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MICROBIAL NITROGEN CYCLING IN SOILS AT PIONEER SITES

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Summary

Pioneer sites such as the forefields of receeding glaciers, mobile sand dunes, and volcanic lava flows, represent ideal environments to study the development of terrestrial ecosystems. Pioneer sites are characterised by scarce vegetation and low nutrient content. Nitrogen (N) is virtually absent in soils at pioneer sites as it is not part of the minerals in the bedrock. Over the years N accumulates and plants colonise these systems, indicating development of the N cycle. Some processes within the N cycle are solely catalysed by archaea, bacteria, and fungi; therefore, microorganisms are essential to the N cycle in soil.

The first objective of this dissertation was to study the development of the N cycle in pioneer soils. Two contrasting pioneer sites were chosen; the forefield of the retreating Damma Glacier in canton Uri, Switzerland and a mobile sand dune near the town of Lieberose in Brandenburg, Germany. In the glacier forefield, a soil chronosequence was established, and samples from 10, 50, 70, 120, and more than 2000 years of soil development were analysed. The climate at the glacier is alpine. The glacier forefield receives 2400 mm rain annually and the mean temperature is between 0 and 5 °C. In contrast, the climate at the Lieberose sand dune is continental with 570 mm rain annually and a mean temperature of 8.9 °C. On the sand dune, three phases of biological soil crust (BSC) were sampled, ranging from thin BSC, dominated by cyanobacteria, to dark BSC, dominated by algae and mosses. For comparison, mobile sand without BSC and the rooting zone of *Corynephorus canescens* were sampled. At both sites, different phases of ecosystem development were investigated and characterised for abundance of microorganisms involved in the N cycle and the activity of the individual process.

In addition, the rhizosphere effect of *Leucanthemopsis alpina* on the microbial N cycling was investigated in two contrasting soils of the soil chronosequence. The objective was to assess the rhizosphere effect in initial (10 years ice-free) versus developed soil (120 years ice-free). Lastly, a novel data analysis method for the quantification of microorganisms by real-time quantitative PCR (qPCR) was developed and tested.

Four major processes dominating the terrestrial N cycle in soil were investigated for gene abundance and enzyme activity. Gene abundance was determined by qPCR, targeting the marker genes (enzyme and gene in brackets) of each of the four processes: N fixation (nitrogenase – *nifH*), mineralisation (protease – *aprA*, chitinase – *chiA*), nitrification (ammonium monooxigenase – *amoA*) and denitrification (nitrite reductases – *nirK* and *nirS*, nitrous oxide reductase – *nosZ*). Enzyme activities were measured by potential enzyme.

At the soil chronosequence of the glacier forefield, the soil development was associated with an increase in the soil nutrients, the abundance of marker genes, and the potential enzyme activities. Potential N fixation activity was only detected in developed soil and ranged between 1 and 3 pmol N g^{-1} h^{-1}. Similarly, potential nitrification and denitrification activity were low in the initial soil but increased in the developed soil. The potential nitrification rate increased from 0.1 to 2.3 nmol NO_2^--N g^{-1} h^{-1}, while the potential denitrification rate increased from 1.8 to 40 nmol N_2O-N g^{-1} h^{-1}. In contrast, chitinase and protease activity were high in the initial soil (14 and 5 nmol MUF g^{-1} h^{-1}) and showed a comparatively minor increase to 72 and 25 nmol MUF g^{-1} h^{-1}, respectively. Gene abundance of protease (*aprA*), chitinase (*chiA*), ammonium monooxygenase (*amoA*) of archaea, and nitrous oxide reductase (*nosZ*) were correlated with the corresponding potential enzyme activities. However, nitrogenase (*nifH*) gene abundance was highest in the 50 year old soil (2×10^2 copies g^{-1}) and did not match the potential enzyme activity.

The rhizosphere effect of *Leucanthemopsis alpina* was associated with an increased abundance of the *nifH* gene in the initial soil (1.7×10^7 copies g^{-1}) compared to the developed soil (10^7 copies g^{-1}). Conversely, the *nosZ* gene was most abundant in the rhizosphere of the developed soil (5.1×10^7 copies g^{-1}). This indicates that the plants in the initial soils containing low amounts of N stimulated the process of N fixation. The presence of plants in the developed soil with high amounts of available nitrate, however, stimulated the denitrification process.

