cytochrome c_M552
cyt	cytochrome
DNA	deoxyribonucleic acid
DNRA	dissimilatory reduction of nitrate to ammonium
DOC	dissolved organic carbon
DON	dissolved organic nitrogen
dw	dry weight
et al.	et alii
e.g.	for example, *exempli gratia*
GlcNAc	poly-β-1,4-acetylglucosamine
mRNA	messenger ribonucleic acid
N_2	dinitrogen
Nap	periplasmic nitrate reductase
napA	gene encoding the catalytic subunit of the nitrate reductase
napB	gene encoding the cytochrome subunit of the nitrate reductase

Nar	membrane bound nitrate reductase
narG	gene encoding the α-subunit of the nitrate reductase
narH	gene encoding the β-subunit of the nitrate reductase
narI	gene encoding the γ-subunit of the nitrate reductase
NH_3	ammonia
NH_4^+	ammonium
N_2H_4	hydrazine
nifD	gene encoding a subunit of the dinitrogenase
nifK	gene encoding a subunit of the dinitrogenase
nifH	gene encoding the dinitrogenase reductase subunit of the nitrogenase
nirK	enzyme encoding the copper containing nitrite reductase
nirS	enzyme encoding the cytochrome cd_1-nitrite reductase
N_{mic}	microbial nitrogen
NO	nitric oxide
N_2O	nitrous oxide
NO_3^-	nitrate
NO_2^-	nitrite
NOB	nitrite oxidising bacteria
nosZ	gene encoding the nitrous oxide reductase
Npr	bacterial neutral metalloprotease
Nxr	nitrite oxidoreductase
nxrA	gene encoding the α–subunit of the nitrite oxidoreductase
nxrB	gene encoding the β–subunit of the nitrite oxidoreductase
O_2	dioxygen
PCR	polymerase chain reaction
pMmo	particulate methane monooxygenase
qNor	NO-reductase receiving electrons from the quinol pool
qPCR	quantitative real-time PCR
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
Ser	bacterial serine protease
spp.	species
TOC	total organic carbon

Acknowledgement

I want to thank the “Deutsche Forschungsgemeinschaft” and the “Brandenburg Ministry of Science, Research and Culture” (MWFK, Potsdam) for funding the Transregional Collaborative Research Centre 38 (CRC/TRR38), which allowed me to perform my Ph.D. thesis in frame of that project .

Moreover, thousand thanks go to Prof. Dr. Michael Schloter, who successfully supported my thesis and gave me the opportunity to work on this topic. He always found time to discuss the experimental planning and associated problems and strongly facilitated contemporary publications of my experiments. The opportunity to actively attend different national and international congresses especially helped me to improve my presentation skills and to become more self-confident.

Furthermore, I am indebted to Prof. Dr. Jean Charles Munch for the possibility to perform my Ph.D. thesis at his institute and to benefit from the provided facilities. Additionally, I am grateful for the recommendations to improve this Ph.D. thesis.

I thank Robert Brankatschk and Prof. Dr. Josef Zeyer from the ETH Zurich for the productive collaboration and especially Prof. Dr. Josef Zeyer for the interesting field trips and the peer review of my Ph.D. thesis.

In addition, I thank Prof. Dr. Ingrid Kögel-Knabner to be willing to verify this thesis.

I would like to thank Andreas Albert for the support of my climate chamber experiment.

Special thanks go to Kristina Kleineidam for the moral and practical support over the whole Ph.D. time and the vigorous assistance at my several sampling trips to the Damma Glacier. In this respect I also thank all remaining “TEGies” for the kind working atmosphere and especially my present and past office members Silvia Gschwendtner, Astrid Bauer, Eva Kastl, Jana Ernst and Brigitte Hai for the energizing coffee breaks and productive discussions. Special thanks go also to Cornelia Galonska and Gudrun Hufnagel for the great assistance in molecular laboratory analyses and soil solution measurements, respectively.

Furthermore, I am very grateful to Joshua Gnizak for the critical review of the manuscript.

Finally, but most importantly, I'd like to give profuse thanks to my parents Rosi and Dieter Töwe, who always supported me on my way, my brothers Frank and Thomas Töwe, who made me feel close while so far away from home. And last but not least I'd like to say thank you to my beloved husband Stephan Schulz for the long standing support and the encouragement especially during hard times.

Publication I

Journal of Microbiological Methods 84 (2011) 406–412

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Improved protocol for the simultaneous extraction and column-based separation of DNA and RNA from different soils

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ARTICLE INFO

Article history:
Received 19 November 2010
Received in revised form 27 December 2010
Accepted 27 December 2010
Available online 21 January 2011

Keywords:
DNA/RNA extraction
DNA/RNA separation
nosZ
qPCR
T-RFLP

ABSTRACT

We developed an improved protocol, allowing the simultaneous extraction of DNA and RNA from soil using phenol-chloroform with subsequent column-based separation of DNA and RNA (PCS). We compared this new approach with the well established protocol published by Griffiths et al. (2000), where DNA and RNA are separated by selective enzymatic digestions and two commercial kits used for DNA or RNA extraction, respectively, using four different agricultural soils. We compared yield and purity of the nucleic acids as well as abundance and diversity profiles of the soil bacterial communities targeting the *nosZ* gene via quantitative real-time PCR and terminal restriction fragment length polymorphism on DNA and RNA level. The newly developed protocol provided purer nucleic acid extracts compared to the used kit-based protocols. All protocols were suitable for DNA- and RNA-based gene quantification, however high variations between replicates were obtained for RNA samples using the original Griffiths protocol. Diversity patterns of *nosZ* were highly influenced by the extraction protocol used both on the DNA and RNA level. Finally, our data showed that the new protocol allows a simultaneous and reproducible extraction and separation of DNA and RNA, which were suitable for reliable analyses of gene and transcript copy numbers and diversity pattern.

1. Introduction

Soils represent one of the most complex environments on earth providing living space for a huge number of different microbes. Enormous progress has been made in recent years by means of molecular methods like quantitative real-time PCR (qPCR), fingerprinting techniques and sequencing to gain information about abundance, composition and diversity of microbial communities in these habitats (Rajendhran and Gunasekaran, 2008; Roose-Amsaleg et al., 2001). Nucleic acid extraction from environmental samples forms the basis for further molecular analyses (McIlroy et al., 2009). However DNA and RNA extraction from complex matrices like soil is highly challenging due to low yields of nucleic acids, difficulties in reproducibility (McIlroy et al., 2009) and contamination of nucleic acid extracts, mainly due to humic substances, which can inhibit PCR amplification (Rajendhran and Gunasekaran, 2008).

In principal, two different approaches for extracting nucleic acids from environmental samples exist (Herrera and Cockell, 2007). The indirect approach, is based on an initially separation of cells from the soil matrix by elution, followed by cell lysis and nucleic acid extraction (Delmotte et al., 2009; Holben et al., 1988), whereas the direct approach is based on a lysis of cells directly in the soil and subsequent extraction of nucleic acids from the environmental sample (Roose-Amsaleg et al., 2001). While numerous articles have described DNA *or* RNA extraction procedures, only very few publications deal with DNA/RNA co-extraction (Costa et al., 2004; Griffiths et al., 2000; Hurt et al., 2001; McIlroy et al., 2009; Ogram et al., 1995; Orsini and Romano-Spica 2001; Yu and Mohn, 1999). However if gene abundance pattern should be linked to transcript rates there is a need for co-extraction of DNA and RNA due to the different types of bias, that is linked to each individual nucleic acid extraction protocol. One of the most cited protocols for DNA/RNA co-extraction has been published by Griffiths et al. (2000). As the DNA/RNA separation is based on DNA or RNA digestion by DNase or RNase treatment, respectively, subsequent purification and separation steps are needed, which in consequence may induce selective ranges of error, despite the same extraction procedure biases. Therefore we developed a protocol for DNA/RNA co-extraction based on nucleic acid precipitation with polyethylene glycol (PEG) (according to Arbeli and Fuentes (2007)) and subsequent DNA and RNA separation via silica-based columns. In this study we compared the new protocol based on a phenol-chloroform extraction with subsequent column-based separation of DNA and RNA (PCS) with the original protocol by Griffiths et al. (2000) and a commercially available DNA and RNA extraction kit, respectively. We used four

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doi:10.1016/j.mimet.2010.12.028

different agricultural soils and compared the amount and purity of the extracted nucleic acids as well as abundance and diversity of the gene *nosZ* which encodes the denitrifying enzyme nitrous oxide reductase (Philippot et al., 2007).

2. Material and methods

2.1. Soil description

The four different soils derived from Germany, the Czech Republic and Burkina Faso and were managed as arable land and pasture, respectively. All soil characteristics are given in Table 1. Soil samples were taken in four replicates and were immediately sieved trough a 2 mm mesh, homogenized and frozen at −80 °C for molecular investigations.

2.2. Nucleic acid extraction and purification

2.2.1. Extraction with kits from MP Biomedicals

DNA and RNA were extracted from 0.5 g soil using the FastDNA® SPIN Kit for Soil and the FastRNA® SPIN Kit for Soil from MP Biomedicals (France), respectively, according to the manufacturers' protocols. Bead beating was performed on the Precellys®24 Homogenizer (Bertin Technologies, France). Extracts were stored at −80 °C until use.

2.2.2. Extraction according to Griffiths et al. (2000)

DNA and RNA from soil samples were co-extracted by phenol-chloroform extraction and subsequently enzymatically digested according to Griffiths et al. (2000). Briefly, 0.5 g of moist soil were extracted in Lysing Matrix E tubes (MP Biomedicals, France) with 0.5 ml of hexadecyltrimethylammoniabromide (CTAB) extraction buffer and 0.5 ml of phenol–chloroform–isoamyl alcohol (25:24:1) (pH 8). The samples were lysed for 30 s using the Precellys®24 Homogenizer and centrifuged at 16100 ×*g* for 5 min at 4 °C. The aqueous layer was removed and mixed with an equal amount of chloroform–isoamyl alcohol (24:1). After centrifugation (5 min, 4 °C), 2 volumes of the precipitation solution containing 30% polyethylene glycol (PEG) 6000 and 1.6 M NaCl were added to the aqueous phase, incubated for 2 h on ice for nucleic acid precipitation, and then centrifuged for 10 min at 4 °C. The nucleic acid pellet was washed in ice-cold 70% ethanol, again centrifuged for 10 min at 4 °C, air dried and re-suspended in 50 ml of pure and nuclease-free water. Afterwards, the extract was divided in two aliquots to obtain pure DNA and RNA, respectively. For pure DNA, 700 ng of the extracted nucleic acids were incubated at 37 °C for 10 min with RNase A (Sigma, Germany) at a final concentration of 100 μg ml^{-1}. Pure RNA was obtained by treating 250 ng of the extracted nucleic acids with DNase (Applied Biosystems, Germany) as recommended by the manufacturer. Afterwards, the digestions were purified with the QIAquick PCR Purification Kit (Qiagen, Germany) and stored at −80 °C until further use.

2.2.3. Extraction according to the improved PCS protocol

The PCS protocol basically rested upon the protocol by Griffiths et al. (2000) but was significantly modified in different steps as specified in the following: (i) The CTAB extraction buffer additionally contained 10 μl ß-mercaptoethanol ml^{-1} to inactivate RNases and DNases, which could be released after cell disruption. (ii) The chloroform extraction step was performed twice to completely remove phenol residues. (iii) Only one volume of the precipitation solution containing however 10% PEG 8000 and 1.2 M NaCl was added to the aqueous phase and incubated on ice for 2 h (according to Arbeli and Fuentes, 2007). (iv) The total DNA/RNA extract was used to separate DNA from RNA by the AllPrep DNA/RNA Mini Kit (Qiagen, Germany) without any further digestions, purification steps or sample divisions. To obtain pure DNA and RNA a maximum of 20 μg of the DNA/RNA mixture could be added to the silica columns.

2.3. Purity and yield of nucleic acids

2.3.1. RNA purity

Contamination of RNA samples with co-extracted DNA was excluded by PCR assays targeting the 16S rRNA genes using the universal primers 986f and 1401r (Nübel et al., 1996). The PCRs resulted in no amplicons for all samples except the $Cambisol_{pasture}$ soil being extracted with the RNA extraction kit. After a reduction of the soil to 0.2 g for extraction weak bands of PCR products were still observed in a few samples. For further analyses these samples were examined critically.

2.3.2. Amount of DNA and RNA

DNA and RNA quantities were determined by ultrasensitive fluorescent nucleic acid staining using the Quant-iT PicoGreen Kit and the Quant-iT RiboGreen Kit (Invitrogen, Germany), respectively, with detection ranges of 0.025–1 ng μl^{-1} for DNA and 0.001–0.05 ng μl^{-1} for RNA. The determined nucleic acid concentrations were used to calculate the DNA/RNA content g^{-1} dry soil. The purity of the DNA and RNA extracts was measured spectrometrically via absorbance at 320 nm (NanoDrop Technologies, USA) (Miller, 2001).

2.4. cDNA synthesis

The cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Germany) in a total volume of 40 μl and was afterwards purified with the QIAquick PCR Purification Kit (Qiagen, Germany).

2.5. Quantitative real-time PCR (qPCR) assay

For the real-time PCR quantification of the single copy gene *nosZ* primers from Henry et al. (2006) were used. The chemical composition and the amplification were conducted as recently published (Töwe et al., 2010). To confirm the specificity of the amplicons after each PCR run a melting curve and a 2% agarose gel were conducted. The amplification efficiencies were calculated by $Eff = [10^{(-1/slope)} - 1] * 100\%$ and resulted in 86.2% (±5.1). The r^2 of the regression amounted to more than 0.99 and the y-intercept value accounted for 35.8 (±2.7).

2.6. T-RFLP assay

Diversity analysis by T-RFLP was also performed targeting the *nosZ* gene. For amplification primer pairs and PCR profiles were performed

Table 1
Soil properties.

Soil type	Land use	Location	Coordinates	% Clay	% Silt	% Sand	% C	% N	pH
Luvisol	Arable	Germany, Munich	48°29′ N, 11°26′ E	15.3	44.6	40.1	1.4	0.13	6.1
$Cambisol_{arable}$	Arable	Germany, Munich	48°29′ N, 11°25′ E	10.2	22.7	67.1	1.1	0.1	5.9
Lixisol	Arable	Burkina Faso, Saria	12°16′ N, 2°9′ W	9.3	–	59.7	0.22	0.015	5.6
$Cambisol_{pasture}$	Pasture	Czech Republic, Budweis	48°52′ N, 14°13′ E	6–14	14–32	60–80	10.6	0.9	8.96

as described for q-PCR, but the forward primer was labeled with 5′-FAM (6-carboxyfluorescein). The PCR products were first purified with the QIAquick PCR Purification Kit (Qiagen, Germany) and afterwards digested using the restriction enzyme *HpyCH4V* (New England Biolabs, Germany) according to the manufacturer's protocol. The restriction enzyme was selected based on *in silico* T-RFLPs using the program REPK (Restriction Endonuclease Picker) (Collins and Rocap, 2007). Digested amplicons (50 ng) were desalted and purified by the MinElute Reaction Cleanup Kit (Qiagen, Germany). One µl was then mixed with 13 µl of Hi-Di formamide (Applied Biosystems, Germany) containing a 400-fold dilution of a 6-carboxy-X-rhodamine-labeled MapMarker 1000 ladder (Bio-Ventures, USA.), denatured (3 min at 95 °C), cooled on ice, and size-separated on a 3730 DNA analyzer (Applied Biosystems, Germany). Electrophoresis was performed with POP-7 polymer in a 50 cm capillary array under the following conditions: 10 s injection time, 2 kV injection voltage, 7 kV run voltage, 66 °C run temperature, and 63 min analysis time. Electropherograms were analyzed using the GeneMapper 3.5 software package (Applied Biosystems, Germany).

2.7. Statistics

Gene abundance data were analyzed by one way ANOVA (SPSS 11.5). Prior to analysis data were tested for normal distribution by Q–Q plots and the Kolmogorov–Smirnoff test. Homogeneity of variances was checked by the Levene test. If the requirements were met pairwise comparisons were conducted by Tukey's test. If homogeneity of the variances was not fulfilled a Games–Howell test was applied. The T-RFLP data set was normalized to percent of the total peak height of a sample (Anderson, 2003) excluding peaks smaller than 0.5%. A permutation-based multivariate analysis of variance was conducted with the PerMANOVA software (Anderson, 2001; McArdle and Anderson, 2001) using an experimental design consisting of the three different treatments (three levels) for each soil with the null hypothesis "no difference between soils" and a significance level of 0.05 ($n=4$ replicates per soil). The different treatments were defined as orthogonal and fixed. For each analysis term, 4999 random permutations of the raw data were conducted to obtain p values (Manly, 1997). Euclidean distance was used as distance measure (Anderson, 2005).

3. Results

3.1. Nucleic acid content and purity

DNA and RNA concentrations measured in the purified extracts were converted to amounts related to g^{-1} dry soil and ranged from 0.02 to 159 µg DNA g^{-1} soil for DNA (Fig. 1A). While the two co-extraction based protocols (Griffiths and PCS) revealed no differences in yield, significantly higher values were obtained with the DNA extraction kit for the Luvisol and $Cambisol_{arable}$. RNA contents ranged from 0.2 to 83 µg RNA g^{-1} soil (Fig. 1B). Significant differences between the extraction methods were only observed for the $Cambisol_{pasture}$ with the highest value for the PCS protocol and lowest for the RNA extraction kit.

Considering the purity of DNA and RNA, significantly (up to 100 times) higher absorption values were obtained for all soil samples extracted with the kits indicating major contaminations with humic substances (Fig. S1; supplemental material).

3.2. Abundance of nosZ genes and transcripts

DNA and RNA extraction efficiencies of the different protocols were evaluated and compared by quantification of *nosZ* genes and transcripts using qPCR. The *nosZ* gene and transcript abundance levels were related to g^{-1} soil (Fig. 2A) as well as to ng^{-1} DNA (Fig. S2). Overall, the gene copy numbers ranged from 3×10^5 (Lixisol, PCS) to 3×10^9 copies g^{-1} soil ($Cambisol_{pasture}$, Griffiths) and reflected approximately the patterns of extracted DNA g^{-1} soil (Fig. 1A). The abundance of *nosZ* genes within one soil differed in part significantly between the protocols, but without favoring a certain protocol. When gene copy numbers were related to ng^{-1} DNA significantly higher gene copies were achieved with the PCS method compared to the DNA extraction kit for the Luvisol and $Cambisol_{arable}$. Highest abundance levels were measured in all soils being extracted according to the Griffiths protocol, but due to high standard deviations, the differences were not significant.

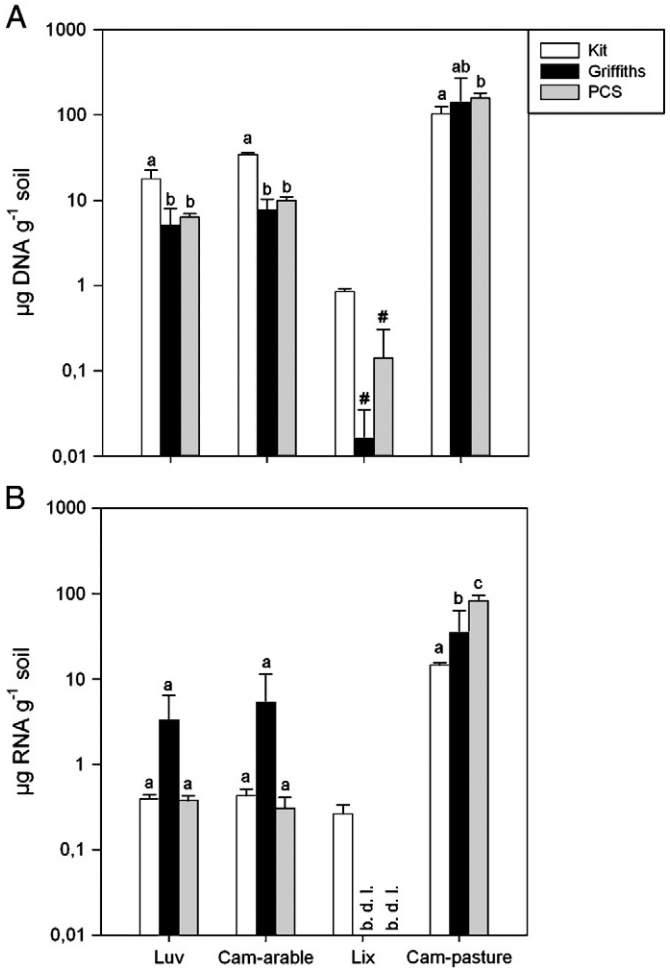

Fig. 1. DNA (A) and RNA (B) contents g^{-1} soil are displayed ($n=4$, error bars represent standard deviations). Significant differences between the three extraction protocols are shown by different letters and were checked by oneway ANOVA. "b.d.l." indicates that all replicates were below the detection limit of 0.001 ng μl^{-1}. #The mean is only composed of two replicates as the DNA concentration was below the detection limit for the remaining samples. Thus, no statistic could be performed for this soil.

For the copy numbers of *nosZ* transcripts, larger differences between the methods were observed compared to *nosZ* genes (Fig. 2B, S2; supplemental material). The use of the Griffiths protocol resulted in higher copy numbers related to g^{-1} soil and ng^{-1} DNA, respectively, but again the differences were not significant due to huge standard deviations.

3.3. Diversity analysis of the extracted DNA and RNA

In order to test whether the different nucleic acid extraction protocols select for different microbial phylotypes, we performed T-RFLP analyses of *nosZ* genes and transcripts. The results are presented in Figs. 3 and 4 with only T-RFs contributing to more than 5% to the total community. Results of the respective Principal Component Analysis (PCA) are shown in Figs. S3 and S4 (supplemental material).

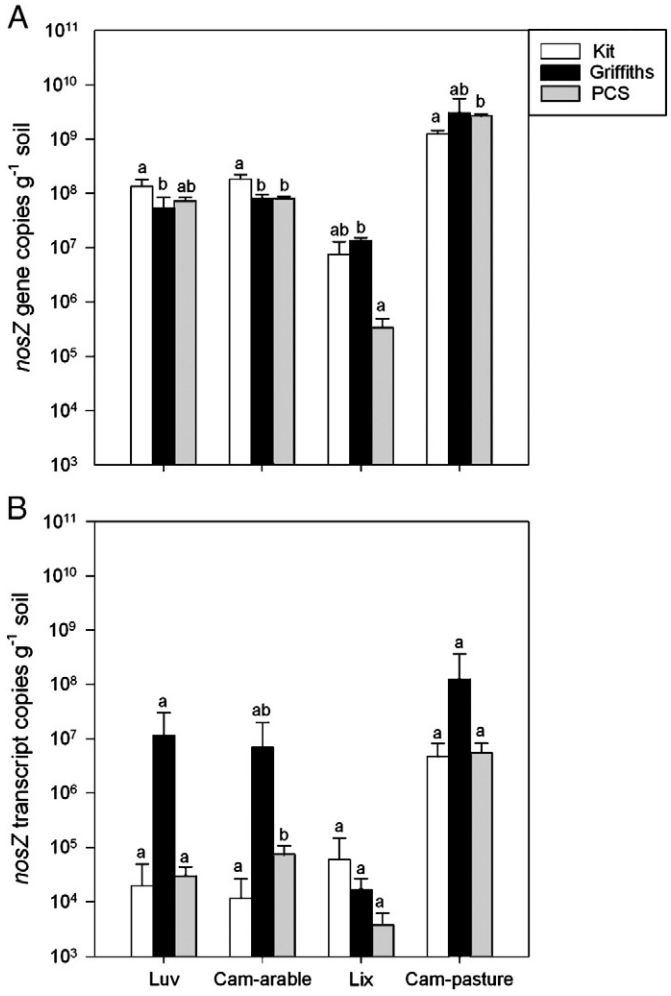

Fig. 2. Copy numbers of the *nosZ* gene per g dry soil are displayed for DNA (A) and cDNA (B) samples ($n=4$, error bars represent standard deviations). Significant differences between the three extraction protocols are shown by different letters and were checked by oneway ANOVA.

Data revealed significantly different communities when comparing the DNA extraction kit and the PCS-method for all investigated soils except the Luvisol (Fig. 3). The number of dominant (>5%) T-RFs obtained by the PCS protocol always exceeded those by the kit, while the Griffiths protocol ranged in between. Only for the Lixisol highest numbers of dominant T-RFs were found in those samples being extracted according to the Griffiths protocol. The Principal Component Analysis revealed a strong clustering of the communities according to the soil type, with the Lixisol being again the only exception.