At the Lieberose sand dune, the BSC development was characterised by an increase in nutrient and chlorophyll content. Similarly, potential enzyme activities and gene abundance increased. In the developed BSCs, nitrification (3.5 nmol NO_2^--N g^{-1} h^{-1}) appeared to be the dominant process but denitrification (1.2 nmol N_2O-N g^{-1} h^{-1}) and mineralisation (1.5 nmol MUF g^{-1} h^{-1}) were also involved in N cycling. Compared to the other processes N fixation was very low in all BSCs (<30 pmol C_2H_4 g^{-1} h^{-1}).

The last part of the study was dedicated to the improving of real-time quantitative PCR (qPCR) data analysis. Currently, the standard curve (SC) method

is commonly used in environmental microbiology. As an alternative absolute quantification method, the One-Point-Calibration (OPC) method was developed and tested. The experiments demonstrated the importance of accounting for amplification efficiency in analysing samples of unknown composition. Quantification by the OPC method were accurate while the SC method over- or underestimated the copy numbers up to 5-fold.

In summary, the N cycle development in the glacier forefield and the sand dune followed similar patterns. As a general rule, at pioneer sites three stages of N cycle development can be distinguished: the heterotrophic stage, the transition stage, and the developed stage. During the heterotrophic stage, a community of opportunistic microorganisms feeds on allochthonous and recalcitrant organic matter, and the process of mineralisation dominates the N cycle. In the transition stage, primary producers such as BSC or plants colonise the system and stabilise the soil surface; N starts to accumulate. During the developed stage, nitrification and denitrification gain importance and a closed N cycle, within which all processes are active, is established.

Zusammenfassung

Pionierstandorte – wie die Vorfelder schmelzender Gletscher, mobile Sanddünen oder vulkanische Lavaströme – stellen ideale Umgebungen dar, um die Entwicklung von terrestrischen Ökosystemen zu untersuchen. Sie zeichnen sich durch karge Vegetation und geringen Nährstoffgehalt aus. Stickstoff (N) ist dort anfangs kaum vorhanden, da dieser kein Mineralbestandteil des Muttergesteins ist. Dennoch reichert sich N innerhalb weniger Jahre im Boden an, und Pflanzen besiedeln den Lebensraum, was auf eine schnelle Entwicklung des Stickstoffkreislaufes hindeutet. Innerhalb des N Kreislaufes werden viele Prozesse nur durch Archaea, Bacteria und Fungi vollzogen. Daher regulieren Mikroorganismen den Stickstoffkreislauf im Boden.

Ziel dieser Arbeit war es, die Entwicklung des Stickstoffkreislaufes in Pionierstandorten zu untersuchen. Dazu wurden zwei gegensätzliche Pionierstandorte betrachtet. Zum einen, das Vorfeld des abschmelzenden Damma Gletschers im Kanton Uri, Schweiz. Zum anderen, eine mobile Sanddüne nahe der Ortschaft Lieberose in Brandenburg, Deutschland. Im Gletschervorfeld besteht eine Bodenchronosequenz von der Bodenproben entnommen und untersucht wurden, die seit 10, 50, 70, 120 und mehr als 2000 Jahren eisfrei sind. Das Standortklima ist alpin. Der Jahrestemperaturdurchschnitt beträgt 0 bis 5 °C und der Jahresniederschlag 2400 mm. Im Gegensatz dazu ist das Klima an der Lieberose Düne kontinental. Der Jahresniederschlag beträgt 570 mm und die Jahresdurchnittstemperatur 8.9 °C. Auf der Düne können verschiedene Stadien von biologischen Bodenkrusten (BSC) beobachtet werden, von initialen Krusten (dominiert von Cyanobakterien) bis hin zu dunklen Krusten (dominiert von Algen und Moosen). Drei Stadien dieser Krusten, mobiler Dünensand und der Wurzelraum von benachbartem Silbergras (*Corynephorus canescens*) wurden untersucht. An beiden Standorten wurde die Entwicklung des Stickstoffkreislaufes durch zwei verschiedene Parameter charakterisiert. Erstens, die Abundanz der am Stickstoffkreislauf involvierten Mikroorganismen. Zweitens, die Aktivität der Stickstoffumwandlungsprozesse.

Eine weitere Fragestellung war der Einfluss der Alpenmargerite (*Leucanthemopsis alpina*) auf die Abundanz der stickstoffumsetzenden Mikroorganismen. Hierbei wurden Unterschiede im Rhizospäreneffekt zwischen initialem Boden

(10 Jahre eisfrei) und entwickeltem Boden (120 Jahre eisfrei) betrachtet. Weiterhin wurde eine neue Datenanalysemethode für die Auswertung von qPCR entwickelt und getestet.

Der terrestrische Stickstoffkreislauf wird durch vier Prozesse dominiert, die mittels Genabundanz und Enzymassays charakterisiert wurden (Enzyme und Gene in Klammern): Stickstofffixierung (Nitrogenase – *nifH*), Mineralisation (Protease – *aprA*, Chitinase – *chiA*), Nitrifikation (Ammonium-monooxygenase – *amoA*), Denitrifikation (Nitritreduktasen – *nirK* und *nirS*, Distickstoffmonoxid-Reduktase – *nosZ*). Die Genabundanzen funktionaler Markergene wurden mit der qPCR Methode quantifiziert. Die zugehörigen potentielle Enzymaktivitäten wurden mit etablierten Methoden gemessen.

Entlang der Chronosequenz stiegen Nährstoffgehalte, Genabundanzen und potentielle Enzymaktivitäten zusammen mit dem Bodenalter an. Die potentielle Stickstofffixierung lag nur in entwickeltem Boden (120 und >2000 Jahre) über der Nachweisgrenze und betrug zwischen 1 und 3 pmol N g^{-1} h^{-1}. Ganz ähnlich waren potentielle Nitrifikation und Denitrifikation von geringer Aktivität in den initialen Böden, die mit zunehmender Bodenentwicklung aber stark anstieg. Die potentielle Nitrifikation stieg von 0.1 auf 2.3 nmol NO_2^--N g^{-1} h^{-1} und die potentielle Denitrifikation stieg von 1.8 auf 40 nmol N_2O-N g^{-1} h^{-1}. Im Gegensatz dazu waren potentielle Chitinase- und Proteaseaktivität in den initialen Böden relativ hoch (14 bzw. 5 nmol MUF g^{-1} h^{-1}) und zeigten nur einen geringen Anstieg auf 72 bzw. 25 nmol MUF g^{-1} h^{-1}. Genabundanzen von Protease (*aprA*), Chitinase (*chiA*), archaeelle Ammonium-monooxygenase (*amoA*) und Distickstoffmonoxid-Reduktase (*nosZ*) korrelierten mit den entsprechenden potentiellen Enzymaktivitäten. Die Abundanz des Nitrogenase Gens (*nifH*) jedoch war am höchsten in demjenigen Boden, der 50 Jahre eisfrei war (2×10^2 Kopien g^{-1}) und korrelierte nicht mit der gemessenen potentiellen Stickstofffixierungsrate.

Der Effekt der Rhizosphäre von *Leucanthemopsis alpina* auf die Abundanz des *nifH* Gens war im initialen Boden (10 Jahre eisfrei) am größten. Dort wurden 1.7×10^7 Kopien g^{-1} gemessen, wohingegen im entwickelten Boden (120 Jahre eisfrei) nur 10^7 Kopien g^{-1} gemessen wurden. Im Gegensatz zum *nosZ* Gen, das am häufigsten in der Rhizosphäre im entwickelten Boden anzutreffen war (5.1×10^7 Kopien g^{-1}). Dies deutet darauf hin, dass Pflanzen die im wenig Nährstoffe enthaltenden initialen Boden wachsen die Stickstofffixierung anregen. Im entwickelten Boden hingegen, der relativ höhere Gehalte an Nitrat aufweist, wurde durch die Pflanzen die Denitrifikation stimuliert.

Die fortschreitende Entwicklung der biologischen Krusten auf der Lieberose Düne ist durch eine Zunahme der Nährstoffgehalte des Chlorophyllgehaltes gekennzeichnet. Genauso nehmen Genabundanzen und potenzielle Enzymaktivitäten zu. In den entwickelten BSC erschien der Prozess der Nitrifikation dominant (3.5 nmol NO_2^--N g^{-1} h^{-1}), jedoch waren auch Denitrifikation (1.2 nmol N_2O-N g^{-1} h^{-1}) und Mineralisation (1.2 nmol N_2O-N g^{-1} h^{-1}) am Stickstoffkreislauf beteiligt. Potentielle Stickstofffixierung war in allen Entwicklungsstadien vergleichsweise gering (<30 pmol C_2H_4 g^{-1} h^{-1}).