In all soils the active bacterial communities markedly differed from the overall community exhibiting different and fewer dominant T-RFs (Fig. 4). Moreover, almost for all combinations of soils and extraction protocols the number of total T-RFs was higher for the *nosZ* transcripts (15–37) than genes (10–22). Both, the lowest (Lixisol) and highest ($\text{Cambisol}_{\text{pasture}}$) number of total T-RFs of *nosZ* transcripts were obtained using the Griffiths protocol. With exception of the Lixisol, the different protocols led to in part significantly different communities within a soil, however without any clear pattern. The PCA analysis revealed a clear separation of the communities received with the PCS protocol for the arable soils from Munich.

4. Discussion

In the last decades many studies focused on the improvement of single steps in nucleic acid extraction like cell lysis, homogenization, precipitation and purification from soils and sediments (Arbeli and Fuentes, 2007; Chandler et al., 1999; de Boer et al., 2010; Inceoglu et al., 2010; Miller et al., 1999). However, only few studies targeted the simultaneous extraction of DNA and RNA from one and the same sample (Duarte et al., 1998; Griffiths et al., 2000; Hurt et al., 2001; Yu and Mohn, 1999). Beside the extraction steps, a crucial point in these protocols is the accurate performance of DNA and RNA separation. The presented PCS protocol allows the complete separation of DNA and RNA in one step. In contrast, many protocols are based on a subdivision of the extract and a subsequent digestion (Griffiths et al., 2000), meaning that a part of the extracted DNA and RNA, respectively, is enzymatically degraded. Alternatively, previous protocols included a separation with resin columns (Hurt et al., 2001), but an additional digestion step was still necessary.

To proof the reliability of newly derived protocols many studies refer to 16S rRNA-based community analysis or gene quantification methods (Krsek and Wellington, 1999; Sagova-Mareckova et al., 2008; Schneegurt et al., 2003; Wang et al., 2009). However, 16S rRNA operon numbers can differ considerably depending on the bacterial strain (Klappenbach et al., 2000), which might be unfavorable for quantitative comparisons. In this study we used instead the widely spread, functional single copy gene *nosZ* (Kandeler et al., 2006) that is known to be congruent with 16S rRNA-based taxonomic classification (Jones et al., 2008).

Extraction and quantification of DNA and RNA in our study were generally possible for all soils with all protocols except the Lixisol, where the reproducibility was only given for the DNA and RNA extraction kit, respectively. Zhou et al. (1996) found out that the carbon content is often positively related to the DNA yield. In our survey, the Lixisol revealed ten times lower carbon and nitrogen contents compared to the other soils, which might have been a reason that the amount of the extractable nucleic acids was remarkably low (Fig. 1A). Nevertheless, it was possible to analyze the gene abundance and diversity of the nitrous oxide reductase gene *nosZ* even in the Lixisol. In contrast, the $\text{Cambisol}_{\text{pasture}}$ was characterized by high microbial biomass and carbon content, which led to RNA being contaminated with DNA when using the RNA kit-based extraction protocol. Furthermore, the obtained RNA yields and *nosZ* transcript copy numbers were similar or lower compared to the other methods. In contrast the PCS protocol enabled a complete separation of DNA and RNA even in soils with high microbial biomass, provided that the loading capacity of the silica columns was not exceeded. Interestingly, replicates extracted with the PCS protocol always revealed comparable RNA yields and *nosZ* transcript copy numbers, whereas samples extracted with the Griffiths protocol resulted in high variations between replicates. This might reflect the drawback of the sample processing with the Griffiths protocol, where repeated purification and sample division result in high standard deviations.

In most cases maximum DNA yields and *nosZ* gene abundance values could be detected within samples that were extracted with the kit-based protocol; however the amount of co-extracted humic substances (revealed by spectrophotometry) was high. This contamination problem frequently arises due to similar physicochemical properties of nucleic acids and humic acids, which bind competitively to the same absorption sites when using the minicolumns for purification (Harry et al., 1999; Lear et al., 2010). However, it was possible to overcome the inhibition by serial dilutions of the DNA extracts.

Regarding the diversity pattern, we could obtain T-RFLP profiles for all soils with all protocols, even if several repetitions of the PCR were necessary for the Lixisol to get sufficient amounts of PCR product due to the overall low nucleic acid yield. Between 10 and 22 *nosZ* T-RFs were obtained for DNA samples, which is in line with previous studies (Enwall and Hallin, 2009; Perryman et al., 2008); for cDNA the numbers ranged between 15 and 37 *nosZ* T-RFs. Interestingly,

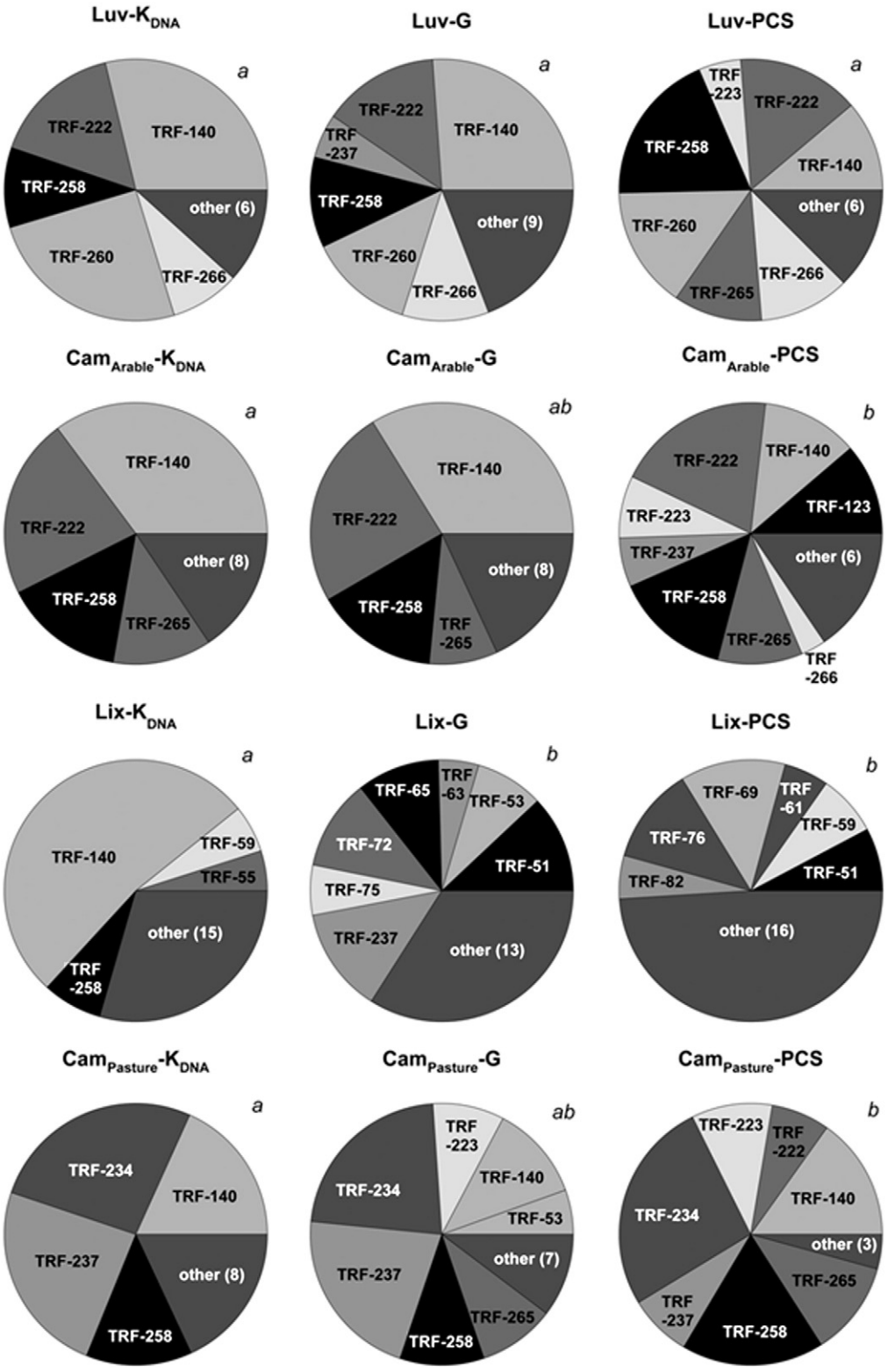

Fig. 3. Contribution of dominant T-RFs to total *nosZ* gene fragment diversity in the DNA samples. T-RFs which contributed to less than 5% were summarized as "other". Significant differences are indicated by different letters ($n=4$).

different dominant T-RFs appeared in the active community compared to the potential community involved in N_2O reduction. The phenomenon that the most dominant microbes are not necessarily the most active ones is corroborated by a study from Bürgmann et al. (2005), who compared the potential and active diazotrophic community. Anyway, no clear preference for one protocol could be revealed by diversity analysis neither for DNA nor cDNA samples. Depending on soil type and extraction protocol, in part significant differences in diversity pattern were revealed. These observations reflect the contradictory opinions about the influence of the extraction method on the community composition (Carrigg et al., 2007; Inceoglu et al., 2010). Moreover, the quite scattered DNA-based

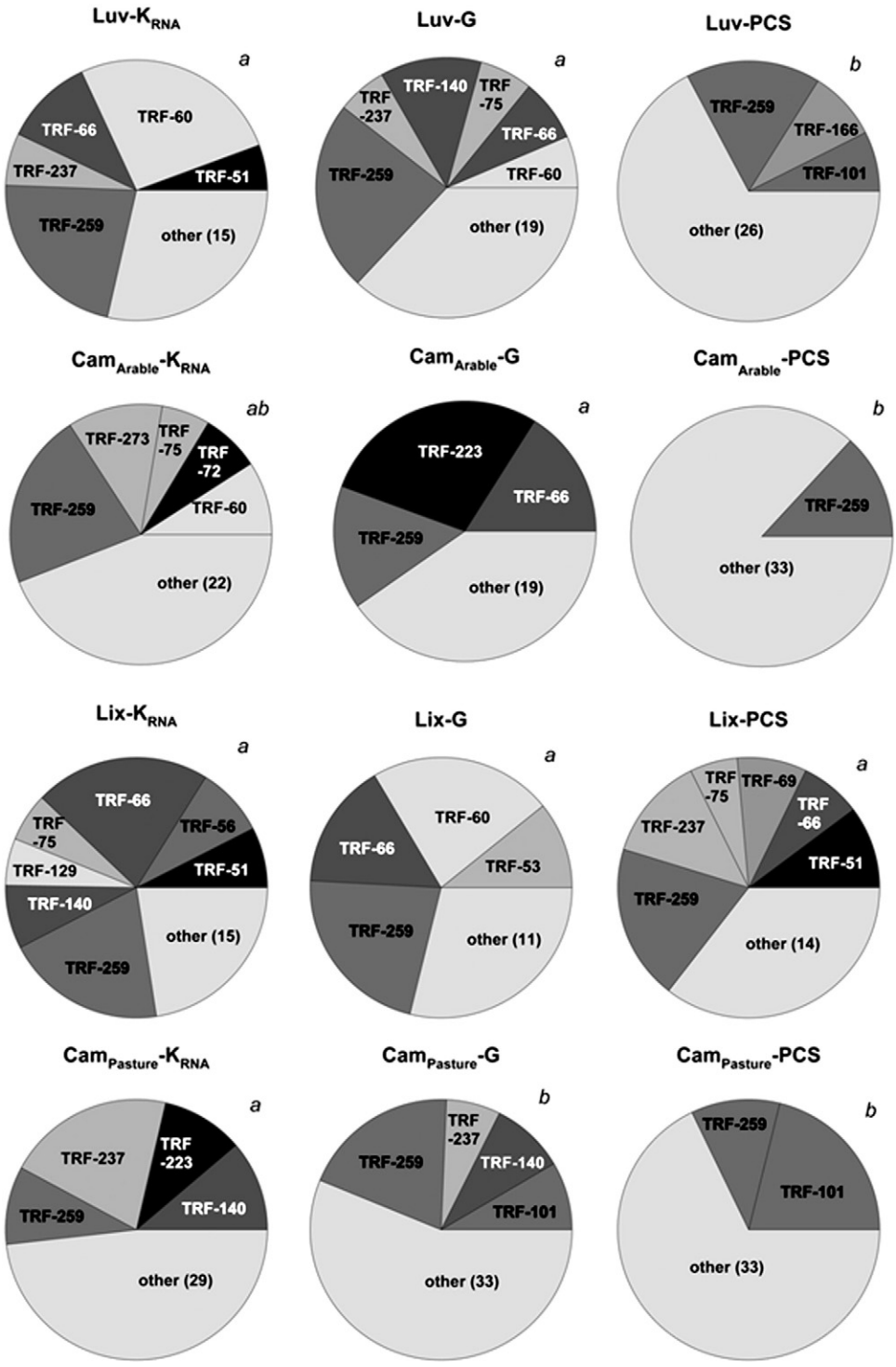

Fig. 4. Contribution of dominant T-RFs to total transcribed *nosZ* gene fragment diversity (cDNA samples). T-RFs which contributed to less than 5% were summarized as "other". Significant differences are indicated by different letters.

diversity pattern of the Lixisol (Fig. S3), underlines the challenge to reproducibly extract DNA or RNA from low biomass environments (Barton et al., 2006).

The results clearly demonstrated that on DNA level quantification and diversity analyses of a functional gene were possible with all protocols. While the RNA extraction kit and the PCS method revealed comparable results for cDNA, samples obtained from the Griffiths protocol were characterized by high standard deviations. However, extraction using the kits had two disadvantages: (i) Two independent extractions for DNA and RNA have to be performed and (ii) the extracts seem to be more contaminated with humic substances. In contrast, it was possible to extract DNA and RNA simultaneously from one and the same sample and to completely separate the DNA and RNA in one additional step with the improved PCS protocol. However, further investigations and adaptations are needed for the reliable nucleic acid extraction of extreme soils, e. g., with low microbial biomass or high clay content. Generally, it is important to stick to one protocol during sample processing and to introduce as less working steps as possible to reduce the method-specific biases especially for quantitative analysis.

Acknowledgement

We thank the German Research Foundation (DFG) for funding. Furthermore, the authors gratefully acknowledge the support of the TUM Graduate School's Faculty Graduate Center Weihenstephan at Technische Universität München, Germany.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.mimet.2010.12.028.

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Supplemental Material

Figure S1. Detection of humic substances at A_{320nm} (n = 4, error bars represent standard deviations). Significant differences between the three extraction protocols are displayed by different letters and were checked by oneway ANOVA.

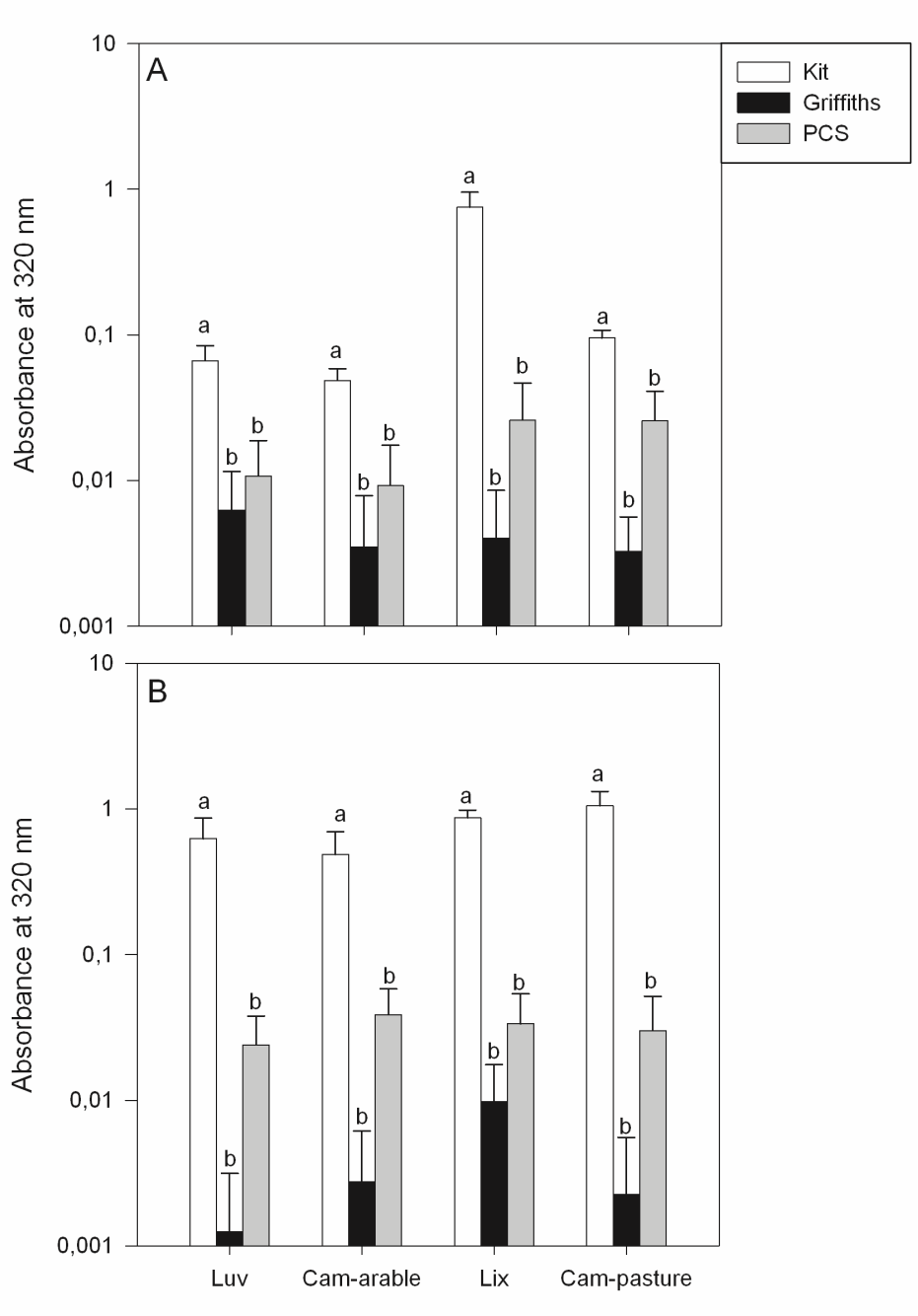
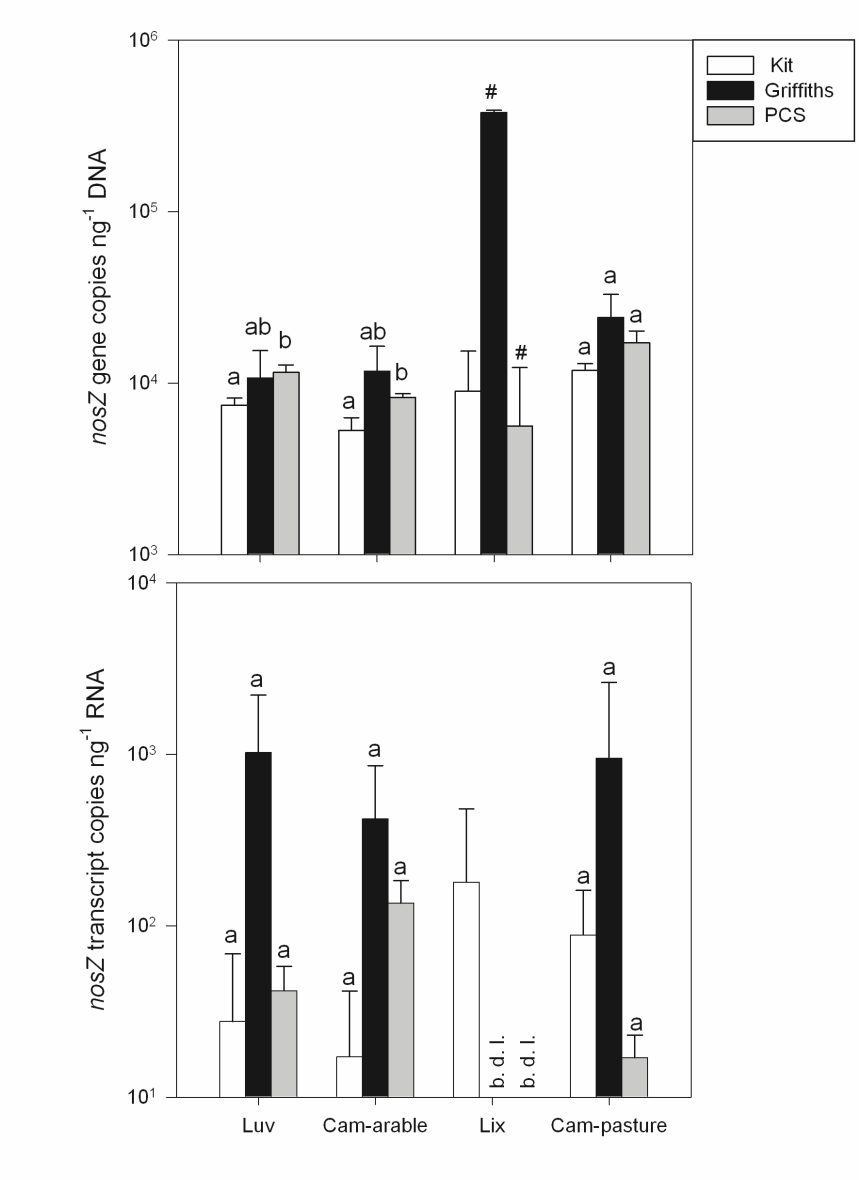


Figure S2. Copy numbers of the *nosZ* gene per ng DNA are displayed (n = 4, error bars represent standard deviations). Significant differences between the three extraction protocols are shown by different letters and were checked by oneway ANOVA. "b.d.l." indicates samples where the RNA concentration was below the detection limit of 0.001 ng μl^{-1} (see Figure 1).



[#] The mean is only composed of two replicates as the DNA concentration below the detection limit for the remaining samples. Thus no statistic was performed for this soil.

Figure S3. Principal Component Analysis of the T-RFLP dataset for *nosZ* gene fragments in the DNA samples. The ordination plots of the first two principal components (PC) show mean values of the replicates per extraction method. The four soils are displayed by four different symbols and each of the three similar symbols per soil stands for an extraction method

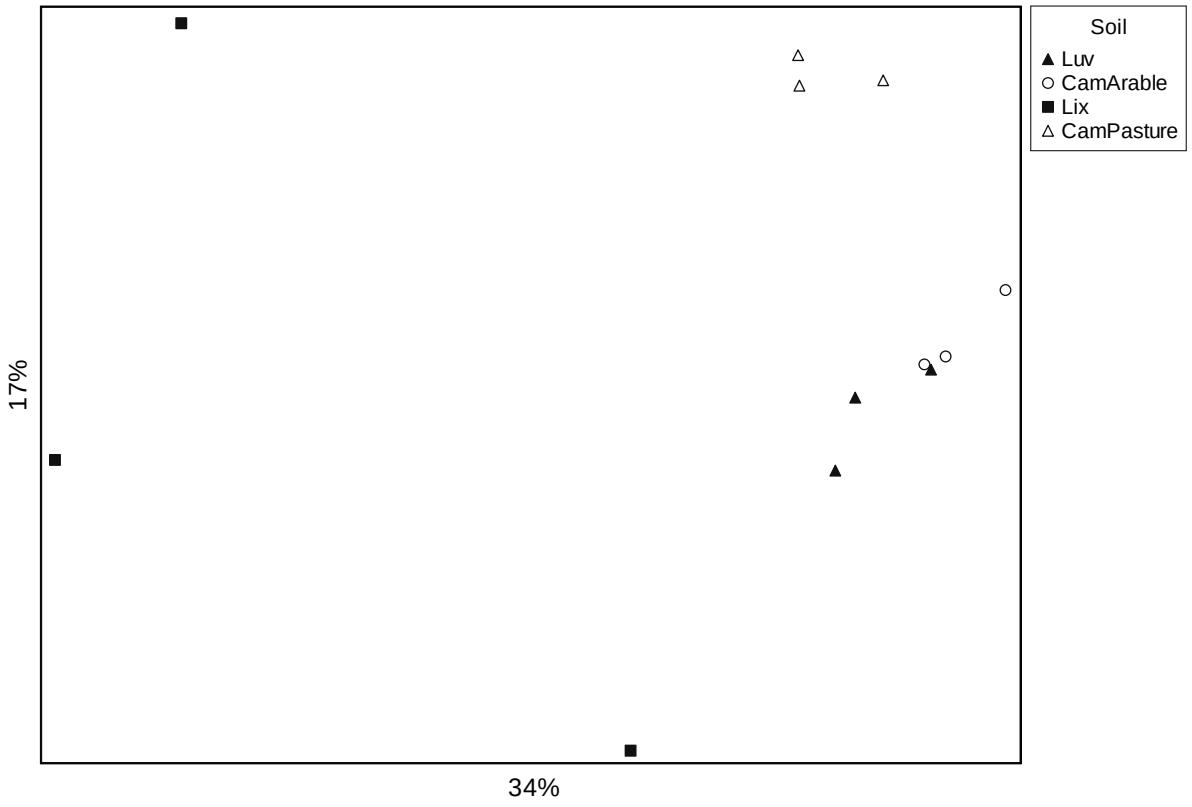
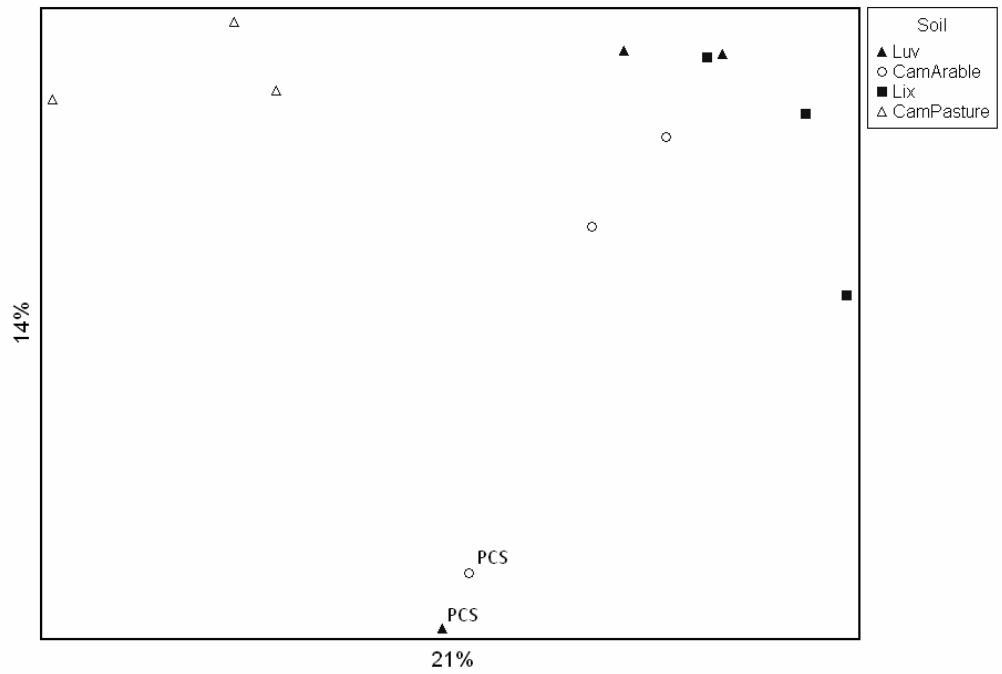


Figure S4. Principal Component Analysis of the T-RFLP dataset for *nosZ* gene fragments in the cDNA samples. The ordination plots of the first two principal components (PC) show mean values of the replicates per extraction method. The four soils are displayed by four different symbols and each of the three similar symbols per soil stands for an extraction method



Publication II

Journal of Microbiological Methods 82 (2010) 338–341

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Note

Differences in amplification efficiency of standard curves in quantitative real-time PCR assays and consequences for gene quantification in environmental samples

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ARTICLE INFO

Article history:
Received 18 June 2010
Received in revised form 4 July 2010
Accepted 4 July 2010
Available online 15 July 2010

Keywords:
Quantitative real-time PCR
Amplification efficiency
Standard curve
LinRegPCR
Nitrogen cycle
Functional genes

ABSTRACT

High and comparable efficiency values are the key for reliable quantification of target genes from environmental samples using real-time PCR. Therefore it was the aim of this study to investigate if PCR amplification efficiencies of plasmid DNA used for the calculation of standard curves (i) remain constant along a logarithmic scale of dilutions and (ii) if these values are comparable to those of DNA extracted from environmental samples. It could be shown that comparable efficiency values within the standards cannot be achieved using log scale serial dilutions and a comparison of gene copy numbers from DNA extracted from environmental samples and standard DNA extracted from plasmids is only possible in a very small interval.

In the last decade quantitative real-time PCR (qPCR) has become a powerful tool in microbial ecology as this approach allowed the quantification of different microbial phyla (Fierer et al., 2005; Ochsenreiter et al., 2003) or selected functional genes (Huic Babic et al., 2008; Mrkonjic Fuka et al., 2008; Schulz et al., 2010) in different environments for the first time. In many cases this knowledge improved our understanding about selected ecosystem services and helped to develop strategies for sustainable land use.

All qPCR methods rely on the measurement of the amplification process in real time after each PCR cycle by using fluorescent reporter molecules (Morrison et al., 1998; Wittwer et al., 1997). The increase in fluorescence intensity of serially diluted plasmids which carry the target gene can be used to generate a standard curve estimating the initial amount of the template molecules in the samples of interest. While it is common practice to adapt the range of standard curves to the respective sample concentration for example when enzyme assays are performed (Cullings et al., 2008; Kreyling et al., 2008; Tabatabai and Bremner, 1969), it is generally accepted to work with logarithmic dilutions covering up to 6 orders of magnitude in qPCR analysis (Hai et al., 2009; Love et al., 2006; Mrkonjic Fuka et al., 2008; Wallenstein and Vilgalys, 2005). This is in fact surprising, as it is well known that only an optimal composition of all PCR components in appropriate proportions to template DNA results in reliable PCR efficiencies (Zhang and Fang, 2006), which are crucial for the analysis of qPCR data (Ramakers et al., 2003; Tichopad et al., 2003). Usually, to obtain efficiency values for PCR amplification, a linear regression line is calculated between the Ct values and the logarithmized concentration of the plasmid DNA, using

$$\mathrm{Eff}_{\mathrm{slope}} = \left[10^{(-1/\mathrm{slope})} - 1\right] \quad (1)$$

for slope calculation. Consequently, this efficiency value (= $\mathrm{efficiency}_{\mathrm{slope}}$) expresses quality of the standard dilution only. Recent studies, however, focused on the development of algorithms estimating PCR efficiencies in each PCR reaction. The LinRegPCR program (Ramakers et al., 2003; Ruijter et al., 2009), e.g., enables the calculation of individual amplification efficiencies per single sample, standard dilution or environmental sample, within a qPCR assay. It determines the log-linear part of the amplification curve in each sample by selecting a lower and upper limit, then performs a linear regression analysis to calculate the efficiency from the regression line that fits best to the log-linear part and results in efficiency values between 1.0 and 2.0 representing 0 and 100% efficiency, respectively.

Using this approach it is now possible to investigate if the PCR amplification efficiencies of plasmid DNA used for the calculation of standard curves (i) remain constant along a logarithmic scale of dilutions and (ii) if these values are comparable to those of DNA extracted from environmental samples. For this purpose qPCR assays with DNA extracted from soil were used. As an example four different functional genes of the microbial nitrogen cycle – the nitrogenase

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0167-7012/$ – see front matter
doi:10.1016/j.mimet.2010.07.005

(*nifH*), the bacterial ammonia monooxygenase (*amoA*), the nitrite reductase (*nirK*) and the nitrous oxide reductase (*nosZ*) – were quantified.

DNA from soil samples was extracted using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Irvine CA) from bulk soil and rhizosphere samples (Töwe et al., 2010) according to the manufactures guidelines. Quantitative real-time PCR was conducted on a 7300 Real-Time PCR System (Applied Biosystems, Germany) using SybrGreen as fluorescent dye. Details on the reaction mixtures used for qPCR as well as qPCR conditions are given in Table 1. DNA samples extracted from soil were diluted 1:64 to overcome inhibitory effects of co-extracted humic acids (data of preexperiments). Standard curves were analyzed in 9 replicates. The reliability of the standard curves and the compliance of the MIQE guidelines (Bustin et al., 2009) were controlled by verifying reproducibility of the y-intercept values, the quality of the dilutions series using the Eq. (1) and ensuring that the r^2 was higher than 0.98 (Table 2). Moreover, the amplification efficiencies from each individual PCR reaction in all qPCR assays were calculated by means of the LinRegPCR program (http://linregpcr.nl/) to compare efficiencies between different dilutions of plasmid DNA within one standard curve and between standards and environmental samples, respectively. Significant differences in amplification efficiencies between the respective dilutions within one standard curve were tested by one way ANOVA, whereas differences between standard dilutions and environmental samples were checked by *t*-test (SPSS 11.5, $p<0.05$).

The results revealed inhomogeneous amplification efficiencies of the different dilution steps of plasmid DNA within the respective qPCR standard curves (Fig. 1). Standard dilutions containing more than 10^3 copies of the target gene μl^{-1} showed significantly lower efficiencies (*nifH* and *nosZ*) and high standard deviations (*nosZ* and *nirK*), respectively. For all analyzed genes except the *nifH* gene, highest amplification efficiencies were detected for the most diluted standards. The *nifH*-based standard curve exhibited, in contrast a stepwise increase of the amplification efficiency from 10^6 to 10^4 copies μl^{-1} and reached a plateau with efficiencies for 10^3–10^1 copies μl^{-1}.

The comparison of amplification efficiencies between dilutions of the plasmid DNA used as standards and environmental samples revealed similar efficiencies in case of comparable copy numbers (Fig. 1) only. This observation could point to clearly altered and suboptimal proportions of PCR chemicals to template concentration in the higher standards (Zhang and Fang, 2006). The qPCR assays are typically optimized for the template amount present in environmental samples and thus, a surplus of template might exist in the higher standards. In case of the *amoA* gene, PCR efficiencies of the standards were always significantly lower compared to the environmental samples. Many studies revealed a niche separation of ammonium-oxidizing bacteria (Carney et al., 2004; Moin et al., 2009) where *Nitrosopira* species are able to outcompete *Nitrosomonas* species in ammonium-limited habitats (Taylor and Bottomley, 2006). As the environmental samples in this evaluation have been derived from a nutrient-poor glacier forefield, *Nitrosospira* might have been the dominant species. Thus a discrepancy between the cloned *amoA* gene fragment of *Nitrosomonas* as standard and the environmental samples could have been existed, which might have been further corroborated by different melting temperatures of the PCR products (data not shown).

Table 2
Amplification efficiencies and y-intercept values calculated as customary via the slope of the linear regression line ($Eff=[10^{(-1/slope)}-1]$) of the respective standard curves with cloned *nifH*, *amoA* AOB, *nirK* and *nosZ* gene fragments in 1:10 serial dilutions and with the cloned *nifH* gene fragment in 1:4 serial dilutions, respectively ($n=9$, standard deviation in parentheses).

	1:10				1:4
	nifH	*amoA* AOB	*nirK*	*nosZ*	*nifH*
$Efficiency_{slope}$	1.806 (0.02)	1.878 (0.016)	1.804 (0.014)	1.886 (0.03)	1.911 (0.025)
y-Intercept	37.4 (0.4)	37.5 (0.7)	38.8 (0.8)	33 (0.8)	39.4 (0.3)

Already Cochran (1950) showed for the "most probable number" method that a 1:10 dilution results in a noticeable standard error, whereas the error was negligible for a 1:5 and even more for a 1:2 serial dilution. However, in microbial ecology it is not always possible to use small scale standard curves, because environmental samples can cover a broad range of gene copies. Moreover, it has been recommended to apply standard curves covering several orders of magnitude for absolute gene quantification by qPCR (Livak, 2001). Thus, we analyzed if a 1:4 serial dilution covering four orders of magnitude resulted in more homogeneous amplification efficiencies than the common 1:10 serial dilution (Fig. 2). We used the *nifH* gene as an example, as it showed the broadest range of amplification efficiencies in the 1:10 dilutions. Statistical evaluations revealed a constant average amplification efficiency of 1.78 for the 1:4 dilution series between 10^1 and 10^4 copies μl^{-1}. In contrast, the dilution containing 10^5 copies μl^{-1} exhibited significantly lower amplification efficiency (1.74), underlining the assumption that the amplification efficiency decreased with increasing template amounts from a certain concentration. Surprisingly, the small scale dilution series entailed lower mean amplification efficiencies than the logarithmic dilution. However, considerably higher standard deviations were observed in the 1:10 dilution series (Cochran, 1950) and general intra-assay variances as demonstrated by Smith et al. (2006) might have contributed to this observation. Nevertheless, most consistent amplification efficiencies within a standard curve were obtained with the 1:4 dilution series.

In summary, we showed that, although the requirements of the MIQE guidelines (Bustin et al., 2009) have been fulfilled for all evaluated assays, efficiencies of 1:10 serially diluted standard curves of plasmid DNA containing the target gene were only comparable to those of DNA from environmental samples within a small range. Thus, we recommend checking routinely the amplification efficiencies calculated for each single PCR reaction and to modify the standard curve to a 1:4 or 1:5 serial dilution covering maximal 4 orders of magnitude, if possible. Furthermore, it seems at least reasonable to

Table 1
Primer and thermal profiles used for real-time PCR quantification of different functional genes. PCR reaction mixture consisted of Power SybrGreen Master Mix (12.5 µl, Applied Biosystems, Germany), BSA (0.5 µl, Sigma, Germany), template DNA (2 µl) as well as primers (Metabion, Germany) as referred in the table.

Target gene	Source of standard	Thermal profile	No. of cycles	Primer	Reference	Primer (10 µM)
nifH	*Sinorhizobium meliloti* DSM 30136	95 °C-45 s/55 °C-45 s/72 °C-45 s	40	nifHF, nifHR	Rösch et al. (2002)	0.5
amoA AOB	*Nitrosomonas* sp. (Pinck et al., 2001)	94 °C-45 s/60 °C-45 s/72 °C-45 s	40	amoA1F, amoA2R	Rotthauwe et al. (1997)	0.75
nirK	*Azospirillum irakense* DSM 11586	95 °C-15 s/63 °C-30 s/72 °C-30 s 95 °C-15 s/58 °C-30 s/72 °C-30 s	5[a] 40	nirK876, nirK5R	Braker et al. (1998), Henry et al. (2004)	0.5
nosZ	*Pseudomonas fluorescens* C7R12 (Henry et al., 2006)	95 °C-30 s/65 °C-30 s/72 °C-30 s 95 °C-15 s/60 °C-15 s/72 °C-30 s	5[a] 40	nosZ2F, nosZ2R	Henry et al. (2006)	0.5

[a] Touchdown: −1 °C cycle^{-1}.

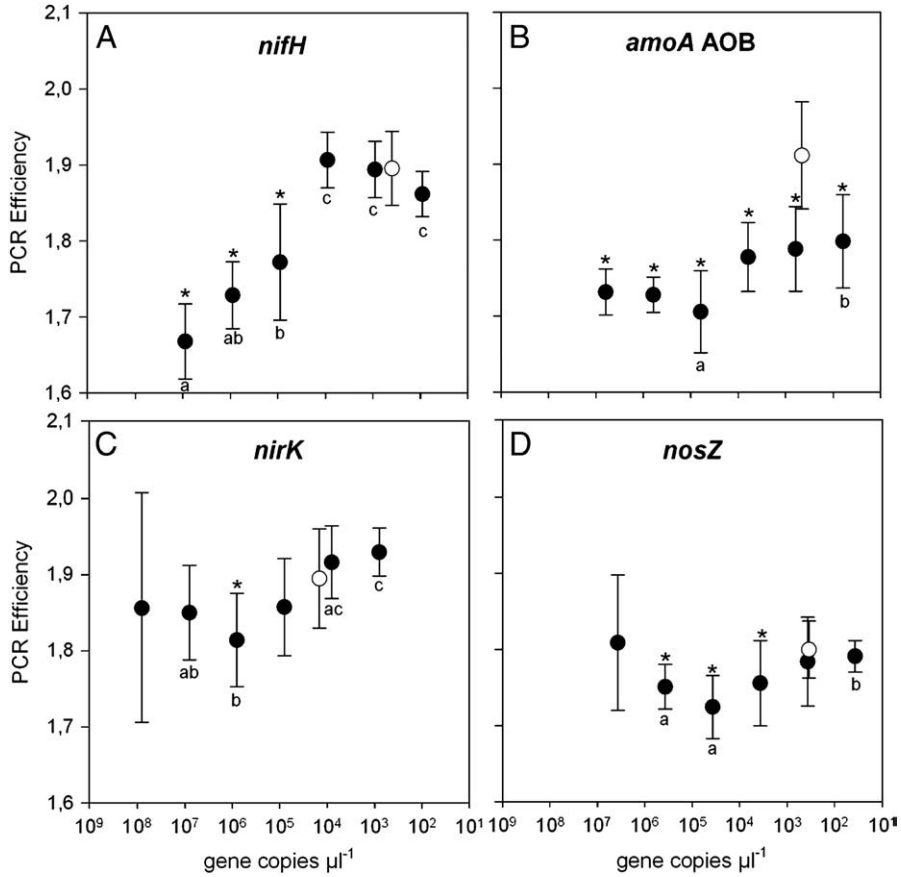

Fig. 1. Copy numbers μl^{-1} are plotted against PCR efficiencies of standards and environmental samples, calculated with the LinRegPCR program. Black circles display efficiency means of the different standard dilution steps from 10^6 to 10^1 copies μl^{-1} ($n=9$ for each dilution) of the investigated functional genes *nifH*, *amoA* AOB and *nosZ* and 10^7 to 10^2 copies μl^{-1} of *nirK*. White circles show efficiency means of the respective gene in all environmental samples ($n=115$, stdev. of Ct 3.7% (*nifH*); $n=108$ stdev. of Ct 6.7% (*amoA* AOB); $n=117$ stdev. of Ct 3.9% (*nosZ*); $n=113$ stdev. of Ct 3.6% (*nirK*)). Error bars represent standard deviations of the PCR efficiencies. Asterisks indicate significant differences in mean PCR efficiencies between a respective standard dilution and the environmental samples as revealed by *t*-test ($p<0.05$). Different lower case letters show significant differences ($p<0.05$) between the dilution steps within a standard curve exhibited by one way ANOVA.

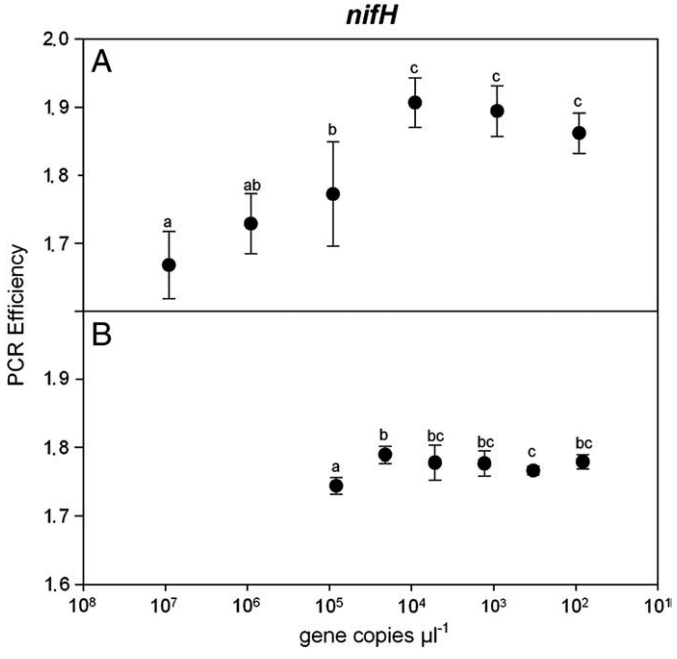

Fig. 2. Comparison of a 1:10 (A) and a 1:4 (B) dilution series of the same *nifH* standard. Different lower case letters indicate significant differences of PCR efficiencies between the dilution steps as revealed by one way ANOVA ($p<0.05$, $n=9$, error bars represent standard deviations).

use the same standard clones or even better the same dilution series for several qPCR assays targeting the same gene in order to obtain similar amplification efficiency pattern of the standard curves.

Acknowledgements

This study is part of the Transregional Collaborative Research Centre 38 (SFB/TRR38) which is financially supported by the Deutsche Forschungsgemeinschaft (DFG, Bonn) and the Brandenburg Ministry of Science, Research and Culture (MWFK, Potsdam).

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Publication III

The ISME Journal (2011) 5, 1025–1037

www.nature.com/ismej

ORIGINAL ARTICLE

Abundances and potential activities of nitrogen cycling microbial communities along a chronosequence of a glacier forefield

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Glacier forefields are ideal ecosystems to study the development of nutrient cycles as well as single turnover processes during soil development. In this study, we examined the ecology of the microbial nitrogen (N) cycle in bulk soil samples from a chronosequence of the Damma glacier, Switzerland. Major processes of the N cycle were reconstructed on the genetic as well as the potential enzyme activity level at sites of the chronosequence that have been ice-free for 10, 50, 70, 120 and 2000 years. In our study, we focused on N fixation, mineralization (chitinolysis and proteolysis), nitrification and denitrification. Our results suggest that mineralization, mainly the decomposition of deposited organic material, was the main driver for N turnover in initial soils, that is, ice-free for 10 years. Transient soils being ice-free for 50 and 70 years were characterized by a high abundance of N fixing microorganisms. In developed soils, ice-free for 120 and 2000 years, significant rates of nitrification and denitrification were measured. Surprisingly, copy numbers of the respective functional genes encoding the corresponding enzymes were already high in the initial phase of soil development. This clearly indicates that the genetic potential is not the driver for certain functional traits in the initial phase of soil formation but rather a well-balanced expression of the respective genes coding for selected functions.

The ISME Journal (2011) **5,** 1025–1037; doi:10.1038/ismej.2010.184; published online 2 December 2010
Subject Category: geomicrobiology and microbial contributions to geochemical cycles
Keywords: real-time PCR; nitrogen cycle; functional genes; soil development; chronosequence

Introduction

Since the end of the Little Ice Age around 1850 many alpine glaciers have retreated (Maisch, 2000), exposing new terrain for soil formation. Consequently, glacier forefields represent chronosequences of different soil development stages offering an ideal system to study the development of functional microbial communities in soil. Initial stages of the glacier chronosequences are characterized by low plant diversity and abundance (Chapin *et al.*, 1994; Tscherko *et al.*, 2005; Hammerli *et al.*, 2007), which increase over time and reach their maxima at sites being ice-free for more than 200 years. Consequently, the amount of available carbon (C) and nitrogen (N) is low during initial ecosystem development. Sigler and Zeyer (2002) reported highest bacterial richness at initial soils (ice-free for 2–10 years). This observation is in agreement with studies reporting highest diversity of functional groups, such as nitrate reducers and N-fixing microbes. (Deiglmayr *et al.*, 2006; Duc *et al.*, 2009; Schmalenberger and Noll, 2009). Moreover, initial sites are often dominated by microbes belonging to the *r*-strategists being able to respond quickly to changing environmental conditions, whereas with continuing succession a shift to *k*-strategists occurs that rather pursue the strategy of maintenance (Sigler and Zeyer, 2002). In contrast to these studies, some reports found no significant correlation between soil age and diversity (Edwards *et al.*, 2006) or increasing diversity (Tscherko *et al.*, 2003; Nemergut *et al.*, 2007).

In the last decades, different studies at glacier forefields focused on the dynamic of selected processes and transformation steps of the N cycle (Deiglmayr *et al.*, 2006; Kandeler *et al.*, 2006; Duc

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Received 9 August 2010; revised 25 October 2010; accepted 25 October 2010; published online 2 December 2010

et al., 2009; Towe *et al.*, 2010a) because N as a macronutrient is essential for ecosystem development, but most bedrocks do not contain any N.

Although many authors have postulated the importance of N fixation at initial stages of soil development (Kohls *et al.*, 1994; Schmidt *et al.*, 2008), the role of mineralization is discussed controversially. For example, Tscherko *et al.* (2003) found a pronounced acceleration of N mineralization only after 50 years of soil development, paralleling plant succession as well as organic-matter assemblage. This is in agreement with the common assumption that autotrophic organisms like cyanobacteria, algae, mosses and lichens appear first (Walker and del Moral, 2003; Schmidt *et al.*, 2008), followed by heterotrophic organisms. However, Bardgett *et al.* (2007) found evidence for a significant mineralization activity also at the initial phases of soil development, which was related to the degradation of ancient and recalcitrant soil organic matter.