Im letzten Teil dieser Arbeit wurde an einer neuen Methode der Datenanalyse für real-time quantitative PCR (qPCR) gearbeitet. Zur Zeit wird in der Umweltmikrobiologie für die qPCR Datenanalyse die Standardkurven-Methode (SC) benutzt. Als alternative Methode zur absoluten Quantifizierung wurde die Ein-Punkt-Kalibrations-Methode (OPC) entwickelt und getestet. Die Experimente machten deutlich, wie wichtig die Beachtung der qPCR-Effizienz ist, wenn Proben unbekannter Zusammensetzung amplifiziert werden.

Zusammenfassend lässt sich festhalten, dass sich der Stickstoffkreislauf sowohl im Gletschervorfeld, als auch in der Sanddüne nach einem ähnlichen Muster entwickelte. Danach können an Pionierstandorten drei Phasen unterschieden werden: die heterotrophe Phase, die Übergangsphase und die entwickelte Phase. Während der heterotrophen Phase besteht eine Gemeinschaft aus opportunistischen Mikroorganismen, die sich von allochthonem und rekalzitrantem organischen Material ernähren. Die Phase wird vom Prozess der Mineralisation dominiert. In der Übergangsphase etablieren sich Primärproduzenten wie BSC oder Pflanzen, die die Bodenoberfläche stabilisieren; somit kann sich Stickstoff im Boden akkumulieren. In der entwickelten Phase gewinnen dann Nitrifikation und Denitrifikation an Einfluss was zur Folge hat, dass sich der Stickstoffkreislauf schließt.

1 General introduction

1.1 Ecosystem development

Pioneer sites, such as sand dunes (Beyschlag et al., 2008), glacier forefields (Chapin et al., 1994), volcanic lava flows (Cutler et al., 2008), and river floodplains (Kalliola et al., 1991) represent the initial starting point of terrestrial ecosystem development and can be found worldwide (Belnap et al., 2001). They are used as model systems to study the process of ecosystem development. First studies on ecosystem development at pioneer sites were conducted on sand dunes. Warming (1902) studied and explained plant community succession on sand dunes on the coast of Denmark during the second half of the 19th century. A few years later, Cowles (1899) followed and described how physical, chemical and biological parameters shape sand dune ecosystems at Lake Michigan, USA.

Ecosystems develop over time from initial to mature states (Odum, 1969). During the development, biological, chemical, and physical properties of an ecosystem change significantly. For example, the biological community structure of the ecosystem changes from a grass and herb dominated plant community to a shrub and tree dominated one. Moreover, species diversity, organic matter content, and biochemical diversity increase, as plant communities establish and supersede one another (Table 1.1, Figure 1.1). The communities shift from a high net productivity and simple linear food webs in the initial state, to low net productivity and complex food webs in the mature states (Odum, 1969).

From these considerations of ecosystem development, fundamental implications for land use practices and agronomy emerge because the two parameters net community productivity and ecosystem stability depend on the development of the ecosystem (Odum, 1969; Schläpfer and Schmid, 1999). This will be illustrated in two examples. In agricultural systems, high productivity is the objective. As productivity is the highest in early phases of ecosystem development, agricultural systems are kept in initial phases of development to maximise the yield (Odum, 1969). In urban systems, one objective might be the constant fresh water supply from the mountains. Since mature ecosystems buffer water