Similarly, the development of denitrification over time remained vague. On the one hand, high gene abundance (copies per nanogram DNA) of *nirS* (nitrite reductase) and *narG* (nitrate reductase) were observed in pioneer soils (Kandeler *et al.*, 2006); on the other hand, the nitrate reductase activity peaked in developed soils (Deiglmayr *et al.*, 2006).

Besides some contradicting data, all these studies focused on a single N transformation step only, but did not consider consequences for other N cycle processes. A complete reconstruction of the microbial N cycle on the genetic as well as potential activity level at different soil development stages is still missing. Therefore, it was the aim of this study to reconstruct the microbial N cycle from bulk soil samples taken from sites that were ice-free for 10 to 2000a and thus represent different soil development stages. The study is restricted to bulk soil, because the initial sites of the Damma glacier forefield are dominated by bare substrate and are only sparsely vegetated.

We postulate that, although high gene copy numbers might occur at the initial sites, nitrification and denitrification activities parallel the succession of plants, whereas mineralization and N fixation are already important at the beginning of the ecosystem development. To test this hypothesis abundance pattern of genes encoding for subunits of enzymes involved in N fixation (*nifH*), proteolysis and chitinolysis (*aprA* and *chiA*) as proxy for N mineralization, nitrification (*amoA* of ammonia oxidizing bacteria and archaea) and denitrification (*nirS, nirK* and *nosZ*) were measured. Gene abundance data was linked to the corresponding potential enzymatic activities and available C and N in soil. In addition, microbial biomass was determined.

Materials and methods

Sampling site

Sampling took place along the forefield of the Damma glacier (46°38′20″N and 8°28′00″E) in canton Uri, Switzerland (Figure 1; Edwards *et al.*, 2006). The forefield is characterized by gneiss as bedrock and silty sandy soil texture (Lazzaro *et al.*, 2009). It has a northeast exposition (Bernasconi, 2008) and an inclination of 25% (Sigler *et al.*, 2002). Two terminal moraines dating from 1992 and 1928, as well as two lateral moraines dating from 1850, characterize the forefield (Figure 1). Mean annual air temperature at the forefield is 0–5 °C; average temperature during summer season is 6–10 °C (Climap, MeteoSchweiz, http://www.meteoswiss.admin.ch/). An annual precipitation of 2400 mm was measured at the forefield (Bernasconi, 2008).

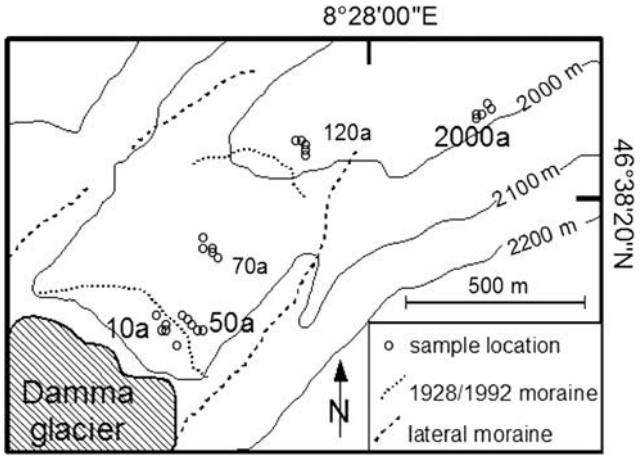

Figure 1 Site map illustrating the Damma glacier forefield. Circles mark the sampling locations. Five circles representing the soil samples of the same developmental stage are labeled with the corresponding sample code: 10, 50, 70, 120 and 2000a.

Chemical and physical soil parameters changed along the forefield. Soil pH values decreased from pH 5.1 in soils being ice-free for 10 years (a) to pH 4.6 in soils being ice-free for 120a and reached a minimum of pH 4.1 outside the forefield, where soils were ice-free for more than 2000a. On the contrary, water-holding capacity slightly increased from 26% (10a) to 33% (2000a). Plant coverage significantly changed from $<10\%$ at site 10 to $>70\%$ and 95% at sites 50 and 70a, respectively. Sites 120 and 2000a were densely covered with plants (Hans Goransson, ETH Zurich, personal communication). Dominant plant species were *Leucanthemopsis alpina, Agrostis gigantea, Rumex scutatus, Salix sp.* and *Lotus alpinus* (Hans Goransson, ETH Zurich, personal communication).

Nitrogen deposition

N depositions in alpine ecosystems of central Switzerland were modeled in different studies (Table 1). Input of total N is in the range between 10–15 kg N ha^{-1} a^{-1}. To estimate the input of organically bound N and C to the forefield of the Damma glacier, we sampled snow patches at the end of the winter season (<1 m snow depth) in the forefields

Table 1 Nitrogen deposition at alpine ecosystems in central Switzerland

Type of deposition	*Area, source of data*	*Deposition (kg N ha^{-1} a^{-1}) or (kg C ha^{-1} a^{-1})*			
		Dry N	*Wet NH_4^+-N*	*Wet NO_3^--N*	*Total N*
Dry and wet deposition within a year (models)	Central Switzerland[a]	—	—	—	10–15
	Central Switzerland[b]	2.2	4.5	3.5	—
	Central Switzerland[c]	—	5–7.5	2.5–3.5	—
Particulate deposition on snow (experimental)		*Particulate N*[d]		*Particulate C*[d]	
	Forefield Damma glacier, this study	0.63 (0.18)		7.5 (1.5)	
	Forefield Stein glacier, this study	0.77 (0.08)		11.8 (0.4)	
	Forefield Tiefen glacier, this study	0.24 (0.05)		4.0 (0.7)	

[a]Ferderal Commission for Air Hygiene (2005).
[b]National Air Pollution Monitoring Network (NABEL) (2008).
[c]Nyiri *et al.* (2009).
[d]Values indicate average of five replicates, s.e.m. in brackets.

of the Damma glacier and two adjacent glaciers: Tiefen glacier and Stein glacier. At the end of May 2009, five replicates of surface snow per sampling site (0.04 m^2) were collected, melted in the field and filtered through 2.7 μm glass fiber filters (Whatman GF/D, Opfikon, Switzerland). Filters were dried, ground in a bead mill and analyzed for C and N content on a microanalyzer (Leco CHNS-932, St Josephs, MI, USA). Average values were used to estimate the annual aerial input of particulate organic matter (Table 1).

Sampling procedure
Sampling took place at the Damma Glacier forefield during the growing season on 15th July 2008. Samples were taken in proximity to an already established and characterized sampling grid, representing sites that have been free of ice for 10–2000a (for details see Bernasconi, 2008 and http://www.cces.ethz.ch/projects/clench/BigLink/). In detail, four differently developed soils were sampled along the chronosequence. As the retreat of the glacier was monitored by the Swiss Glacier Monitoring Network (http://glaciology.ethz.ch/swiss-glaciers/), the sites chosen for sampling can be attributed to ice-free times of 10, 50, 70 and 120a (for details see Duc *et al.*, 2009). The distance from the glacier terminus was 100 m for the 10a site and 650 m for the 120a site. In addition, a grassland soil, adjacent to the glacier forefield, was sampled as reference for a developed soil (Figure 1). This site is approximately 500 m away from the 120a site and was not glaciated for more than 2000a (Stefano Bernasconi, ETH Zurich, personal communication). Bulk soil samples from 0 to 2 cm depth were collected in five independent replicates from each site and treated separately. Each of the replicate samples consisted of five subsamples taken within an area of 1 m^2, avoiding plant roots. The distance between the sampling areas for each replicate was 5–10 m. In the field, the soil was sieved (2 mm) and kept on ice for chemical analyses and enzyme assays (Schutte *et al.*, 2009); soil for molecular analysis was stored on dry ice.

Soil carbon and nitrogen content
Fresh soil and fumigated soil (see below) was extracted in 0.01 M calcium chloride solution by 45 min horizontal shaking. Soil-to-liquid ratio was 1:2 for the 10–120a samples and 1:3 for the 2000a samples. The following parameters were measured for each soil filtrate (Whatman 595 $\frac{1}{2}$, Dassel, Germany): dissolved organic carbon and nitrogen on DIMA-TOC 100 (Dima Tec, Langenhagen, Germany), nitrate (NO_3^-) using Spectroquant Nitrate-Test kit (Merck, Darmstadt, Germany) and ammonium (NH_4^+) using Nanocolor kit (Macherey-Nagel, Düren, Germany). Microbial carbon (C_{mic}) and microbial nitrogen (N_{mic}) were determined by the fumigation-extraction method (Vance *et al.*, 1987; Joergensen, 1996). Total organic carbon of air-dried ground soil was determined on a CN-analyzer (Leco CNS2000, St Josephs, MI, USA). Total organic carbon was measured as total C, as the soil contains no carbonates. Total nitrogen was determined by the persulfate oxidation method as described for soil extracts (Cabrera and Beare, 1993). Approximately 200 mg (2000a) to 500 mg (10a) of ground soil was autoclaved (60 min at 121 °C) in 10 ml of oxidizing reagent. Total nitrogen was then measured as nitrate by ion chromatography (Dionex DX-320, IonPac AS11-HC column, Sunnyvale, CA, USA). All analyses for soil C and N content were performed in technical triplicates.

Enzyme assays
To measure the potential N fixation activity in soil, the protocol by Tsunogai *et al.* (2008) was modified. Briefly, 2 g of fresh soil was weighed into a 20 ml serum bottle. The headspace was flushed with

helium for 15 min (Helium 5.0, Pangas, Switzerland) and 1 ml $^{15}N_2$ gas (98%+, Cambridge Isotope Laboratories, Andover, MA, USA) was added. After 2 weeks of incubation at 12 °C in the dark, 10 ml of oxidizing reagent (Tsunogai *et al.*, 2008) and 0.5 g (samples 10, 50 and 70a) or 1.0 g (samples 120 and 2000a) of low-N potassium persulfate (Fluka 60489, Buchs, Switzerland) was added to convert total N to nitrate. Then, the serum bottles were autoclaved (Tsunogai *et al.*, 2008). After freeze-drying, 30–50 mg of the nitrate-containing sulfate salts were analyzed on a Thermo Fisher Scientific FlashEA (Waltham, MA, USA) coupled with a ConFlo IV (Thermo Fisher Scientific) interface to a Delta V IRMS system (Thermo Fisher Scientific). The instrument was calibrated with the international standards IAEA N1, IAEA N2 and IAEA N3. Samples only flushed with helium served as control. All samples were analyzed in technical quintuplicates.

As proxy for N mineralization activity, chitinase and protease potential activity were measured. Chitinase and protease catalyze the initial breakdown of the two most abundant natural macromolecules that contain N, chitin and protein. Chitinase and protease potential activity were determined using a slightly modified protocol by Hendel and Marxsen (2005). The modifications were: 100–200 mg fresh soil was incubated in 2 ml Eppendorf tubes, containing 50 mM fluorescence substrate in 1.6 ml sterilized stream water from the forefield. As fluorogenic substrates, L-Leucine 7-amido-4-methyl coumarin hydrochloride (Fluka 61888, Buchs, Switzerland) for protease activity and 4-Methylumbelliferyl *N*-acetyl-β-D-glucosaminide (Sigma M2133, Buchs, Switzerland) for chitinase activity were used. All samples were incubated at 12 °C for 6 h in the dark on a rotating shaker and analyzed in triplicate. Fluorescence was determined from 200 µl aliquots in 96-well microtiter plates and compared with 4-Methylumbelliferone (MUF) standards (Aldrich M1381, Buchs, Switzerland). An incubation temperature of 12 °C was chosen, because it is the approximately average soil temperature during summer season (unpublished results).

Potential nitrification activity was performed as a microtiter plate assay following the method described by Hoffmann *et al.* (2007). Briefly, 2.5 g soil was incubated in 10 ml 1 mM ammonium sulfate solution amended with 50 µl of 1.5 M sodium chlorate solution. Samples were incubated for 5 h in triplicates and reaction was stopped by adding 2.5 ml of a 2 M KCl solution. Nitrite concentrations of non-incubated samples served as controls. Nitrite measurements were performed colorimetrically on a Spectramax 340 (Molecular Devices, Ismaning, Germany) (Hoffmann *et al.*, 2007).

Potential denitrification activity was determined as reported by Luo *et al.* (1996). Three replicates of 10 g fresh soil were saturated with 0.1 mM glucose and nitrate solution. The headspace of the serum bottle was flushed with helium and 10% acetylene was added. N_2O was analyzed by gas chromatography on Shimadzu GC-14B (Düsseldorf, Germany). The soil was incubated at 20 °C and headspace samples were taken after 3 and 6 h. Preliminary experiments showed that in all soils considered, the N_2O production was a linear function of time from 0 to 6 h (data not shown). It was also found that in these assays the denitrification rates were identical in the presence and absence of chloramphenicol (data not shown).

DNA Extraction and quantification

DNA was extracted from thawed soil using Fast DNA SPIN Kit for Soil (MP Biomedicals, Illkirch, France). In the final step, DNA was eluted two times from the DNA binding column, incubating the column with 30 µl water (supplied) for 5 min. To ensure quantitative DNA extraction, preceding tests stipulated to extract ~500 mg of initial and transient soils (10, 50 and 70a) and ~250 mg of developed soil (120 and 2000a). Then there was a linear relationship between DNA yield and amount of soil extracted. DNA yields were quantified using Sybr green I (Invitrogen, Basel, Switzerland) assay as described by Matsui *et al.* (2004).

Real-time PCR

Sybr Green-based quantification assays using Power Sybr Green (Applied Biosystems, Darmstadt, Germany) and Kapa Sybr Fast (Kapa Biosystems, LabGene, Chatel-St-Denis, Switzerland) PCR master mixes were run on a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Reaction volumes were 25 µl containing onefold PCR master mix. PCR conditions, efficiencies, primers and calibration standards used are summarized in Table 2. Preceding tests with dilution series of the extracted DNA showed no inhibitory effects at a 1:16 dilution. Each PCR run included triplicate sample templates, calibration standard series and no template controls.

Statistical analysis

One-way analysis of varience (ANOVA) on ranks was performed using SigmaPlot 11.0 package (Systat Software Inc., San Jose, CA, USA). Before regression analysis, potential enzymatic activities and copy numbers were log-transformed according to $\log_{10}(x+c)$, where c is the 2.5% quartile of the measured parameter.

Results

Soil parameters along the chronosequence

Measurements of the C and N pools indicate that soil-nutrient status changed substantially along the glacier chronosequence (Table 3). All C and N pools increased by one order of magnitude from

Table 2 Reaction composition, thermal profiles, sources of calibration standards and primer references of qPCR reactions used in this study

Target gene	*Reaction conditions*								*Source of calibration standard*	*Primer name and reference*
	F- and R-primer (pmol µl^{-1})	*BSA (µg µl^{-1})*	*DMSO (µg µl^{-1})*	*Denaturation time at 95 °C (s)*	*Annealing time and temperature*	*Elongation time at 72 °C (s)*	*Number of cycles*	*qPCR efficiency (%)*[a]		
nifH[b]	0.2	0.2	0	15	25 s at 53 °C[c]	45	43	88–90	*Paenibacillus azotofixans* DSM 5976	nifH-F, nifH-R, Rosch *et al.*, 2002
ChiA[b]	0.2	0.1	0	10	90 s at 65 °C	30	40	95–96	*Streptomyces coelicolor* DSM 41189	chif2, chir, Xiao *et al.*, 2005
Apr[b]	0.2	0.2	0	10	20 s at 54 °C	30	42	72–74	*Pseudomonas fluorescens* DSM 50090	FR apr I, RP apr II, Bach *et al.*, 2001
nirK[d]	0.2	0.6	25	15	30 s at 58 °C[e]	30	45	83–88	*Azospirillum irakense* DSM 11586	nirK 876, Henry *et al.*, 2004, nirK 5R, Braker *et al.*, 1998
nirS[d]	0.2	0.6	25	45	45 s at 57 °C	45	40	88–95	*Pseudomonas stutzeri*	nirS cd3af, Michotey *et al.*, 2000, nirSR3cd Throback *et al.*, 2004
nosZ[d]	0.2	0.6	0	15	30 s at 60 °C[f]	30	45	92–95	*Pseudomonas fluorescens* C7R12[g]	nosZ2F, nosZ2R, Henry *et al.*, 2006
amoA[d] (AOA)	0.2	0.6	0	45	45 s at 55 °C	45	40	85–87	Fosmid clone 54d9[h]	19F, Leininger *et al.*, 2006, CrenamoA616r48x, Schauss *et al.*, 2009
amoA[d] (AOB)	0.3	0.6	0	45	45 s at 60 °C	45	40	78–79	*Nitrosomonas* sp.	amoA 1F, amoa 2R, Rotthauwe *et al.*, 1997

Abbreviation: AOB, ammonia-oxidizing bacteria.
[a]Efficiency was calculated after Babic *et al.* (2008).
[b]Amplified using Kapa SYBR Fast (Kapa Biosystems) master mix.
[c]Touch down starting at 63 °C temperature decrease of 2 °C per cycle.
[d]Amplified using Power SYBR Green (Applied Biosystems) master mix.
[e]Touch down starting at 63 °C temperature decrease of 1 °C per cycle.
[f]Touch down starting at 65 °C temperature decrease of 1 °C per cycle.
[g]Eparvier *et al.* (1991).
[h]Treusch *et al.* (2005).

10 to 2000a. Total organic C increased from 0.7 to 40 mg C g^{-1} Similarly, microbial carbon increased from 58 to 902 µg C g^{-1} and was significantly correlated to the amount of DNA that was extracted from the soil ($n=25$, $R^2=0.53$, $P<0.001$). Cell counts and abundance of *rpoB* gene copy numbers were less well-correlated to microbial carbon ($n=25$, $R^2=0.31$, $P=0.004$ and $n=25$, $R^2=0.11$, $P=0.099$; respectively) (unpublished data). Total N and microbial N contents were one order of magnitude lower than C contents. The resulting C/N ratios ranged from 12 to 18, whereby samples 50 and 70a displayed the highest C/N ratio. Nitrate concentration was five times higher in soils from the 10a site (0.13 µg N g^{-1}) compared with ammonium; however, the ammonium content increased in proportion to the nitrate concentration, reaching one order of magnitude higher concentrations (12.8 µg N g^{-1}) at site 2000a.

Potential enzyme activities

Potential enzyme activity was related to soil dry weight as well as the amount of extracted DNA to obtain a biomass-independent parameter (Figure 2). Generally, potential enzyme activities related to dry soil increased along the chronosequence. N-fixation activity was below the detection limit in samples 10–70a (detection limit 0.2 pmol N h^{-1} g^{-1}) but ranged between 1 and 3 pmol N h^{-1} g^{-1} in samples 120 and 2000a. Relative N-fixation activity was similar in samples 120 and 2000a. As proxy for potential mineralization activity, protease and chitinase assays were performed. Significant increases of protease activity from 5 to 25 nmol MUF h^{-1} g^{-1} as well as of chitinase activity from 14 to 72 nmol MUF h^{-1} g^{-1} were observed along the chronosequence. At the same time, relative mineralization activity tended to decrease, however, the trend was not significant. Potential nitrification activity increased significantly from 0.1 (10a) to 2.3 nmol NO_2^--N h^{-1} g^{-1} (2000a). The same pattern was observed for potential denitrification activity, where activities increased from 1.8 to 40 nmol N_2O-N h^{-1} g^{-1} The relative nitrification and denitrification activities resulted in similar pattern but did not reach significant levels in most cases.

Abundance of functional genes

Gene copy numbers were related to gram soil as well as nanogram DNA (Figure 3). Abundance of *nifH* gene was lowest in sample 10a (2×10^6 copies per gram soil) and increased up to 2×10^7 copies per

Table 3 Carbon, nitrogen and DNA content of the soil. Numbers give the average of five replicates, in brackets standard error of means

	Site				
	10a	*50a*	*70a*	*120a*	*2000a*
Carbon ($\mu g\,C\,g^{-1}$)					
TOC ($\times 10^3$)[a]	0.7 (0.2)	4.7 (1.2)	3.0 (0.5)	12.0 (4.2)	39.6 (12.4)
DOC[b]	7.1 (2.3)	18.2 (8.3)	6.6 (1.2)	42.4 (14.3)	77.8 (16.7)
C_{mic}[c]	58 (11)	122 (26)	73 (7)	241 (71)	902 (215)
Nitrogen ($\mu g\,N\,g^{-1}$)					
TN ($\times 10^3$)[d]	0.07 (0.02)	0.25 (0.05)	0.17 (0.03)	0.73 (0.24)	2.67 (0.83)
DON[e]	0.3 (0.1)	0.9 (0.2)	0.6 (0.1)	3.0 (2.3)	12.2 (6.1)
Ammonium	0.03 (0.01)	0.18 (0.12)	0.15 (0.08)	6.69 (5.21)	12.81 (6.32)
Nitrate	0.13 (0.05)	0.23 (0.08)	0.18 (0.08)	1.31 (0.32)	0.81 (0.10)
N_{mic}[f]	6 (1)	19 (5)	13 (2)	29 (8)	115 (28)
C/N ratio[g]	12.8 (3.1)	18.0 (1.2)	18.5 (1.6)	15.9 (1.0)	15.0 (0.6)
Extracted DNA ($\mu g\,g^{-1}$)	2.3 (0.8)	12.0 (2.9)	7.8 (2.2)	17.4 (4.6)	28.0 (8.5)

[a]Total organic carbon.
[b]Dissolved organic carbon.
[c]Microbial carbon.
[d]Total nitrogen.
[e]Dissolved organic nitrogen.
[f]Microbial nitrogen.
[g]Ratio of TOC to TN.

gram soil in soil samples from 50a. In the soil samples from sites that have been ice-free for a longer period *nifH* gene abundance decreased. Similar results were obtained when data were related to nanogram extracted DNA.

Abundance of genes coding for enzymes involved in mineralization significantly increased (Figure 3): *aprA* genes from 6×10^6 to 4×10^7; *chiA* genes from 7×10^5 to 9×10^6 copies per gram soil. In contrast, relative abundances (related to nanogram of DNA) of both genes involved in mineralization were constant (Figure 3b) at all sites.

Abundance of the nitrification marker gene *amoA* was studied for ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB). Although *amoA* AOB gene abundances showed no significant differences related to gram of soil, AOA significantly increased from 3×10^4 to 8×10^5 copies per gram soil. Generally, AOB abundances were up to 80-times higher compared with AOA at the 10, 50, 70 and 120a site, whereas the AOB to AOA ratios were close to two in soil samples 2000a. Relative *amoA* AOA gene abundances remained stable, whereas relative *amoA* AOB gene abundances significantly decreased from 1.4×10^3 to 7.2×10^2 copies per nanogram DNA along the chronosequence.

To quantify the denitrifying bacteria, genes coding for the nitrite reductases (*nirK* and *nirS*) and nitrous oxide reductase (*nosZ*) were measured. Generally, *nirK* gene abundances were two orders of magnitude higher than *nirS*. Gene abundances of *nirK* decreased from 1.5×10^8 to 3×10^7 copies per gram soil along the chronosequence. The *nosZ* gene copy numbers were lowest in sample 10a with 9×10^5 copies per gram soil but equaled *nirK* gene abundance at site 2000a. Relative gene abundances showed comparable patterns for denitrification genes except *nirS*, which decreased along the chronosequence.

Correlation of abundances and activities

Correlations between gene abundance pattern (copies per gram soil) and potential enzyme activities (per gram soil) were assessed. In order to reduce variability and to include all available data points, both data sets were log-transformed before regression analysis (Figure 4). Although *nifH*, *amoA* AOB, *nirS* and *nirK* gene abundances did not correlate with the corresponding potential enzyme activities, significant correlations with the potential enzymatic activities were found for *chiA*, *aprA*, *amoA* AOA and *nosZ* gene copy numbers significantly correlated with the potential enzyme activities ($\alpha = 0.1$).

Discussion

The initial state

Initial ecosystems are characterized by low nutrient content and scarce vegetation (Sigler and Zeyer, 2002; Tscherko *et al.*, 2004; Yoshitake *et al.*, 2007). For the Damma glacier it was shown that the initial sites (10a) have less than 10% vegetation cover, whereas the developed sites (120–2000a) have a closed plant cover (Hammerli *et al.*, 2007; Hans Goransson, personal communication). Thus, the input of organic material by root exudates or plant litter might be low at the initial sites of the glacier forefield. Hence, N fixation and autotrophic CO_2 incorporation may have a crucial role for ecosystem development (Kohls *et al.*, 1994). Interestingly, our

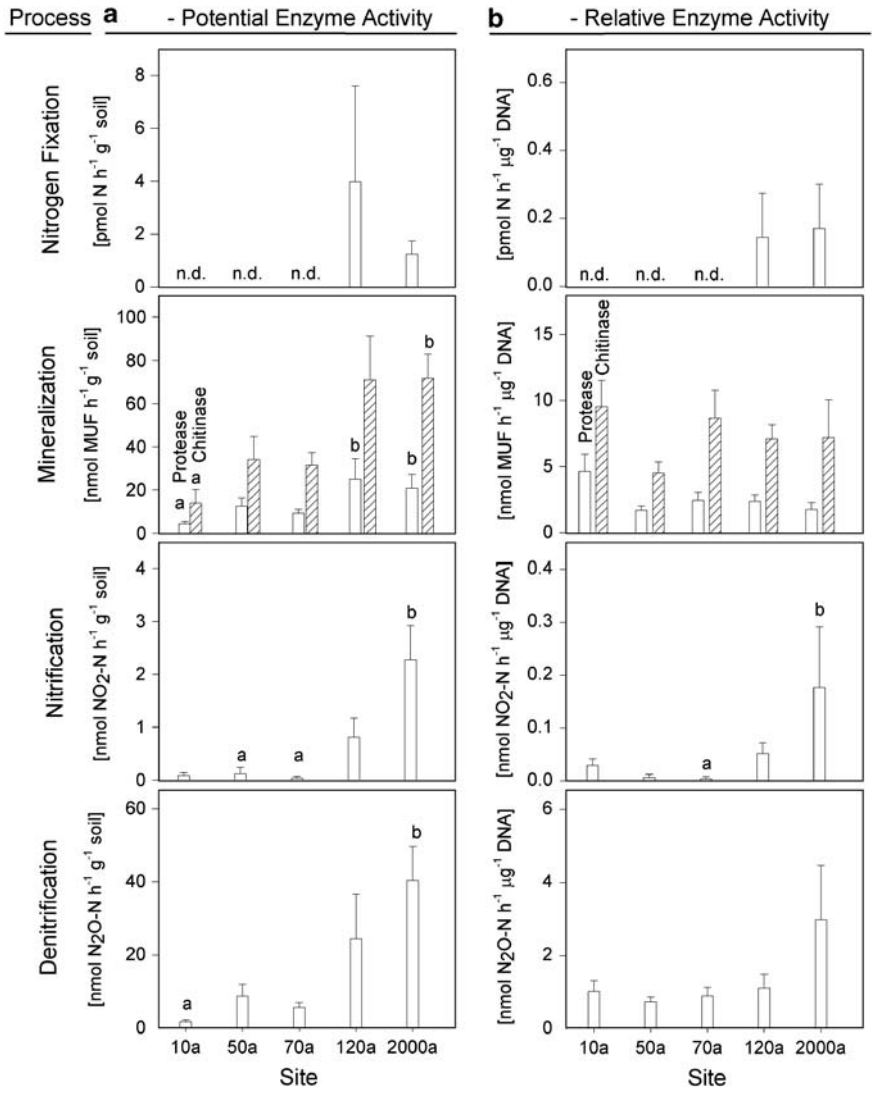

Figure 2 Potential (**a**) and relative enzyme activities (**b**) for nitrogen fixation, mineralization, nitrification and denitrification are displayed ($n = 5$, error bars represent standard error of means). Only significant differences as revealed by one way ANOVA on ranks ($P < 0.05$) are indicated by different letters. n.d. indicates that all five replicates of potential N fixation were below the detection limit of 0.2 pmol N h^{-1} g^{-1} soil.

data revealed no potential N-fixation activity and the lowest *nifH* gene copy numbers per gram soil in the 10a soil. This is supported by the studies of Duc *et al*. (2009) and Nemergut *et al*. (2007) in which the lowest N fixation rate was measured in the bulk soil of the initial stage of the Damma glacier (Switzerland) and Puca glacier (Peru) forefield, respectively. In contrast, mineralization of organic material seemed to be the driver for N cycling at the initial stage of ecosystem development, indicated by the highest potential activities for proteases combined with highest relative *aprA* gene copy numbers (based on the amount of extracted DNA). Although other genes like *nirK* and *amoA* AOB showed similar or even higher gene copy numbers at the initial sites, the potential denitrification and nitrification rates were extremely low. This implies the presence of inactive nitrifying and denitrifying populations, attributed to the shortage of ammonium and nitrate. Obviously, at the initial stage of the glacier forefield, sufficient N and C are provided for mineralization: (i) allochthonous organic material (plant debris, insects and so on) is deposited in the forefield, as revealed by our and other measurements (Hodkinson *et al.*, 2002; National Air Pollution Monitoring Network (NABEL), 2008; Nyiri *et al.*, 2009), (Table 1), (ii) the cryoconite holes in the glacier ablation zone possibly inhabit small food-webs of *cyanobacteria* and heterotrophs, which were relocated to the glacier forefield by the glacial stream after snowmelt (Schmalenberger and Noll, 2009; Xiang *et al.*, 2009) and (iii) microbes might feed on ancient recalcitrant C (Hodkinson *et al.*, 2002; Bardgett *et al.*, 2007). In addition, significant inputs of nutrients may be due to atmospheric dry and wet deposition of N and C species (Table 1).

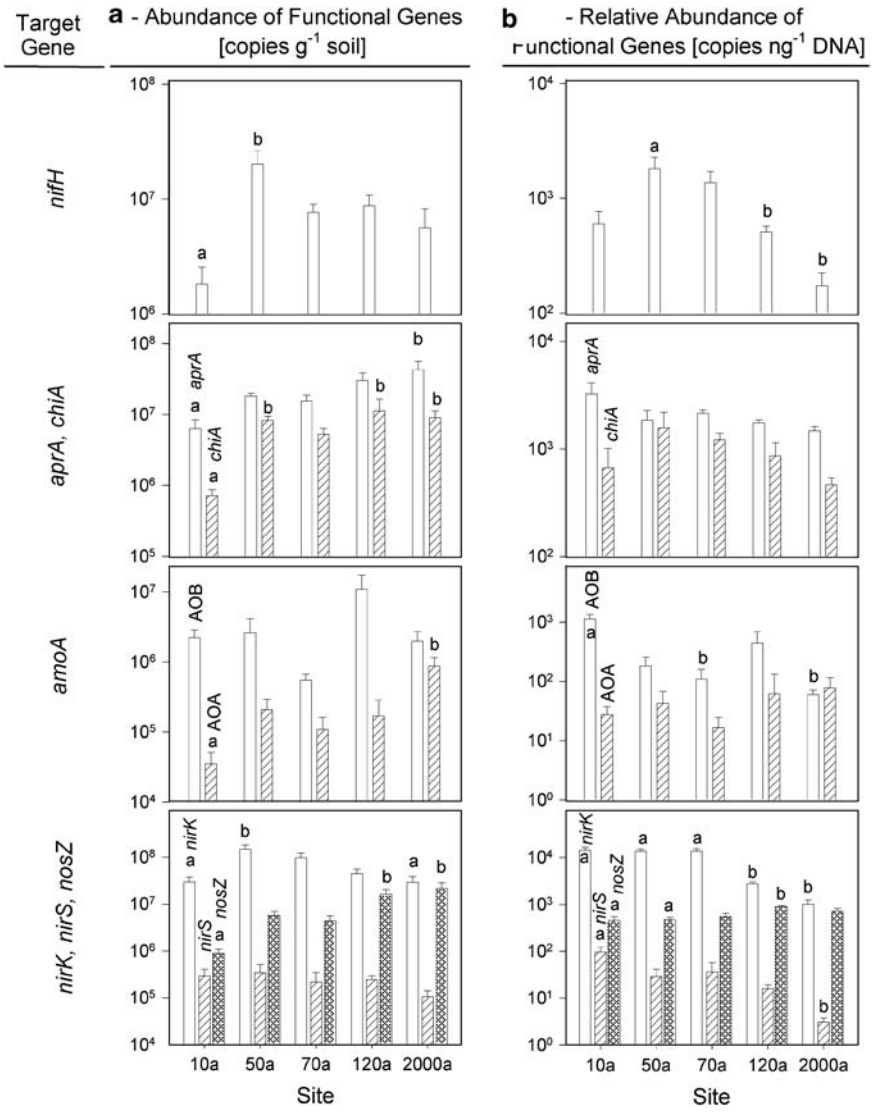

Figure 3 Gene copy numbers of functional genes involved in major steps of the nitrogen cycle (*nifH*, *aprA*, *chiA*, *amoA* AOB, *amoA* AOA, *nirK, nirS* and *nosZ*) are displayed related to gram soil (**a**) and nanogram DNA (**b**) ($n=5$, error bars represent standard error of means). Only significant differences as revealed by one way ANOVA on ranks ($P<0.05$) are indicated by different letters.

Plants as competitors for nutrients

With the first appearance of plant patches it could be expected that C will be provided in moderate quantities by rhizodeposition and exudation. However, it has rather been postulated for developing ecosystems that plants and microbes compete for nutrients, like N and phosphorus, as on the one hand nutrient availability is low and on the other hand plant performance and exudation is reduced (Chapin *et al.*, 1994; Hammerli *et al.*, 2007). Although ammonium and nitrate concentrations increased two- to sixfold from site 10a to the transient soils (50 and 70a), these values are still more than 10 times lower than values from pasture sites (Chronakova *et al.*, 2009), confirming the assumption that strong competition for N still exists. Thus, it is likely that with the first occurrence of plant patches at site 50a, the *nifH* gene abundance reached its maximum even if the potential N-fixing activity was still negligible. However, it has to be taken into account that the used assay only covered the performance of free-living heterotrophic N fixers and did not consider symbiotic N fixation by *Rhizobia*, plant-associated N fixation or phototrophic N fixation. The observation that the activity of free-living N fixers is low at this stage of ecosystem development is not surprising, as the amounts of dissolved organic carbon in the bulk soil of the 50 and 70a sites were low and consequently, C sources were missing for the energy-consuming process of N fixation (Zehr *et al.*, 2003). In this regard Duc *et al.* (2009) showed that once C-rich nutrients were delivered like exudates in the rhizosphere of plants, N-fixation activity significantly increased. Hence, it can be speculated that mainly symbiotic and plant-associated N fixing

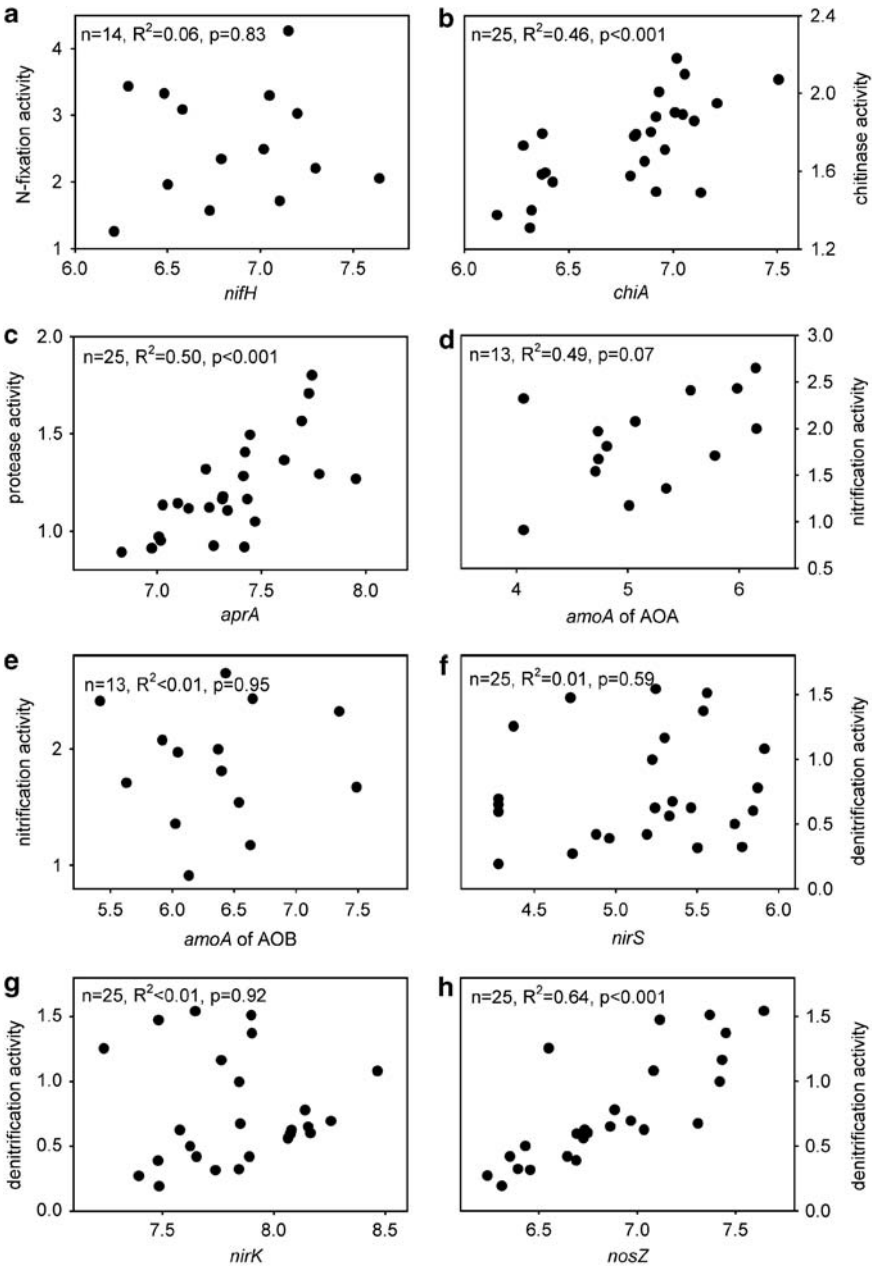

Figure 4 Correlations of the gene copy numbers and the corresponding potential enzyme activities are displayed for nitrogen fixation (**a**), mineralization (**b**, **c**), nitrification (**d**, **e**) and denitrification (**f**, **g** and **h**). The graphs plot log-transformed copy numbers vs log-transformed enzyme activities. In the top left corner of each plot the parameters of the regression analysis, including *P*-values of *t*-tests, are provided.

microbes might have a role for N input at these sites.

Along with the increase in plant abundance and diversity (Hammerli *et al.*, 2007) also other organisms establish in the developing soils. Especially the abundance of fungi increased along glacial chronosequences (Ohtonen *et al.*, 1999; Bardgett and Walker, 2004). Thus, the observed increase in *chiA* gene abundance and potential chitinase activity were not surprising. These findings are further in-line with data presented by Tscherko *et al.* (2004) who revealed highest *N*-acetyl-β-glucosaminidase activity in the 75a transient soils of the Rotmoosferner glacier. On the contrary, *aprA* gene abundances did not change, underlining the high relevance of chitin as alternative C and organic N source (Olander and Vitousek, 2000), which might result from the deposition of dead insects (Hodkinson *et al.*, 2002).

Although potential mineralization was also prominent at the transient stages of ecosystem development (50 and 70a), potential nitrification and denitrification activity remained low. That is not surprising as nitrification causes the transformation of ammonium to highly mobile nitrate and in

connection with denitrification, it would result in a substantial loss of N. Although *amoA* AOA, *nirK* and *nosZ* gene abundances increased with increasing biomass, their relative abundances in the microbial community were constant. Interestingly, that was not true for *amoA* AOB because their relative abundance significantly dropped towards site 70a, which might be a response to the decreasing pH values along the chronosequence with highest pH values of 5.1 at the initial sites. De Boer and Kowalchuk (2001) found that *Nitrosospira* species, which are the most common AOB in soil (Kowalchuk *et al.*, 2000), were not able to oxidize ammonium at pH values below 5.5. In contrast, several studies indicate that AOA are able to oxidize ammonium in a broad pH range (Leininger *et al.*, 2006; Nicol *et al.*, 2008; Reigstad *et al.*, 2008) and appear to be better adapted to ammonium-poor environments (Di *et al.*, 2009, 2010) and soils with low pH (Nicol *et al.*, 2008).

Plants as driver for nitrogen turnover

After 120a of soil development at the Damma glacier forefield the soil surface is densely covered with plants. The pronounced root penetration in the developed soils brings along an enhanced water retention potential resulting in less oxygen diffusion and reduced partial oxygen pressure (Rheinbaben and Trolldenier, 1984; Deiglmayr *et al.*, 2006). In combination with the sevenfold increase of the nitrate concentrations, developed soils (120 and 2000a) provide good conditions for denitrification. That development is also reflected in our potential denitrification measurements, which showed highest activity in the 2000a soil. Our data showed a negligible role of *nirS* genes at the Damma glacier forefield for nitrite reduction. Obviously, nitrite reduction appeared to be driven by *nirK*-harboring bacteria. Similarly, Kandeler *et al.* (2006) found a dominance of *nirK* and *nosZ* genes along the Rotmoosferner glacier forefield, Austria. However, if comparing gene abundances of different genes, one should keep in mind that the quantitative PCR approach suffers from some biases such as unspecific primers or varying PCR amplification efficiencies. Thus, the displayed gene copy numbers did never reflect the actual *in situ* abundance. Nevertheless, the use of degenerated primers (Henry *et al.*, 2004) and the accurate performance of the measurement (Towe *et al.*, 2010b) allow the discussion of solid tendencies.

Beside highest potential enzyme activities, gene copy numbers (per gram of soil) of *chiA, aprA* and *amoA* AOA revealed significantly highest values in the 2000a soil. These data are congruent with observations from Frank *et al.* (2000) who found a positive correlation between nitrification, denitrification and N mineralization processes in Yellowstone Park grasslands. Additionally, associations between mineralizing and nitrifying organisms were described in previous studies (Schimel and Bennett, 2004).

Linking potential enzymatic activities to gene abundance

We found correlations between gene abundance and enzymatic activities for chitinolytic and proteolytic microbes, as well as for N_2O reducers. This indicates that over the whole chronosequence no shifts in the physiology of the corresponding functional groups have occured, as turnover rates have not changed under optimal conditions (potential activities) in relation to the gene copy numbers. This is not surprising, as microbes that are able to mineralize N are restricted to a few specialized microbes that are often taxonomically closely related. For example, many proteolytic bacteria belong to different species of *Bacillus* (Fuka *et al.*, 2008). For chitinases it has been postulated that horizontal gene transfer might have an important role for transfer of this functional trait, thus operon structures and induction pathways might be comparable (Cohen-Kupiec and Chet, 1998). In contrast to N-mineralizing microbes, N_2O reducers occur in many different groups of microorganisms with different physiological backgrounds. However, induction of the N_2O reductase is mainly linked to the presence of nitrate and thus similarly regulated for all groups of N_2O reducers (Zumft, 1997; Hallin *et al.*, 2009), which might explain the good correlation between gene abundance and enzymatic activities for this functional group.

Vice versa low or not existing correlations between gene abundance and enzymatic activities might indicate shifts in the diversity pattern and the physiology of the dominant functional groups along the chronosequence. This is obvious for N-fixing microbes: In the first phase of soil development N fixation is driven mainly by *cyanobacteria*, which have relatively low N-fixation activity. In contrast, N-fixing symbionts, who aquire carbon from the plant, fix N at higher rates. However, it must also be taken into account that the conditions of the enzymatic assays have been only optimal for a subgroup of each functional group. If environmental conditions change along the chronosequence (for example, pH values) these changing conditions have not been reflected in the assays performed. It might be speculated that this fact could explain the missing correlation between ammonia-oxidizing bacteria and potential nitrification, as some well adapted AOBs mainly at the developed sites (120 and 2000a) with lower soil pH (4.6–4.1), could respond differently to the conditions of the nitrification assay performed compared with AOBs from the initial and transient soils.

Overall it must be clearly stated that potential activities measured under laboratory conditions cannot be related to *in situ* fluxes and turnover rates (Roling, 2007). To address this type of question a different experimental setup is needed.

The chronosequence approach
Although a chronosequence approach might be biased by, for example, different microclimatic conditions along the slope of hill or the big spatial heterogeneity, it was shown in several studies that the influence of the soil age outcompetes minor influences of other site characteristics (Nemergut *et al.*, 2007; Duc *et al.*, 2009). Thus, it displayed a proper approach to compare and pursue N cycle processes during different soil development stages. The study did not intend to assess seasonal effects and therefore, all samples were taken at one single time-point.

Conclusion
In conclusion, our data revealed that initial (10a) and transient soils (50 and 70a) were dominated by processes, which were responsible for N input and release from organic matter, that is, N fixation and mineralization. Denitrification and nitrification that lead to N losses from the system were reduced at these stages. As soon as a closed plant cover developed and microbial associations are established, nitrification and denitrification potential activity increased. Moreover, we demonstrated that potential enzyme activities correlated with *amoA* AOA, *nosZ, chiA* and *aprA* gene abundance only. To corroborate these results further studies are needed with the aim to measure *in situ* enzyme activities and link these data to the corresponding gene transcript abundances. However, the enormous spatial heterogeneity and the low enzymatic activities mainly at the initial sites require sophisticated sampling strategies. In addition, future studies dealing with N fixation, should allow assessing the individual contributions of free-living heterotrophs, phototrophs, symbionts and plant-associated N-fixing microbes to the N budget.

Acknowledgements

The authors thank Stefano Bernasconi for stable isotope measurements and critical reviewers for their helpful comments on the manuscript. This study is part of the Transregional Collaborative Research Centre 38 (SFB/TRR 38), which is financially supported by the Deutsche Forschungsgemeinschaft (DFG, Bonn) and the Brandenburg Ministry of Science, Research and Culture (MWFK, Potsdam).

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Publication IV

Microb Ecol (2010) 60:762–770
DOI 10.1007/s00248-010-9695-5

ENVIRONMENTAL MICROBIOLOGY

Abundance of Microbes Involved in Nitrogen Transformation in the Rhizosphere of *Leucanthemopsis alpina* (L.) Heywood Grown in Soils from Different Sites of the Damma Glacier Forefield

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Received: 14 January 2010 / Accepted: 31 May 2010 / Published online: 12 June 2010

Abstract Glacier forefields are an ideal playground to investigate the role of development stages of soils on the formation of plant–microbe interactions as within the last decades, many alpine glaciers retreated, whereby releasing and exposing parent material for soil development. Especially the status of macronutrients like nitrogen differs between soils of different development stages in these environments and may influence plant growth significantly. Thus, in this study, we reconstructed major parts of the nitrogen cycle in the rhizosphere soil/root system of *Leucanthemopsis alpina* (L.) HEYWOOD as well as the corresponding bulk soil by quantifying functional genes of nitrogen fixation (*nifH*), nitrogen mineralisation (*chiA*, *aprA*), nitrification (*amoA* AOB, *amoA* AOA) and denitrification (*nirS*, *nirK* and *nosZ*) in a 10-year and a 120-year ice-free soil of the Damma glacier forefield. We linked the results to the ammonium and nitrate concentrations of the soils as well as to the nitrogen and carbon status of the plants. The experiment was performed in a greenhouse simulating the climatic conditions of the glacier forefield. Samples were taken after 7 and 13 weeks of plant growth. Highest *nifH* gene abundance in connection with lowest nitrogen content of *L. alpina* was observed in the 10-year soil after 7 weeks of plant growth, demonstrating the important role of associative nitrogen fixation for plant development in this soil. In contrast, in the 120-year soil copy numbers of genes involved in denitrification, mainly *nosZ* were increased after 13 weeks of plant growth, indicating an overall increased microbial activity status as well as higher concentrations of nitrate in this soil.

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Introduction

In ecosystems which are relatively rich in nutrients like many natural grasslands or forests in Central Europe, beneficial plant–microbe interactions in the rhizosphere can be described as a give and receive situation. The plants provide up to 40% of their photosynthetically fixed carbon to the microbes [34] and enhance microbial biomass and activity compared to bulk soil, known as the "rhizosphere effect" [5, 36]. That occurs either due to uncontrolled leakage or controlled exudation of organic substances like malate, citrate or oxalate [19]. In return, microbes supply the plant with nitrogen, phosphate or other nutrients and additionally protect them against herbivores or parasites. Previous studies have shown that plants are even able to select for functional bacterial groups like nitrogen fixers [7] or ammonium oxidizers [4] to create an optimal environment for their growth.