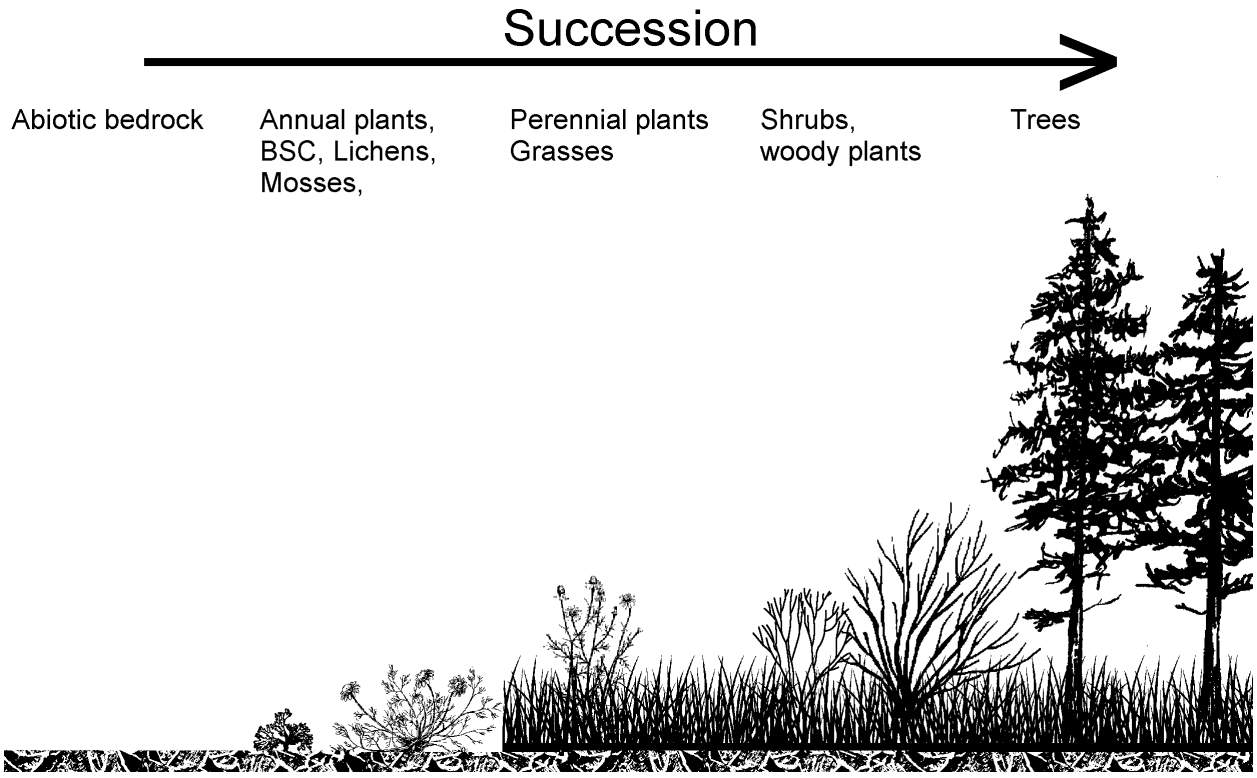

Figure 1.1: Plant community changes during succession (modified after Chapin et al., 1994).

flow considerably better than initial ecosystems, a mature phase of ecosystem development, i.e. a forest, is desired in order to achieve this objective (Körner, 2004).

1.2 Global nitrogen budget

Nitrogen is a macronutrient that is essential to all living organisms (Francis et al., 2007). However, N is often the limiting nutrient in terrestrial and marine ecosystems (Elser et al., 2007) and plays a critical role in controlling the primary production (Gruber and Galloway, 2008). Anthropogenic fertilizer production via the Haber-Bosch process enabled to increase the world food production and to sustain the increasing world population. However, the production of fertilisers contributes to an additional 100 Tg N per year to the global N budget. On the global basis this is in the range of the terrestrial biological N fixation of 140 Tg N per year (Figure 1.2) and accelerates the global N turnover. This has negative consequences, such as, acidification, eutrophication, and loss of species diversity (Vitousek et al., 2002; Gruber and Galloway, 2008). Moreover, denitrification might be stimulated by the increase in N availability, contributing to the release of the greenhouse gas nitrous oxide (N_2O). In the past 60,000 years

Table 1.1: Definitions of ecological and biological concepts.