In contrast, it has been postulated that in developing ecosystems like glacier forefields, plants and microbes compete for nutrients as, on the one hand, nutrient availability is low and, on the other hand, plant performance on exudation rates is reduced [6, 14]. However, recent studies have shown local patches of enhanced nutrient availability created by pioneering plants along different glacier forefields [6, 9, 12, 50–52]. In this respect, especially microbial nitrogen fixation may display a selective advantage for the pioneering plant [21]. Jacot et al. [18] showed that 70–95% of nitrogen requirements of legumes in the Swiss Alps were provided by symbiotic nitrogen fixers. Furthermore, Duc et al. [12] assigned a key role in initial plant-free ecosystems to free-living diazotrophs, from whom plants benefit indirectly. However, this requires a repression of nitrification in the rhizosphere of plants from initial ecosystems to avoid the formation of highly mobile nitrate. This example illustrates that for an improved understanding of nitrogen turnover in less developed soils, a detailed understanding of the major nitrogen cycling processes is needed as the comprehension of the kinetics of one single transformation step does not explain the overall nitrogen status of the soil.

Therefore, we aimed to reconstruct the microbial nitrogen cycle in the rhizosphere of a typical alpine plant growing in soils of different development stages. We postulate that plants from less developed soils strongly depend on the nitrogen delivered by microbes via nitrogen fixation. In contrast, a balanced relationship concerning nitrogen exchange is expected in more developed soils with higher abundance of microbes involved in nitrification and denitrification. To test the hypothesis, two different soils of the Damma glacier forefield (Canton Uri, Switzerland) being ice-free for respectively 10 and 120 years were planted in a greenhouse with *Leucanthemopsis alpina* (L.) HEYWOOD [27], which is distributed along the whole glacier forefield independent from the soil development stage. The climatic conditions were simulated according to the natural situation at the forefield. Samples were obtained from the rhizosphere soil/root complex and the respective bulk soil after 7 and 13 weeks of plant growth. To reconstruct the microbial nitrogen cycle, gene abundances of the nitrogenase reductase (*nifH*), the bacterial group a chitinase (*chiA*), the alkaline metalloprotease (*aprA*), the ammonium monooxygenase (*amoA*), the nitrite reductase (*nirS*, *nirK*) and the nitrous oxide reductase (*nosZ)* were linked to the carbon and nitrogen content of the plant biomass and the nitrate and ammonium concentrations of the soil.

Materials and Methods

Experimental Procedures

Soil Description

The experiment was performed using soils from the Damma Glacier forefield, being located in the canton Uri, Switzerland (46°38′13″ N, 8°27′15″ E, 2,000 m above sea level). Soil material was collected from two differently developed sites which correspond to an ice-free period of 10 (10a) and 120 years (120a). Generally, the forefield is built up from bedrock material. The 10a site is characterised by a vegetation cover of <20% [14] dominated by *L. alpina* (L.) HEYWOOD. The soil is characterised by a pH value of 5.1 (0.01 M $CaCl_2$), a total nitrogen content of <0.15 g kg^{-1}, an ammonium content of 0.06 mg kg^{-1}, a nitrate content of 0.8 mg kg^{-1}, a total carbon content of 0.7 g kg^{-1} and a maximum water holding capacity of 26%. The soil texture according to the method of Dumig et al. [13] consists of 86% sand, 10% silt and 4% clay. In comparison to that, the 120a site is fully covered by vegetation, dominated by *L. alpina*, *Agrostis gigantea*, *Rumex scutatus*, *Salix* sp. and *Lotus alpinus*. The soil has a pH value of 4.6, a total nitrogen content of 0.9 g kg^{-1}, an ammonium content of 6.95 mg kg^{-1}, a nitrate content of 15.8 mg kg^{-1}, a total carbon content of 12 g kg^{-1} and a maximum water holding capacity of 29%. The soil texture is characterised by 80% sand, 17% silt and 3% clay. A total amount of 70 kg soil per site was taken from the upper 5 cm, air-dried and sieved through a 2-mm mesh.

Experimental Design

The experiment was carried out in plastic pots (8× 8× 9 cm) containing approximately 500 g of each soil and three plants per pot. The water content was adjusted to 60% of maximum water holding capacity for each soil [48].

Before the experiment started, the air-dried soils have been equilibrated for 2 weeks at 4°C. Irrigation was performed every 2 days by a semi-automatic dropping system (Gardena, Germany) with deionised water supplemented with 0.27 µM K_2HPO_4, 6.31 µM Na_2SO_4, 4.4 µM $NaNO_3$, 4.8 µM KNO_3, 4.5 µM $CaCl_2{\cdot}2\ H_2O$ and 1.2 µM $MgCl_2$, which displayed the natural rain composition at the Damma glacier.

As model plant *L. alpina* was chosen because it is a typical pioneering plant distributed along the whole glacier forefield [27]. The seeds were purchased from Sandeman Seeds (Lalongue, France). Before sowing, seeds were washed three times with sterile distilled water. The experiment was performed using a random block design, resulting in a total number of ten pots per soil type.

After sowing, pots were placed in a greenhouse. The photobiological environment of the glacier forefield was simulated using a combination of four types of lamps to obtain a natural balance of simulated global radiation throughout the ultraviolet (UV) to infrared spectrum [11, 46]. The light intensity at plant level was stepwise increased from 250 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR, 400–700 nm) and 1.5 W m^{-2} UV-A radiation (315–400 nm) for the first 16 days, followed by 2 days with 450 µmol m^{-2} s^{-1} PAR, 3.3 W m^{-2} UV-A radiation and 3 days with 650 µmol m^{-2} s^{-1} PAR, 4.5 W m^{-2} UV-A radiation. After this time without UV-B radiation, the plants were treated with 800 µmol m^{-2} s^{-1} PAR, 9.0 W m^{-2} UV-A and 0.41 W m^{-2} UV-B radiation. The pots were exposed to radiation for 14 h per day. The climatic conditions were kept constant during the period of acclimatisation (14 days) at 15°C and 90% relative air humidity and were adjusted to a night–day cycle from 8°C to 16°C and 90–50% relative air humidity, respectively.

Sampling

From each treatment, five pots were taken at two time points of plant development, after 7 (T1) and 13 (T2) weeks of plant growth, and treated as independent replicates. From each pot, bulk soil (BS) material of the root-free upper 2 cm, the rhizosphere soil/root complex (RRC) [17] and the aboveground biomass of the plants were taken. All samples were divided into two subsamples; one was stored at −80°C for nucleic acid extraction and the other one was immediately used for ammonium and nitrate measurements.

Soil Ammonium and Nitrate Measurement

Nitrate (NO_3^-–N) and ammonium (NH_4^+–N) concentrations of the RRC and BS samples were determined in $CaCl_2$ extracts by a colorimetric method using the commercial kits NANOCOLOR Nitrat50 (detection limit, 0.3 mg N kg^{-1} dry weight) and Ammonium3 (detection limit, 0.04 mg N kg^{-1} dry weight; Macherey-Nagel, Germany). For the extraction, 0.2 g RRC and 20 g BS samples were overhead shaken for 45 min with 4 and 20 ml of 0.01 M $CaCl_2$, respectively. Finally, the extracts were filtered through a Millex HV Millipore filter (pore size, 0.45 µm) and measured as described by the manufacturer.

Carbon and Nitrogen Content in Plants

The plant material was dried at 65°C for 2 days. Afterwards, it was ball-milled (Retsch MM2, Retsch GmbH, Haan, Germany) and 1.5 mg was weighted into 3.5× 5-mm tin capsules (HEKAtech GmbH, Wegberg, Germany). The total carbon and nitrogen contents were measured with the Elemental-Analysator 'Euro-EA' (Eurovector, Milano, Italy) [25].

Nucleic Acid Extraction

DNA was extracted from 0.3 g RRC and 0.5 g BS, respectively, using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Irvine, CA) and the Precellys24 Instrument (Bertin Technologies, France). Quality and quantity of the extracted DNA were checked with a spectrophotometer (Nanodrop, PeqLab, Germany). Afterwards, extracts were stored at −20°C until use.

Real-Time PCR Assay

Quantitative real-time PCR was conducted on a 7300 Real-Time PCR System (Applied Biosystems, Germany) using SybrGreen as fluorescent dye. The 25 µl reaction mixture was composed as referred in Table 1. As standards, serial plasmid dilutions ranging from 10^1 to 10^6 gene copies per microlitre were used. Samples exceeding the standard curve were discarded. The Power SybrGreen Master Mix was obtained from Applied Biosystems, primers were synthesised by Metabion (Germany), and bovine serum albumin (BSA) and dimethyl sulfoxide (DMSO) were purchased from Sigma (Germany). In order to avoid inhibition of the PCR reaction, an experiment with dilution series of all samples was performed in advance, resulting in an optimal dilution of 1:64. Quantitative real-time PCR was performed in 96-well plates (Applied Biosystems) for all eight target genes as described in Table 1. Each PCR run started with a hot start for 10 min at 95°C. All samples and standards were analysed in triplicates and, additionally, several negative controls were included. To confirm the specificity of the amplicons after each PCR run, a melting curve and a 2% agarose gel were conducted. The amplification efficiencies were calculated with the equation Eff=[$10^{(-1/\text{slope})}$ − 1] and resulted in the following values: *nifH* 84–85%,

Table 1 Thermal profiles and primer used for real-time PCR quantification of different functional genes

Target gene	Source of standard	Thermal profile	No. of cycles	Primer	Primer (10 µM)	DMSO
nifH	*Sinorhizobium meliloti*	95°C–45 s/55°C–45 s/72°C–45 s	40	nifHF, nifHR [38]	0.5	–
amoA AOA	Fosmid clone 54d9 [49]	94°C–45 s/55°C–45 s/72°C–45 s	40	amo19F, CrenamoA16r48x [23, 40]	0.5	–
amoA AOB	*Nitrosomonas* sp.	94°C–45 s/60°C–45 s/72°C–45 s	40	amoA1F, amoA2R [39]	0.75	–
nirS	*Pseudomonas stutzeri*	95°C–15 s/57°C–30 s/72°C–30 s	40	cd3aF, R3cd [26, 47]	0.5	0.625
nirK	*Azospirillum irakense*	95°C–15 s/63°C–30 s/72°C–30 s	5[a]	nirK876, nirK5R [3, 15]	0.5	0.625
		95°C–15 s/58°C–30 s/72°C–30 s	40			
nosZ	*Pseudomonas fluorescens*	95°C–30 s/65°C–30 s/72°C–30 s	5[a]	nosZ2F, nosZ2R [16]	0.5	–
		95°C–15 s/60°C–15 s/72°C–30 s	40			
chiA	*Streptomyces griseus*	95°C–30 s/60°C–30 s/72°C–60 s	40	chif2, chir [54]	1.0	–
apr	*Pseudomonas aeruginosa*	95°C–20 s/53°C–30 s/72°C–60 s	40	FR aprI, RP aprII [1]	2.0	–

PCR reaction mixture consisted of Power SybrGreen Master Mix (12.5 µl), BSA (0.5 µl), template (2 µl) as well as primer and DMSO as referred in the table (in microlitres)

[a] Touchdown: −1°C per cycle

chiA 84–88%, *aprA* 82–85%, *amoA* ammonium-oxidising bacteria (AOB) 88%, *amoA* ammonium-oxidising archaea (AOA) 77–83%, *nirS* 87–90%, *nirK* 80–82% and *nosZ* 88–91%. The detection limit of the method was assigned to 10 gene copies per microlitre according to the manufacturer's instructions. In correspondence, gene copy numbers below 10^4 copies g^{-1} dry weight of RRC and BS, respectively, were not detectable.

Statistical Analysis

Statistical analyses were carried out with the R environment (http://www.r-project.org). Prior to analysis, data were tested for normal distribution by Q-Q plots and the Kolmogorov–Smirnoff test. Homogeneity of variances was checked by the Levene test. To look for overall effects and interactions of the three factors soil age, incubation time and soil compartment, a three-factor ANOVA was performed for gene abundance data and a two-factor ANOVA for plant C and N contents. As complex interactions were revealed, the data sets were divided by soil age to allow a reasonable data interpretation. In the case of ammonium and nitrate, a two-factor ANOVA was conducted for the 120a soil only. Due to the high amount of values below the detection limit for the 10a soil, data contingency tables were built and the exact Fisher test for count data was performed to expose influences of soil compartment and incubation time.

Results

Nitrogen and Carbon Content of Plant Biomass

Mean values of plant nitrogen and carbon contents are presented in Table 2. Whilst the carbon content did not differ between both sampling time points and both treatments, the nitrogen content significantly increased in the plants cultivated in the 10a soil from 1.28% (T1) to 3.94% (T2). On the contrary, the nitrogen content remained stable in the plants grown in the 120a soil. Consequently, the C/N ratio dropped in the 10a samples to 10.3, whereas it remained at a constant average level of 22 in the plants grown in the 120a soil (Table 2). Total nitrogen content increased four and fivefold in the plants from the 10a and the 120a soil, respectively. Absolute values of carbon and plant biomass revealed a twofold increase of the plants from T1 to T2 planted in the 10a soil and a ninefold increase of the plants cultivated in the 120a soil (data not shown).

Ammonium and Nitrate Concentrations in BS and RRC Samples

The ammonium values were very low or even below the detection limit (<0.04 mg NH_4^+–N kg^{-1}) for all BS and RRC samples of the 10a soil. In contrast, a time-dependent decrease of the ammonium concentration was observed in

Table 2 Nitrogen and carbon contents of the harvested aboveground plant biomass of *L. alpina* after 7 (T1) and 13 weeks (T2) of plant growth in the 10a and 120a soil (n = 5; standard deviation in parentheses)

	10 years		120 years	
	T1	T2	T1	T2
%C	39.68 (0.56)	39.47 (1.01)	38.99 (2.61)	38.75 (0.67)
Statistic	No effect		No effect	
%N	1.28 (0.15)	3.94 (0.52)	1.96 (0.63)	1.86 (0.2)
Statistic	Effect of incubation time		No effect	
C/N Ratio	33.04 (4.69)	10.34 (1.81)	22.77 (9.8)	21.33 (2.93)
Statistic	Effect of incubation time		No effect	

The significant impact of time of plant growth was checked by ANOVA (p<0.05)

the BS and RRC samples of the 120a soil. Nitrate concentrations also differed between the two soils, but were further significantly influenced by the soil compartment. Thus, values were much higher in RRC than in BS, peaking in 49.33 mg kg^{-1} (RRC, T1) in the 10a soil and 108.26 mg kg^{-1} (RRC, T1) in the 120a soil, respectively. Data are summarised in Table 3.

Abundances of Functional Genes of the Nitrogen Cycle

In general, all quantifiable functional genes were significantly influenced by the soil compartment with higher gene copy numbers in the RRC compared to BS samples, except the *nirS* gene (Fig. 1 and Table 4).

Concerning nitrogen fixation, interestingly, highest *nifH* gene copy numbers were detected in RRC samples of the 10a soil with 1.7×10^7 copies g^{-1} dry weight (dw) after 7 weeks of plant growth. As the *nifH* gene abundance was significantly influenced by the incubation time in the 10a soil (p = 0.008), gene abundance was strongly reduced in RRC after 13 weeks to a level of 8×10^6 copies g^{-1} dw. A similar trend of a time-dependent decrease of *nifH* gene copy numbers was observed in the bulk soil samples of the 10a soil. In contrast, *nifH* gene abundance was neither influenced by the incubation time nor by the soil compartment in the 120a soil.

In terms of mineralisation, gene abundance of *chiA* was significantly higher in samples of the 120a soil compared to those of the 10a soils. In the 10a samples, a strong rhizosphere effect (p = 0.0) was visible at both sampling time points, with six times higher *chiA* abundances in RRC compared to BS. However, no time-dependent changes were observed in the 10a soil. In the 120a soil, a significant interaction of the factors soil compartment and incubation period was revealed (p = 0.021), which is revealed by rather similar gene abundances (1×10^8 copies g^{-1} dw) in BS and RRC at T1 and dispersing gene abundances at T2. Gene copy numbers of a*prA* were below the detection limit in all cases ($<10^4$ *apr* gene copies g^{-1} dw, data not shown).

With regard to nitrification, independent of sampling time point and soil compartment AOB clearly dominated in numbers over AOA, which were in all cases below the detection limit (10^4 *amoA* gene copies g^{-1} dw soil, data not shown). Whilst a strong rhizosphere effect (p = 0.0) on *amoA* (AOB) gene abundances was detected in the 10a soil, it was negligible in the 120a soil. Accordingly, up to five times higher values were measured in RRC of the 10a soil, peaking in 8.9×10^6 copies g^{-1} dw after 7 weeks of plant growth compared to the bulk soil. Contrary, RRC and BS samples of the 120a soil showed no significant differences and revealed an average gene abundance of 1.3×10^7 *amoA* (AOB) copies g^{-1} dw.

Table 3 Ammonium and nitrate concentrations (mg N kg^{-1} dw) were measured in BS and RRC of *L. alpina* after 7 (T1) and 13 weeks (T2) of plant growth in the 10a and 120a soil (n = 5; standard deviation in parentheses)

	10 years				120 years			
	T1		T2		T1		T2	
	BS	Rhi	BS	Rhi	BS	Rhi	BS	Rhi
NH_4^+–N	b. d. l. (b. d. l.)	0.7 (1.6)	0.02 (0.03)	b. d. l. (b. d. l.)	5.92 (1.9)	8.14 (3.1)	2.29 (1.3)	0.91 (2.0)
Statistics	No effect				Effect of incubation time			
NO_3^-–N	0.07 (0.15)	49.33 (17.0)	b. d. l. (b. d. l.)	27.01 (11.8)	38.56 (23.5)	108.26 (74.3)	11.22 (9.9)	76.14 (17.6)
Statistics	Effect of compartment				Effect of compartment			

"b.d.l." indicates that all five replicates were below the detection limit of 0.04 mg NH_4^+–N kg^{-1} and 0.3 mg NO_3^-–N kg^{-1}, respectively. The significant impact of time of plant growth and soil compartment was checked by contingency tables and exact test after Fisher for the 10a soil and two-factor ANOVA for the 120a soil (p<0.05)

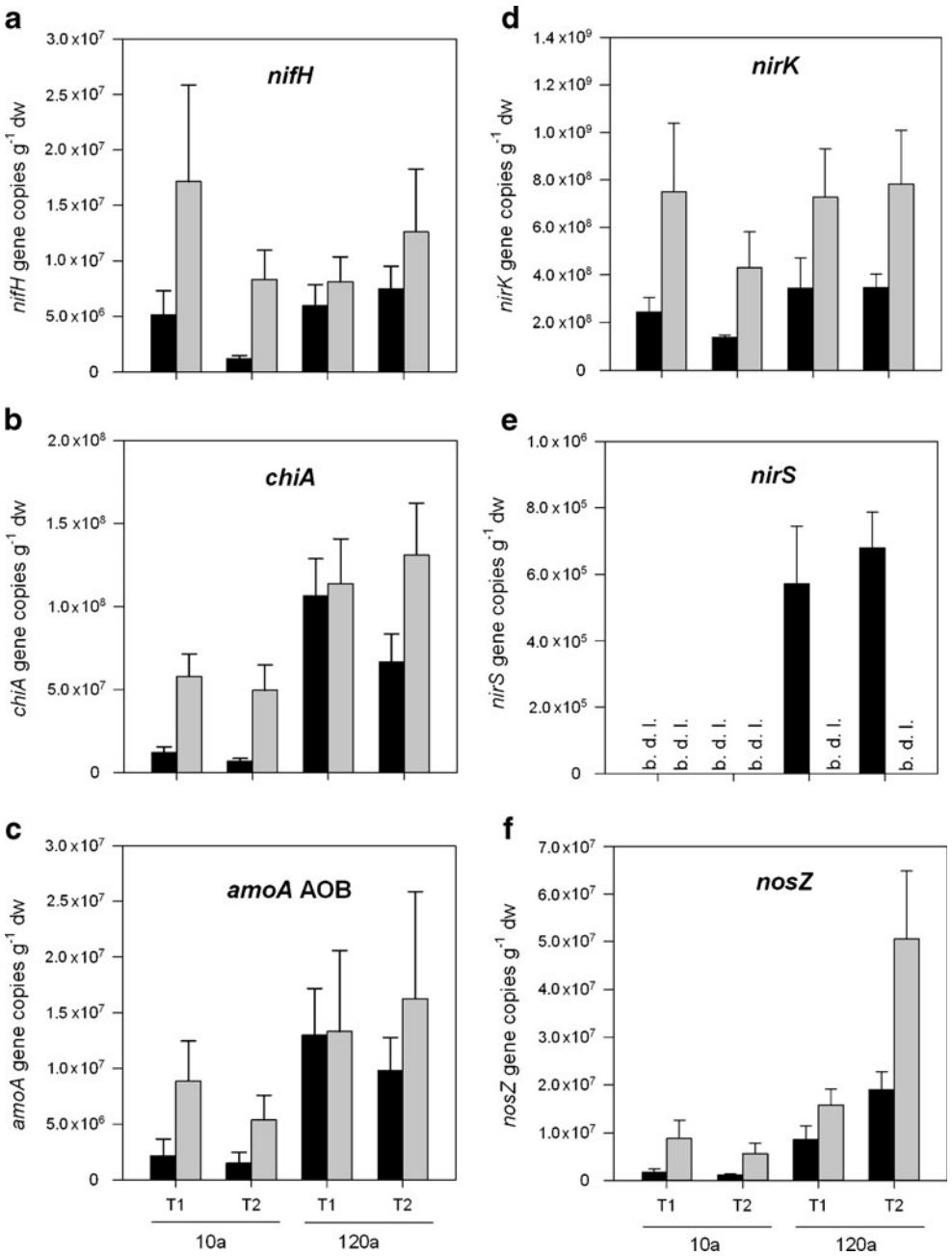

Figure 1 Copy numbers of functional genes involved in the nitrogen cycle (*nifH*, *chiA*, *amoA* AOB, *nirK*, *nirS* and *nosZ*) are displayed for RRC (*gray bar*) samples of *L. alpina* and the corresponding BS (*black bar*) after 7 (T1) and 13 weeks (T2) of plant growth in the 10a and 120a soil (n=5, *error bars* represent standard deviations). "*b.d.l.*" indicates that samples were below detection limit of 10^4 gene copies g^{-1} dw

In respect of denitrification, *nirK* gene copy numbers ranged between 1.1×10^8 (10a, BS, T2) and 7.8×10^8 g^{-1} dw (120a, RRC, T2), whereas *nirS* genes were only observed in the bulk soil of the 120a soil (5.7 (T1) and 6.8×10^5 copies g^{-1} dw (T2)). The abundance of *nirK* genes was strongly impacted by the soil compartment (p = 0.0) in both soils. Additionally, incubation time had a significant (p = 0.011) influence in the 10a soil, resulting in a decrease of gene abundance from 7 to 13 weeks of RRC and BS samples as well. In contrast to *nirK*, *nosZ* gene copies remained constant over time in the 10a soil, with average gene abundances of 1.4 and 7.2×10^6 copies g^{-1} dw in BS and RRC, respectively. In the 120a soil, the *nosZ* gene abundance was significantly influenced by incubation time (p = 0.0) and soil compartment (p = 0.0). Thus, the *nosZ* gene copy numbers significantly increased in RRC of the 120a soil, peaking in 5.1×10^7 copies g^{-1} dw after 13 weeks of plant growth. This is in contrast to all other genes measured which were more influenced by the soil compartment than the incubation time in the 120a soil.

Discussion

The aim of this study was to investigate the impact of two differently developed soils on the formation of plant–

Table 4 Statistical evaluation of gene abundance data by multi-factorial ANOVA

Factor	ANOVA p values				
	nifH	*chiA*	*amoA* AOB	*nirK*	*nosZ*
Total					
Age (10a, 120a)	0.648	0.000*	0.000*	0.005*	0.000*
Time (T1, T2)	0.207	0.141	0.489	0.088	0.000*
Compartment (BS, RRC)	0.000*	0.000*	0.009*	0.000*	0.000*
Age × time	0.001*	0.704	0.53	0.027*	0.000*
Age × compartment	0.028*	0.486	0.557	0.921	0.001*
Time × compartment	0.707	0.032*	0.588	0.442	0.004*
Age × time × compartment	0.133	0.018*	0.154	0.214	0.001*
10a					
Time (T1, T2)	0.008*	0.163	0.059	0.011*	0.078
Compartment (BS, RRC)	0.000*	0.000*	0.000*	0.000*	0.000*
Time × compartment	0.256	0.732	0.183	0.165	0.217
120a					
Time (T1, T2)	0.058	0.321	0.973	0.703	0.000*
Compartment (BS, RRC)	0.027*	0.006*	0.26	0.000*	0.000*
Time × compartment	0.331	0.021*	0.304	0.735	0.003*

The p values describe the impacts and interactions of soil age, time of plant growth and soil compartment on functional genes (*nifH*, *chiA*, *amoA* AOB, *nirK* and *nosZ*) involved in the nitrogen cycle. Significant impacts or interaction were marked by asterisks ($p<0.05$). *aprA*, *amoA AOA* and *nirS* genes could not be statistically evaluated due to copy numbers below the detection limit

microbe interactions in the rhizosphere of the pioneering plant *L. alpina*. We used a 10a and a 120a ice-free soil from the Damma Glacier forefield which we replanted with *L. alpina* and sampled BS and RRC after 7 and 13 weeks of plant growth, respectively. As nitrogen is highly relevant for plant growth, we investigated microbial communities involved in nitrogen transformation as model.

Interestingly, only in the 10a soil did the plant nitrogen concentration increase from T1 to T2, and simultaneously, the plant C/N ratio decreased. Two possible sources of nitrogen can be considered: firstly, more ammonium was allocated by nitrogen-fixing microbes in the 10a soil, as indeed their abundance was highest in the rhizosphere in this soil (T1). Secondly, recent studies showed [24, 41] that plants under nitrogen limitation do not only rely on microbially derived ammonium or nitrate but are also able to assimilate low weight organic nitrogen like amino acids.

Besides nitrogen input via N_2 fixation, the soil nitrogen pool is also supplied by nitrogen mineralisation. Chitin is, amongst others, one of the dominant forms of organic N entering the soil [32], which is part of the cell walls of fungi and invertebrates. Since the availability of organic substrates and hence chitin is an important driver for chitinases, it is thus reasonable that *chiA* gene abundance paralleled the succession of fungi, which increased along glacier forefield chronosequences [2, 30]. Also, Tscherko et al. [50] revealed a high relevance of *N*-acetyl-β-glucosaminidase in mature soils of the Rotmoosferner glacier. Therefore, we measured *chiA* gene abundances in our study as marker of mineralisation potential and observed smaller differences between BS and RRC in the 120a soil compared to the 10a soil. This was probably due to a higher soil organic matter content in the 120a soil [12, 45]. Correspondingly, highest *chiA* gene abundance values were detected in the 120a soil. In contrast to *chiA* genes, *aprA* gene copies were not detectable, indicating indirectly low amounts of available proteins in the soils [14].

Although *nifH*, *chiA* and *amoA* AOB gene abundances did not change between the two sampling time points in the 120a soil, ammonium concentrations significantly decreased. It might be speculated that ammonium concentrations decreased due to higher nitrification rates, elevated plant uptake or a combination of both. The assumption of higher nitrification rates is underlined by the study of Schimel and Bennett [41] who stated that nitrification activity is repressed in pioneer ecosystems, whereas it is favoured in developed ones where nitrifying and mineralizing microorganisms live in closer association to each other. Thus, although *amoA* AOB and *chiA* gene abundances did not increase significantly over time, mineralizing and nitrifying microbes could have developed a stronger interaction. Interestingly, gene abundance levels of *amoA* AOA were below the detection limit in all samples, although many studies assigned a major role in nitrification to archaea [23, 29, 40, 43]. Taking into account that AOB exhibit much higher turnover rates (32–86 fmol NO_2 per cell per hour) [31, 35, 53] than AOA (0.3–0.14 fmol NO_2 per cell per hour) [8, 22], it is possible that AOA might have been outcompeted by AOB. Accordingly, Sigler et al. [44] revealed a shift from r- to k-strategists along the Damma glacier forefield.

Besides N_2 fixation, N mineralisation and nitrification, also denitrification as a process of nitrogen loss was under investigation. Two steps of the denitrification pathway were studied: on the one hand the reduction of nitrite and on the other hand the reduction of nitrous oxide by quantifying the respective functional genes *nirK*, *nirS* and *nosZ*. Denitrification is closely linked to nitrate availability and partial oxygen pressure; consequently, higher values of denitrification genes in the rhizosphere of *L. alpina* could be assumed. Interestingly, the assumption applied to *nirK* and *nosZ* genes but not for *nirS* genes, which were only detectable in the 120a bulk soil in considerably lower amounts than *nirK* genes. Although these results are in contrast to the observations of Kandeler et al. [20] at the Rotmoosferner glacier, this phenomenon was already found in a previous study [42] where only *nirK* was detected in the rhizosphere of three different grain legumes. Possibly, the two functionally redundant genes *nirK* and *nirS* occur in different niches. However, the *nirK* gene showed the highest abundance of all investigated genes, peaking in 8×10^8 gene copies g^{-1} dw. In contrast to the relatively constant appearance of *nirK*, *nosZ* gene abundance was very dynamic. As the nitrous oxide reductase is most sensitive to low amounts of oxygen [28, 33], it was not surprising that highest *nosZ* gene abundances were detected in the rhizosphere of *L. alpina* after 13 weeks of development where a more pronounced root system was developed, presumably entailing a lower oxygen partial pressure [37]. Additionally, the N_2O/N_2 ratio of denitrification products is positively related to nitrate concentrations [10]. Thus, the declining nitrate concentrations in all samples might have boosted the growth of the *nosZ*-harbouring microbial community.

In conclusion, the data revealed a stronger rhizosphere effect in the 10a soil in comparison to the 120a soil, reflected by much higher gene abundances in the rhizosphere compared to the bulk soil. Interestingly, only plants cultivated in the 10a soil were able to accumulate remarkable amounts of nitrogen. Thus, the hypothesis corroborated that in less developed soil ecosystems, plants depend more on microbially delivered nitrogen via N_2 fixation than in more developed soils. However, the measured gene abundance patterns only reflect a microbial potential for nitrogen transformation and do not describe actual turnover rates in soils. Therefore, investigations of gene expression, enzyme activity and stability remain to be performed to compare the presence of functional groups with their activities and actual turnover rates. Furthermore, as a special case of microbe–plant interaction, the mycorrhiza–plant interaction displays a particular advantage for both partners. Therefore, also the role of mycorrhiza should be a subject of future work if the overall performance of developing ecosystems should be understood.

Acknowledgement This study is part of the Transregional Collaborative Research Centre 38 (SFB/TRR38) which is financially supported by the Deutsche Forschungsgemeinschaft (DFG, Bonn) and the Brandenburg Ministry of Science, Research and Culture (MWFK, Potsdam). We also thank Franz Buegger for excellent technical support in measuring C and N contents of the plant biomass.

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Publication V

MINIREVIEW

Nitrogen turnover in soil and global change

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Received 23 January 2011; revised 18 May 2011; accepted 20 June 2011.
Final version published online 29 July 2011.

DOI:10.1111/j.1574-6941.2011.01165.x

Editor: Ian Head

Keywords
nitrogen cycle; nitrification; denitrification; nitrogen fixation; mineralization.

Abstract

Nitrogen management in soils has been considered as key to the sustainable use of terrestrial ecosystems and a protection of major ecosystem services. However, the microorganisms driving processes like nitrification, denitrification, N-fixation and mineralization are highly influenced by changing climatic conditions, intensification of agriculture and the application of new chemicals to a so far unknown extent. In this review, the current knowledge concerning the influence of selected scenarios of global change on the abundance, diversity and activity of microorganisms involved in nitrogen turnover, notably in agricultural and grassland soils, is summarized and linked to the corresponding processes. In this context, data are presented on nitrogen-cycling processes and the corresponding microbial key players during ecosystem development and changes in functional diversity patterns during shifts in land use. Furthermore, the impact of increased temperature, carbon dioxide and changes in precipitation regimes on microbial nitrogen turnover is discussed. Finally, some examples of the effects of pesticides and antibiotics after application to soil for selected processes of nitrogen transformation are also shown.

Introduction

Nitrogen is one of the crucial nutrients for all organisms (LaBauer & Treseder, 2008), as it is an essential component of important biopolymers. However, most of the N in nature occurs as dinitrogen gas or is fixed in organic compounds, like proteins or chitin, both of which cannot be directly used by plants and animals. Only specialized microorganisms are able to transform the gaseous dinitrogen into ammonia or to make organically bound N bioavailable by mineralization. Not surprisingly, N input by fertilization has always been a key factor for high crop yields and plant quality. Therefore, crop production is by far the single largest cause of human alteration of the global N cycle (Smil, 1999). Whereas in preindustrial times exclusively organic fertilizers had been used, the invention of the Haber Bosch procedure in the 20th century made huge amounts of mineral fertilizer available. The doubling of world food production in the past four decades could only be achieved with a strong land-use intensification including an almost sevenfold increase of N fertilization (Tilman, 1999) as well as wide-ranging land reclamations. These developments have contributed to the doubling of N loads to soil since the beginning of the 20th century (Green *et al.*, 2004). The total global N input in the year 2000 was about 150 TgN (Schlesinger, 2009), whereas supply in croplands via mineral fertilizer was the single largest source accounting for almost half of it. Surprisingly, N entry from N-fixation was the second largest factor and contributed to 16%, while manure and recycled crop residues provided similar amounts and each accounted for only 8–13% of the total global supply. Remarkably, the entry of N via atmospheric deposition was in the same range. In regions with high mineral fertilizer application, the highest N accumulation potential in ecosystems could be observed, whereas the accumulation of N leads to high impacts on environmental quality like loss of diversity (Cragg & Bardgett, 2001), dominance of weed species (Csizinszky & Gilreath, 1987) and soil acidification (Noble *et al.*, 2008). Additionally, land-use intensification also results in an increased use of bioactive chemicals, like pesticides and herbicides as well as antibiotics, which enter the environment via manure (Lamshöft *et al.*, 2007).

According to Liu *et al.* (2010), 55% of the global applied N was taken up by crops. The remainder was lost in leaching

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(16%), soil erosion (15%) and gaseous emission (14%). Such N depletion of soils leads to eutrophication (Stoate *et al.*, 2009), surface- and groundwater pollution (Spalding & Exner, 1993) and emission of the greenhouse and ozone-depleting gas nitrous oxide (N_2O) (Davidson *et al.*, 2000), impacting on human health and climate change (Fig. 1).

To reduce these threats, Schlesinger (2009) suggested that policy makers and scientists should focus on increasing N-use efficiency in fertilization, reducing transport of reactive N fractions to rivers and groundwater and maximizing denitrification to N_2.

Because of the use of advanced molecular tools (Gabriel, 2010) and stable isotopes (Baggs, 2008) in recent years, scientists have been able to identify new key players of N turnover for selected processes like nitrification (Leininger *et al.*, 2006) or N-fixation (Chowdhury *et al.*, 2009) as well as completely new processes like anammox (Op den Camp *et al.*, 2006). All these findings have revolutionized our view of N transformation processes in soils, although the relevance for the overall understanding of N transformation is not entirely clear yet and discussed controversially in the literature. However, despite numerous studies and a large amount of collected data, we have to admit that N turnover and factors driving the corresponding populations are not yet completely understood.

Furthermore, according to the *UN Millenium Ecosystem Assessment* (http://www.maweb.org/), global change will highly affect N turnover in soils to a so far unknown extent. According to the definition given in Wikipedia, the term 'global change' encompasses interlinked activities related to population, climate, the economy, resource use, energy development, transport, communication, land use and land cover, urbanization, globalization, atmospheric circulation, ocean circulation, the C cycle, the N cycle, the water cycle and other cycles, sea ice loss, sea-level rise, food webs, biological diversity, pollution, health, overfishing and alteration of environmental conditions including climate change as well as land-use changes and effects of xenobiotic substances. Therefore, there is a need for experimental approaches to study the consequences of altering environmental conditions including climate change as well as land-use changes and the effects of xenobiotic substances on N turnover in soil. In the following review, state-of-the-art knowledge is summarized concerning the impact of selected global change scenarios on microbial N turnover as well as the abundance and diversity of key players. Additionally, implications for future research strategies and priorities are given.

Ecosystem development

Natural and anthropogenic activities lead to new terrain for soil development. In this context, different chronosequences of ecosystem development like glacier forefields, sand dunes, volcanoes or restoration sites have emerged. These are interesting aspects to study the development of N-cycling processes as well as the contributing functional microbial groups. Overall, three phases can be postulated: initial, intermediate and mature phases. Depending on the investigated ecosystem, these phases can range from a few days or weeks (Jackson, 2003) to hundreds of years (Kandeler *et al.*, 2006; Brankatschk *et al.*, 2011), respectively.

Most of the initial ecosystems are characterized by nutrient shortage, barren substrate and scarce vegetation (Crews *et al.*, 2001; Nemergut *et al.*, 2007; Smith & Ogram, 2008; Lazzaro *et al.*, 2009; Brankatschk *et al.*, 2011). The total N concentrations are often far below 0.1% and only traces of ammonia and nitrate can be measured (Brankatschk *et al.*, 2011). Additional N input by the weathering of bedrock material is unlikely as it only contains traces of N. Thus, the colonization with N-fixing microorganisms seems to be the only way for N input, despite the high energy demands for the transformation of N_2 into ammonium. Crews *et al.* (2001) demonstrated that the total N input in young lava flows was mainly driven by N fixation, although fixation rates were low. This has been confirmed in several other studies, which demonstrated a high abundance of nonheterocystous N-fixing cyanobacteria like *Microcoleus vaginatus* (Yeager *et al.*, 2004; Nemergut *et al.*, 2007; Abed *et al.*, 2010). It is obvious that in initial ecosystems, cyanobacteria play a prominent role in ecosystem engineering. They not only improve the N status of soils by N-fixation, but also secrete a polysaccharide sheath, resulting in the formation of soil crusts. This leads to a stabilization of substrates, capture of nutrients and an increase of the water-holding capacity, which paves the way for other organisms and processes (Garcia-Pichel *et al.*, 2001; Schmidt *et al.*, 2008). Therefore, at early stages of soil development heterotrophic microorganisms, which are able to mineralize the N derived from air-driven deposition (e.g. chitin) or ancient and recalcitrant materials are able to find their niches and stimulate N turnover (Bardgett *et al.*, 2007; Brankatschk *et al.*, 2011). However, this process is highly energy demanding and thus the turnover rates typically low. Obviously, as only limited competition for N resources exists at this stage (due to a lack of plants), the amount of ammonia is sufficient for the development of microbial communities involved in nitrification. This process results in the formation of nitrate, which leaves the ecosystem mainly by leaching. Therefore, N accumulation rates at initial sites are low (Tscherko *et al.*, 2004).

If the total N concentrations in soil exceed 0.2%, plant development starts and cyanobacterial soil crusts are displaced by shadowing by plant growth (Brankatschk *et al.*, 2011). Therefore, the intermediate stage of ecosystem development is characterized by increasing plant coverage and surface stabilization resulting in an increased C input

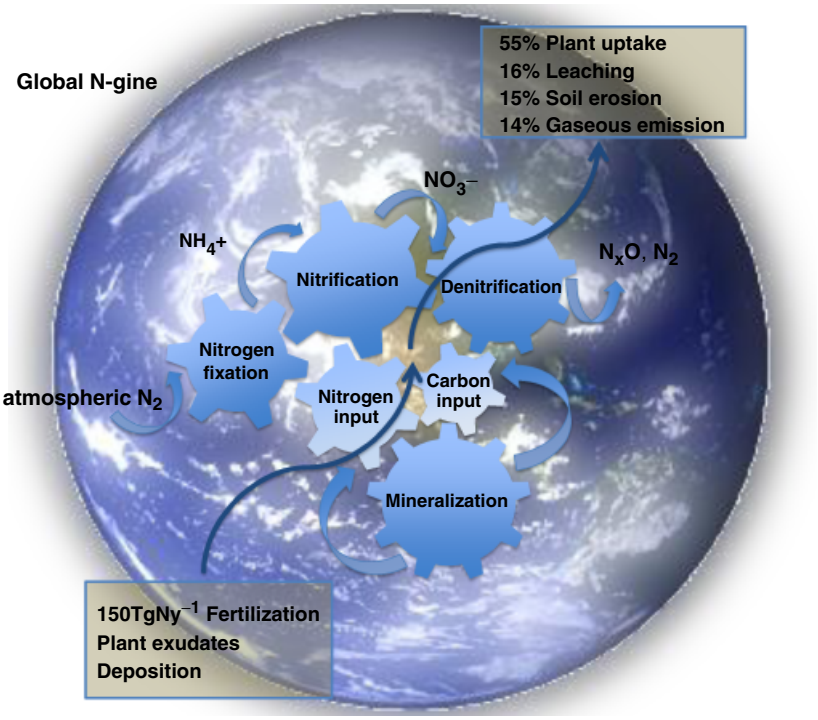

Fig. 1. Nitrogen turnover at the global scale.

via exudation and litter material. However, ammonium and nitrate contents are still much lower (Kandeler *et al.*, 2006; Brankatschk *et al.*, 2011) than in well-developed grassland sites (Chronáková *et al.*, 2009). Although it has been argued that this stage of ecosystem development is characterized by a competition between microorganisms and plants for N (Schimel & Bennett, 2004; Hämmerli *et al.*, 2007), associative or symbiotic networks between N-fixing microorganisms (mainly bacteria) and plants become a central element at this stage (Duc *et al.*, 2009). This results in an increased N-fixation activity in the rhizosphere and a highly efficient share of nutrients between plants and microorganisms. Because of the patchy distribution of C and N concentrations at those sites, many studies have revealed the highest microbial diversity at intermediate stages of ecosystem development by targeting functional genes like *nifH* (Duc *et al.*, 2009) or general microbial diversity by 16S rRNA gene (Gomez-Alvarez *et al.*, 2007). This fits with the intermediate-disturbance hypothesis, postulating that medium disturbance events cause the highest diversification (Molino & Sabatier, 2001). However, besides the development of plant–microorganism interactions, the intermediate phase of ecosystem development is also characterized by highly efficient degradation of litter and subsequent N mineralization (Esperschütz *et al.*, 2011) as well as an increase in fungal biomass (Bardgett & Walker, 2004), probably also of arbuscular mycorrhiza, which may contribute to a better distribution of the N in soil with ongoing succession. At this stage, the abundance and activity of nitrifiers (Nicol *et al.*, 2005) and denitrifiers (Smith & Ogram, 2008) is still low due to the high N demand of the plants. Whether typical plants at those sites are able to produce nitrification inhibitors to better compete for ammonium might be a highly interesting question for future research (Verhagen *et al.*, 1995).

In contrast, when total N concentrations above 0.7% are reached in soils at well-developed sites and vegetation is no longer dominated by legumes, nitrification becomes a highly significant process. Interestingly, in ecosystems of glacier forefields, nitrification activity seems to be driven by ammonia-oxidizing archaea (AOA), although being lower in abundance than their bacterial counterpart [ammonia-oxidizing bacteria (AOB)]. This might be due to the better adaptation to relative ammonium-poor environments (Di *et al.*, 2009) and low pH (Nicol *et al.*, 2008). In combination with pronounced root penetration resulting in increased exudation, enhanced water retention potential and less oxygen diffusion (Deiglmayr *et al.*, 2006), denitrification becomes a key process for the overall N budget at those sites in soil. Interestingly, Brankatschk *et al.* (2011) only found a good correlation of a part of the functional genes of the denitrification cascade, for example, *nosZ* (nitrous oxide reductase) gene abundance and potential denitrification activity, whereas *nirK* and *nirS* (nitrite reductases) gene abundance did not correlate with the rates of potential activity. Moreover, the highest relative gene abundance of *narG* was observed in early development stages of soils (Kandeler *et al.*, 2006), while the nitrate reductase activity peaked at late stages of soil development (Deiglmayr *et al.*,

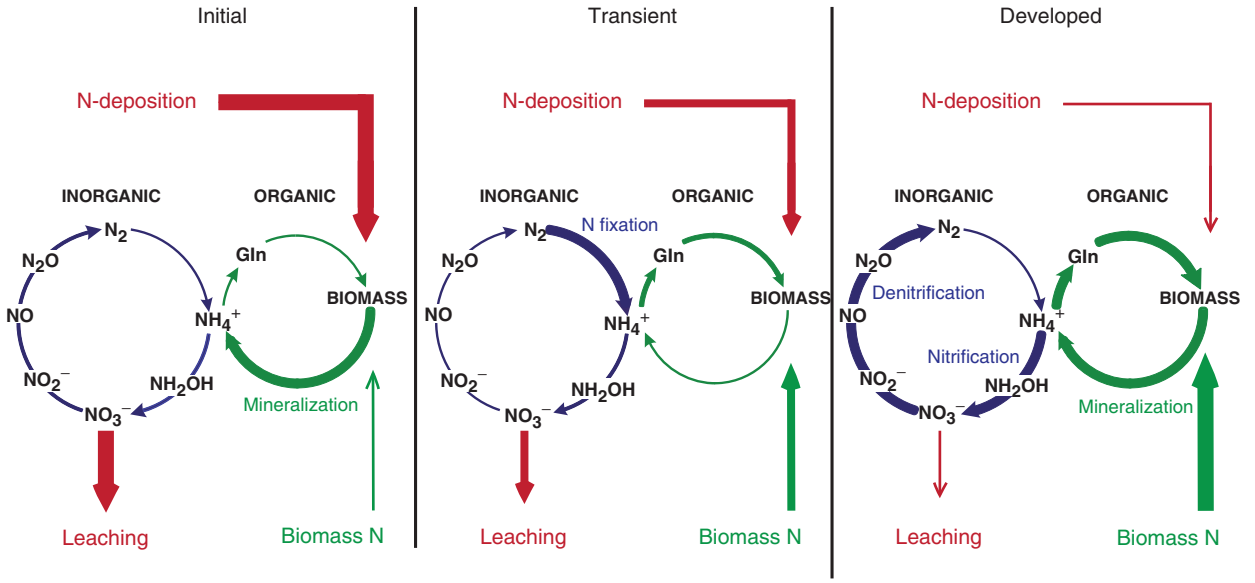

Fig. 2. Scheme of the development of the nitrogen cycle during ecosystem development (initial, transient and developed). The size of the arrows represents the impact of the corresponding process for nitrogen turnover.

2006). Similar observations were made by Smith & Ogram (2008) along a restoration chronosequence in the Everglades National Park. The mechanistic bases for these observations are still not clear. In addition to high activities of nitrifiers and denitrifiers at well-developed sites, the highest values of mineralization activity have been observed there in several studies (Tscherko *et al.*, 2004; Brankatschk *et al.*, 2011). These data are congruent with the observations of Frank *et al.* (2000), who found a positive correlation between nitrification, denitrification and N mineralization processes in Yellowstone Park grasslands.

Overall, the studies performed so far using the chronosequence approach to describe ecosystem development have revealed surprisingly similar patterns of the participation of different functional groups of microorganisms involved in N cycling at the three different phases (Fig. 2). In summary, all systems described were characterized by very low C and N concentrations in soil as well as less pronounced organismic networks of interaction at the initial stages of soil development.

Changing land-use patterns

A generalization of the results described above to other scenarios of global change related to ecosystem development, for example, in response to natural disasters (earthquakes), after manmade destructions (clear cuts of forest sites) or due to land-use changes is not possible. This is due to the different quality and amount of C and N present in soil as well as the biodiversity, mainly related to soil animals and plants at the initial stages in these disturbed systems. Whereas the consequences of natural disasters for N turnover have been rarely addressed, the impact of land-use changes on N turnover and the corresponding functional communities has been studied extensively. However, in this context, it is difficult to identify *one* main driver for shifts in the microbial population structure, as land-use changes often encompass a combination of different forms of management. For example, the use of extensively used grassland for crop production will not only change aboveground biodiversity, but will also result in changes in pesticide application, tillage and fertilizer management.

Overall, the conversion of forests or grasslands to agricultural land has an impact on almost all soil organisms (Postma-Blaauw *et al.*, 2010). Therefore, the functional diversity of microorganisms involved in N cycling is also highly influenced by land-use changes. This has been well documented for nitrifiers and denitrifiers, whereas surprisingly for N-fixing bacteria, clear response patterns have been described in only a small number of cases. In some cases, even no response of *nifH* towards land-use changes was detected (Colloff *et al.*, 2008; Hayden *et al.*, 2010), which might be related to the high concentrations of ammonium and nitrate before land-use change. In terms of nitrification, good correlations between gene abundance and land use have been described for AOB in several studies. Colloff *et al.* (2008) found higher gene abundance of the bacterial *amoA* gene in agricultural soils compared with soils from rainforests. By contrast, Berthrong *et al.* (2009) observed consistently reduced nitrification rates in soils that were converted from grassland into forest. These trends were also confirmed by Bru *et al.* (2010) comparing land-use changes between forests, grassland and agricultural soils in different

parts of the world. The authors found a strong correlation between AOB and the form of land use. Interestingly, in the same study, no differences were observed for archaeal ammonia oxidizers (AOA) in relation to the investigated land-use types. Hayden *et al.* (2010) almost consistently observed a greater abundance of AOB *amoA* genes in managed compared with remnant sites. The good correlation between AOB and land use might be related to the different ammonium concentrations in soil in response to different land-use types. AOB often colonizes habitats with high ammonium concentrations, whereas for AOA abundance, so far, no general dependency on ambient ammonium concentration has been documented. Furthermore, the results might be related to the high sensitivity of AOB towards low pH, which is often present in forest soils and leads to low availability of ammonia.

It was reported that land-use changes from forest to grassland soils are often accompanied by high N losses from soil (reviewed by Murty *et al.*, 2002). However, no clear trends are visible so far, if these losses occur in general due to increased denitrification rates or leaching of the nitrate formed during nitrification, as both observations have been described in the literature. This might be explained by the different soil types under investigation in the various studies. Whereas in loamy soils, which tend to have more anoxic microsites, denitrification might be stimulated (Rich *et al.*, 2003; Boyle *et al.*, 2006), in sandy soils, the nitrate formed may leach fast to the groundwater (Murty *et al.*, 2002). For denitrifiers, land-use changes overall influence the abundance and diversity patterns of selected functional groups. Attard *et al.* (2010) described, for example, higher potential denitrification rates in grassland soils compared with soils under cropping management. This was in accordance with a 1.5–5-fold higher abundance of denitrifiers (based on the abundance of *nirK* genes) in grassland soils than arable soils found in various studies (Baudoin *et al.*, 2009; Attard *et al.*, 2010), including shifts in the diversity patterns of *nirK*-harboring bacteria. Whereas a strong correlation between gene copy numbers of *nirK* and potential denitrification rates has been described, no correlation was found between the diversity patterns of *nirK* and turnover of nitrate. This indicates highly similar ecophysiological patterns of nitrite reducers of the *nirK* type.

Agricultural management

Not only changes in land-use patterns, but also shifts in agricultural management practice can result in alterations of functional microbial communities involved in N cycling. In general, there is consensus that an intensification of agriculture and subsequent increased fertilization regimes result in higher nitrification and denitrification rates as well as an increase of both functional groups (Le Roux *et al.*, 2003, 2008; Patra *et al.*, 2006). In the case of ammonia oxidizers, mainly AOB benefit from the increased availability of ammonium in soil (Schauss *et al.*, 2009a). For N-fixing prokaryotes, several studies have indicated a reduction based on the abundance of *nifH* and consequently also lower N-fixation activity in highly fertilized soils (Coelho *et al.*, 2009). Interestingly, the inoculation of seeds from legumes with rhizobia, which is a common practice in low-input farming to enhance N-fixation, does not only increase *nifH* abundance in the rhizosphere, but also leads to higher abundance of nitrifiers and denitrifiers (Babic *et al.*, 2008). This indicates that at least a part of the fixed N is released into soil, despite the symbiotic interaction (Babic *et al.*, 2008). As the use of monocultures and the intensification of agriculture *per se* (including the transformation of sites, which are less suited for agriculture, for the production of renewable resources) is often accompanied by a loss in nutrients (Malézieux *et al.*, 2009), which is primarily compensated by the application of inorganic fertilizers, changes in N turnover and the corresponding microbial communities might be primarily a result of changed fertilization regimes, as described by Drury *et al.* (2008). It has been confirmed in several studies that the type of fertilizer (mineral vs. organic fertilizer) has a clear influence on the N budget of soils and the corresponding functional microbial groups (Hai *et al.*, 2009; Ramirez *et al.*, 2010). As expected, the application of a mineral fertilizer based on ammonia-nitrate increases the nitrification and denitrification patterns in soil shortly after application, when the fertilizer is not taken up by the plant due to increased availability of the corresponding substrates. In contrast, the application of an organic fertilizer leads to higher abundance of microorganisms involved in mineralization and only relatively slight increases of nitrifiers and denitrifiers and their activity in the long run. Because of the overall more balanced N budget in soil when organic fertilizers are applied, N-fixing microorganisms are favored by this practice (Pariona-Llanos *et al.*, 2010). Not surprisingly, the effects observed in soils that have been used for grazing can be compared with those where manure has been applied, including clear shifts mainly in the diversity patterns of ammonia- and nitrite-oxidizing microorganisms as well as denitrifiers (Chroňáková *et al.*, 2009) Furthermore, grazing also induces shifts in root exudation patterns (Hamilton & Frank, 2001), which may further influence the abundance and activity of microorganisms involved in N turnover.

In the last decades, the influence of tillage management on N turnover has been studied in several projects, as nontillage systems have been described to be of advantage in terms of nutrient supply and are very popular in organic farming (Hansen *et al.*, 2011). Overall, changes in nitrification activity after modifying the tillage practice were well

explained by the accumulation of ammonium in the top soil due to nontillage and the corresponding changes in the abundance of nitrifiers (Attard *et al.*, 2010). In most studies, performed so far, a higher nitrification activity and subsequent higher nitrate concentrations in soil were linked to increased denitrification rates in the top soil layer in nontillage compared with tillage treatments (Petersen *et al.*, 2008; Baudoin *et al.*, 2009; Attard *et al.*, 2010). This is due to tillage-induced higher C concentrations in top soils and a stronger formation of aggregates with anoxic microsites due to a lack of tillage-induced mixing. In addition, tillage results in a merging of the surface soil layers with the lower layers, the latter being characterized by lower denitrification potential (Attard *et al.*, 2010), which causes overall lower denitrification rates and abundance of the corresponding functional genes (especially *nirK*). However, as stated above, in most cases, changes in tillage management are accompanied by changes in pest management and cropping sequences. The changes observed in long-term studies therefore cannot be linked conclusively to tillage management alone. Thus, most studies performed so far in this area were linked to short-term perturbations. They may not reflect the typical response patterns of the soil microorganisms to the new conditions after the change of the tillage management, as they do not account for microbial adaptation, in the context of the intermediate-disturbance hypothesis (Molino & Sabatier, 2001) as well as the increasing C contents in the top soils over time where nontillage practice has been performed.

Changing climatic conditions

Because of ongoing climate change, various modifications in land use and agricultural management have been implemented. Thereby, climate and land management are highly interlinked and cannot be separated. In addition, it is well accepted that climatic conditions notably influence microbial performance in soil. Thus, several studies have been performed to estimate the consequences of increased atmospheric temperature or carbon dioxide (CO_2) concentrations as well as shifts in precipitation on N turnover and the corresponding functional communities.

In general, it is difficult to simulate increased temperature scenarios in experiments, as an increase of the average temperature of 3 °C over the next 50 years would at most result in an annual increase of < 0.2 °C. Therefore, experiments comparing soils with ambient temperature with soils increased in temperature by 2–5 °C do not simulate climate change, but are more appropriate to understand the overall stress response of the soil microbial community. An air temperature increase of 3 °C for example, induced shifts in the AOB community structure, decreased AOB richness and concurrently increased potential nitrification rates in the rhizosphere of legumes. It remains open whether AOA adopted the ability to transform ammonia, while their bacterial counterparts were sensitive to the elevated temperature (Malchair *et al.*, 2010a). Besides questioning the relevance for studying climate change effects, it is unclear whether the observed shifts were a direct effect of the temperature or were rather related to changes of the plant performance, for example, increased exudation, in response to the increased temperature.

More relevant in the context of temperature-related effects are questions addressing changes in soils of permafrost regions, as here, only a slight increase of air temperature results in a prolonged period in which soils are unfrozen during the summer time. In these studies, the focus has mostly been on C turnover and methane emission, although clear effects on N transformation have been described. There is broad agreement that thawing of permafrost soils leads to a rapid increase of denitrification and hence high N_2O emissions, due to the high water saturation and the availability of easily degradable C and nitrate in those soils (Repo *et al.*, 2009; Elberling *et al.*, 2010). Measured emissions were comparable to values from peat soils (0.9–1.4 g N_2O m^{-2} and year). In contrast, nitrifying communities did not benefit from the changed environmental conditions in the short run. Metagenomic analysis and clone library studies revealed a low diversity and a relatively low abundance for ammonia oxidizers (AOA and AOB) (Liebner *et al.*, 2008; Yergeau *et al.*, 2010). Obviously, the high concentrations of available C as well as the anoxic conditions do not favor the growth of AOA and AOB. Therefore, not surprisingly, in permafrost soils, clear evidence for anaerobic ammonia oxidation has been obtained (Humbert *et al.*, 2010), in contrast to many other soil ecosystems. N-fixing microorganisms did not play a major role in the investigated sites and did not change in abundance and diversity after thawing (Yergeau *et al.*, 2010).

However, also in moderate climatic zones, small shifts in the temperature affect freezing and thawing regimes in soil during winter time and increased numbers of freezing–thawing cycles are expected. Therefore, this topic is of interest for agricultural management practice, notably when intercropping systems are used over winter. Like in permafrost regions, soil thawing is mainly accompanied by an accelerated release of nutrients, but also by the emission of greenhouse gases, such as N_2O and nitric oxide (NO), as well as CO_2 and methane. Considerable research was focused on gaseous N losses and the N_2O/N_2 ratio in the last two decades (Philippot *et al.*, 2007). A modeling study by De Bruijn *et al.* (2009) indicated that N_2O emissions resulting from freezing–thawing are not monocausal and mainly depend on the amount and quality of available C and N, the microbial biomass and the redox conditions in soil after thawing. Although N_2O emissions were reported from

soils that are generally characterized by a low temperature (< 15 °C), these values are far lower than the N_2O concentrations emitted from thawing soils (Koponen & Martikainen, 2004). Wolf *et al.* (2010) could show that up to 70% of the annual N_2O emissions from agricultural fields might occur in the winter period. Peak emissions of N_2O were reported from arable soils during or shortly after thawing (Dörsch *et al.*, 2004) and could only be attributed in part to N_2O physically trapped in soil aggregates during freezing (Teepe *et al.*, 2001). A large part of N_2O arises from the microbial denitrification process, which fits with decreased oxygen and increased C and N availabilities in soils that were subject to freezing–thawing cycles (Öquist *et al.*, 2004). Sharma *et al.* (2006) observed an increase in transcripts of the nitrate and nitrite reductase genes *napA* and *nirK*, respectively, straight after thawing began. Other studies have shown a significant increase in N mineralization compared with nonfrozen soils (De Luca *et al.*, 1992). In contrast to permafrost soils, where aerobic ammonium oxidation did not play an important role, increased nitrification rates were measured after thawing in soils from moderate climatic zones. Su *et al.* (2010) demonstrated that bacterial ammonia oxidizers were impaired by freezing and thawing, whereas their archaeal counterparts even increased in abundance. This is in accordance with the hypotheses by Schleper *et al.* (2005) and Valentine (2007), who presumed that archaea are more tolerant to stress conditions than bacteria. Therefore, archaea could be the main contributors to ammonia oxidation after freezing and thawing.

Studies on the effects of changes in precipitation on microbial N turnover are rare, notably when questions about the effects of extreme weather events are addressed, although it is well accepted that the increased variability in precipitation and the resulting soil water dynamics directly alter N cycling in terrestrial ecosystems (Corre *et al.*, 2002; Aranibar *et al.*, 2004). Not surprisingly, irrigation increased, on the one hand, nitrate leaching rates mainly in sandy soils (Olson *et al.*, 2009). On the other, increased denitrification activities were measured. For example, scenarios simulating high rainfall events resulted in 2.4–13-fold increases in ammonia, nitrate, NO and N_2O fluxes in clay loam, whereas NO and N_2O fluxes decreased in sandy soils in response to water drainage (Gu & Riley, 2010). Ruser *et al.* (2006) found maximum N_2O emission rates in differently compacted soils after rewetting of dry soil that increased with the amount of water added. Muhr *et al.* (2008) postulated that rather than the intensity of rewetting, the length of the drought period might be more important for the process patterns and the microbial communities involved in N_2O and NO emissions. Again, the effects of precipitation depend on other factors like agricultural management. For example, it could be shown that the effects of irrigation depend on the type of cover crop in soil (Kallenbach *et al.*, 2010). Overall, studies mainly focused on the effects of precipitation on denitrification rates. Other processes of the N cycle as well the corresponding communities have been rarely studied so far. It must also be assumed that these processes are also highly affected directly or indirectly by dryness and precipitation, respectively. Interestingly, Zavaleta *et al.* (2003) demonstrated changes in plant diversity patterns in different grasslands in response to different precipitation regimes, which may indicate indirect effects of different precipitation regimes on nitrifiers as well as on N-fixing microorganisms.

The same authors could show that enhanced CO_2 concentrations in the atmosphere decrease plant diversity at grassland sites. However, C input into the soil via exudation was enhanced, which resulted in an overall stimulation of most microorganisms. Mainly N-fixing bacteria benefited from the additional C input, as their abundance was increased at grassland sites with increased CO_2 (He *et al.*, 2010). As expected, enhanced CO_2 concentrations also stimulated denitrifiers in soil due to a general reduction of the redox potential in soil as a result of the increased microbial activity (Pinay *et al.*, 2007). Furthermore, a stimulation of N mineralization has been proven (Muller *et al.*, 2009). Consequently, elevated CO_2 values in the atmosphere resulted in reduced abundance of autotrophic microorganisms like ammonia oxidizers (Horz *et al.*, 2004) in combination with reduced activity patterns (Barnard *et al.*, 2006) due to competition from heterotrophs as well as lower and lower activity in grassland soils. In addition, several studies have described a positive correlation between plant species richness and AOB richness in grassland soils. Malchair *et al.* (2010b) hypothesized that this link could be due to the spatial heterogeneity of ammonia, promoted by the plant species richness. In contrast, AOB were unaffected by increased atmospheric CO_2 (Nelson *et al.*, 2010) in soils under intensive agricultural use (e.g. soybean or maize cultivation), probably as the present ecotypes in these soils are already adapted to higher C input into the soil, for example, by manuring, litter application and intensive exudation by the cultivated crop. However, when relating those results to ongoing climate change, it must be considered, as described above for temperature effects, that we are challenged with an continuous increase in CO_2 concentrations in the atmosphere and not with a doubling from 1 day to another as simulated in most experiments.

Xenobiotics

New climatic conditions and changed agricultural practice have led to an emerging pressure from weeds and phytopathogens, which complicates farming practice and has resulted in the increased use of (new) chemical substances

worldwide. Pesticides, i.e. herbicides, fungicides and insecticides, can exert collateral effects on soil microorganism and important functions such as N cycling. Some of these compounds also represent a source of N to microbial communities through mineralization. For example, the ability of microorganism to use atrazine as a sole N source has been demonstrated (Mandelbaum *et al.*, 1995; Struthers *et al.*, 1998). As bioavailability of pesticides depends on the formulation as well as on diverse crop and soil factors (e.g. percentage crop cover of the soil surface, soil type, structure, pH, N and C contents, pore volume, water-holding capacity) determining sorption, leaching and degradation of the compound, the response of the microbial biomass is expected to be linked to both the soil type and the pesticide used. Moreover, herbicides are typically applied onto bare soil while fungicides and insecticides are used on dense crops and the exposure of the soil is consequently lower (Johnsen *et al.*, 2001).

The effects of pesticides on bacterial groups involved in N transformation have been thoroughly studied using cultivation-dependent methods in the past, for example, *Rhizobium* fixing N in symbiosis with leguminous plants (Aggarwal *et al.*, 1986; Kishinevsky *et al.*, 1988; Mårtensson, 1992; Revellin *et al.*, 1992; Ramos & Ribeiro, 1993; Singh & Wright, 2002), free-living diazotrophs *Azotobacter* and *Azospirillum* (Banerjee & Banerjee, 1987; Jena *et al.*, 1987; Martinez-Toledo *et al.*, 1988) and nitrifying bacteria (Doneche *et al.*, 1983; Banerjee & Banerjee, 1987; Martinez-Toledo *et al.*, 1992a, b). On the contrary, only a few recent studies have used culture-independent approaches to better gain insight into the effects on the structure and function of soil microbial communities (Engelen *et al.*, 1998; Rousseaux *et al.*, 2003; Seghers *et al.*, 2003; Devare *et al.*, 2004; Saeki & Toyota, 2004; Bending *et al.*, 2007). In many cases, pesticides applied at the recommended field rate concentration did not have a significant impact on the structure and function of the soil microbial communities (Saeki & Toyota, 2004; Ratcliff *et al.*, 2006). Seghers *et al.* (2003) demonstrated that the community structure of AOB in bulk soil of a maize monoculture was unaltered by 20 years of atrazine and metolachlor application. Some other studies have indicated more pronounced effects. Thus, Chang *et al.* (2001) observed a severe impact of atrazine on both the abundance and the community structure of AOB. However, in this study, short-term microcosm experiments were performed with high herbicide concentrations (*c.* three orders of magnitude higher than the field rates). There is also increasing evidence that chloropicrin and methyl isothiocyanate can stimulate N_2O production (Spokas & Wang, 2003; Spokas *et al.*, 2005, 2006). For other herbicides like prosulfuron, glyphosate and propanil as well as the fungicides mancozeb and chlorothalonil, decreased N_2O emissions were observed, possibly because the compounds inhibited nitrification and denitrification (Kinney *et al.*, 2005). Cernohlávková *et al.* (2009) confirmed this hypothesis and demonstrated that mancozeb and dinocap can impair nitrification at a field rate in an arable and a grassland soil.

Besides pesticides, antibiotics are also extensively used in agricultural production systems, predominantly in livestock husbandry. As slurry and manure are usually applied as organic fertilizers in agricultural farming, a substantial fraction of the administrated compounds enters the environment (Lamshöft *et al.*, 2007). Unlike pesticides, antibiotics are explicitly designed to affect microorganisms. The impact of, for example, sulfadiazine, a broad-spectrum bacteriostatic agent, has been intensively evaluated due to its frequent use, high excretion rate and persistence in soil (Thiele-Bruhn, 2003; Lamshöft *et al.*, 2007; Schauss *et al.*, 2009a). Similar to pesticides, soil and crop characteristics are major factors influencing the response patterns of the microbial communities toward antibiotics in soil (Heuer & Smalla, 2007; Hammesfahr *et al.*, 2008; Kotzerke *et al.*, 2008; Schauss *et al.*, 2009a; Ollivier *et al.*, 2010). Potential nitrification activity remained unchanged under low sulfadiazine concentration conditions in bulk soil when applied in combination with manure (Kotzerke *et al.*, 2008). This might have been due to a substitution of the highly affected AOB by their archaeal counterparts (Schauss *et al.*, 2009b). Similar observations concerning sulfadiazine effects on the abundance patterns of AOB and AOA were made in the rhizosphere of maize and clover (Ollivier *et al.*, 2010). Also, both functionally redundant groups of nitrite reducers were negatively influenced by antibiotic addition to manure. Hence, not surprisingly, potential denitrification rates decreased in treatments where sulfadiazine was applied (Kotzerke *et al.*, 2008). While nitrite reducers harboring the *nirS* gene increased in abundance after bioavailable sulfadiazine had declined, the abundance of *nirK*-harboring nitrite reducers remained on the level of the nonmanured control treatment (Kleineidam *et al.*, 2010). Clearly, pronounced effects of sulfadiazine on the denitrifying bacteria were also observed in the rhizosphere of maize and clover, where the dominating *nirK*, but also the *nirS* nitrite reducers as well as the *nosZ*-harboring N_2O reducers were significantly impaired (Ollivier *et al.*, 2010). Furthermore, the abundance of *nifH* genes, coding for key enzyme of N fixation, was significantly impacted by sulfadiazine in the rhizosphere of both plant types, but to a greater extent in the rhizosphere of the legume.

Conclusions and outlook

The research over the last two decades linking N transformation processes in soil to the corresponding functional microbial communities has improved our knowledge

significantly about the factors driving the abundance, diversity and activity mainly of microorganisms involved in the inorganic N cycle as well as the dynamics of the corresponding turnover processes and nutrient fluxes. Overall, most studies that addressed questions linked to the consequences of land-use changes or agricultural management included data for nitrifiers, denitrifiers and N-fixing microorganisms, whereas studies in the area of climate change in most cases focused only on consequences for denitrification and N_2O emissions. This reflects well the areas of interest of the various scientific communities involved in the different research areas. However, it must be taken into account that the processes of the N-cycle are closely interlinked and thus influence each other. Thus, even if the focus is on trace gas emissions from soil, knowledge of processes like nitrification and N-fixation is of key importance too. In general, data on the diversity and abundance of N-mineralizing microorganisms are rare in microbial ecology, due to the huge variety of different biochemical pathways, which are so far mostly unknown. Therefore, not surprisingly, in most studies that are of relevance for consequences of global change on N-transformation, this functional group of microorganisms has been excluded from analyses. Nevertheless, it is generally accepted that the amount of mineralized nitrogen is one major driver for the inorganic nitrogen cycle mainly in nonfertilized natural soils.

From the recently published data, the following conclusions can be drawn generally: (1) global change-related modifications of environmental factors affect nitrifiers, denitrifiers and N-fixing microorganisms and alter the corresponding processes. (2) The abundance of the autotrophic ammonia oxidizers and nitrite oxidizers in soil is negatively correlated with additional C input by plants as a result of land-use changes towards agricultural land or a more intensive agriculture as well as enhanced CO_2 concentrations in the atmosphere. This results in soils, where no inorganic fertilizer has been applied, in reduced nitrate concentrations and consequently, despite the presence of easily degradable carbon sources, in reduced denitrification activity under anoxic conditions. Although N-fixing microorganisms benefit from the additional carbon input, their activity is only increased under low ammonia concentrations in soil, for example, conditions where most of the ammonia is taken up by the plant or by soil microorganisms for biomass production. Overall, plants might benefit from this scenario due to reduced competition for ammonium with ammonia-oxidizing microorganisms in soil. Furthermore, such conditions may reduce the amount of leached nitrate as well as emissions of N_2O. (3) By contrast, ammonia oxidizers might benefit from the application of xenobiotics as AOA in particular seems to tolerate a number of compounds that, like antibiotics, are toxic for other prokaryotes (Schauss *et al.*, 2009a, b). This may result in increased nitrification rates if enough ammonia is available and consequently in the formation of nitrate. As denitrifiers might be reduced in their activity under the given scenario, nitrate could leach to the ground water, if it is not taken up by the plants. (4) Water conditions and the oxygen content in soil highly influence nitrifiers and denitrifiers. Under anoxic conditions, however, the activity of denitrifiers again depends on the amount of available nitrate and, therefore, either on fertilization regimes or the activity of nitrifiers in non-water-logged habitats in soil.

As stated in the introduction, 'global change' encompasses *interlinked* activities of the different scenarios described above. Because each scenario results in a different response pattern of the investigated microbial communities, a prediction of what happens if two or more scenarios are mixed is almost impossible. For example, whether the addition of xenobiotics and increased carbon inputs by increased atmospheric CO_2 concentrations will lead to higher or lower concentrations of nitrate in soil cannot be predicted from currently available data. However, these types of predictions are needed to transform scientific results into concrete recommendations for practice. Another important aspect of research linked to global change is to understand the long-term consequences of changes in the environment for microbial life in soil. As yet, most studies in the past have concentrated on short-term effects using sometimes highly unrealistic predictions of future conditions. Therefore, in many cases, results represent data more relevant for disturbance ecology than for global change research. As described above, this is true for many experimental setups in the frame of climate change. Finally, the different scales of relevance must be taken into account. Microorganisms act on the μm^2 scale; however, the scales that need to be addressed in terms of political recommendations are at regional or even at a global scale. And conceptual approaches to overcome the scale problem are far from being 'on the market'. This holds true for 'upscaling' from 1 g of soil to the ha or km^2 scale, but also for 'downscaling' 1 g of soil to microsites of μm^2, where microbial life occurs. In this respect, research addressing questions about the relevant scale that must be considered for different scenarios of global change is currently absent.

Authors' contribution

J.O. and S.T. contributed equally to this work.

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