Concept	Definition[a]
Species diversity	The number of different species in an ecosystem. Different approaches exist to calculate indices of species diversity, e.g., Shannon index, Simpson index.
Biochemical diversity	Characterises the number of different biochemicals and biochemical interactions in an ecosystem. For example the number of degradation pathways of a certain compound that are catalysed in the ecosystem.
Genetic diversity	The total number of genetic characteristics in a species. On the population level, genetic diversity allows to adapt to changing environmental conditions.
Ecological niche	Describes the relational position and role of an individual, a species, or a population in its ecosystem. The term is theoretical and encompasses a multitude of aspects. Therefore, a niche can not be characterised completely. However, at a sub level, the niche concept proved valuable, e.g., food niche, nesting niche.
Ecological stability	Describes the capability of an ecosystems to remain despite changing external conditions, e.g., environmental conditions, disturbance. Stability is differentiated into resilience (to quickly return to the previous state) and persistence (to constantly persist in one state).
Food web	Represents the feeding connections in an ecological community. It illustrates the complex feeding interactions and relations of the various organisms.
Limiting nutrient	The nutrient that controls the growth of an individual organism, a species, or a population.
Net community productivity	The gross production of the community, mainly by phototrophs, minus the respiration of the entire community, i.e. phototrophs and heterotrophs.

[a] modified after Kuttler, 1995.

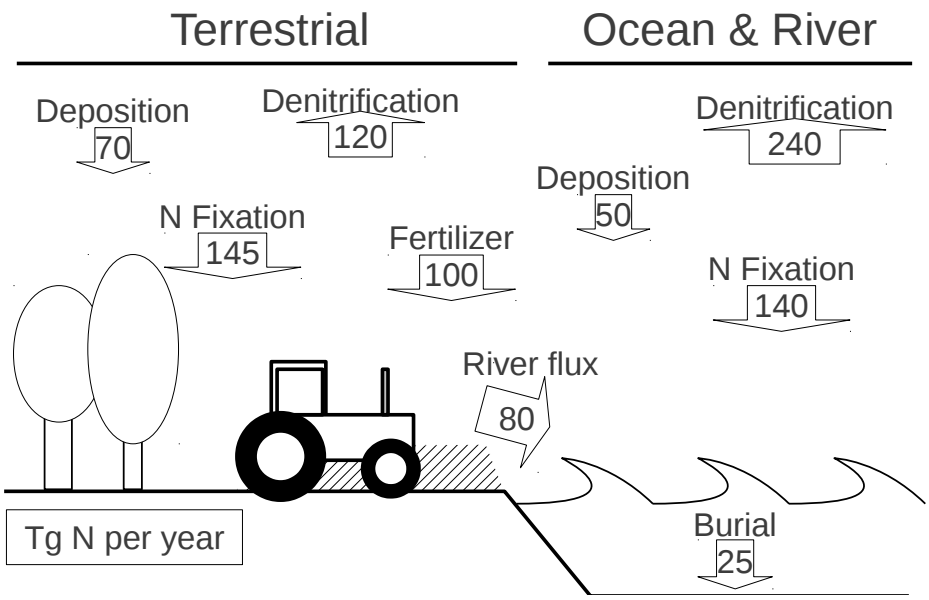

Figure 1.2: Estimated global nitrogen fluxes in Tg N a^{-1} (after Gruber and Galloway, 2008).

climate record, high N_2O concentrations have been linked to warm periods; the underlying mechanism, however, has not yet been identified (Gruber and Galloway, 2008).

1.3 Nitrogen cycle

Microorganisms, plants and animals, use different N species as their N source (Schimel and Bennett, 2004). Depending on the organism, the N source can range from complex organic molecules, such as amino acids and amino sugars to inorganic molecules, such as ammonium and nitrate. Naturally all different N species are transformed into one another by chemical and biochemical processes, which are summarised in Figure 1.3. The reduction of elemental N (N_2) to ammonium or amino acids is called **N fixation**. It is one of the most important processes of the N cycle, since it contributes atmospheric N to the ecosystem (Vitousek and Howarth, 1991). The process of **mineralisation** releases freely available N species that can be taken up by organisms (Schimel and Bennett, 2004). Therefore, mineralisation refers to the decomposition (and release) of any complex organic matter into small molecules that contain reduced N, i.e. amino sugars, amino acids, and ammonium. This definition is important, as the term mineralisation has been used differently, e.g., for the release of ammonium from the breakdown of organic matter (Schimel and Bennett, 2004). In the process of **nitrification**, ammonium is oxidised to nitrate (De Boer and Kowalchuk, 2001). The counter reaction is **nitrate reduction**,

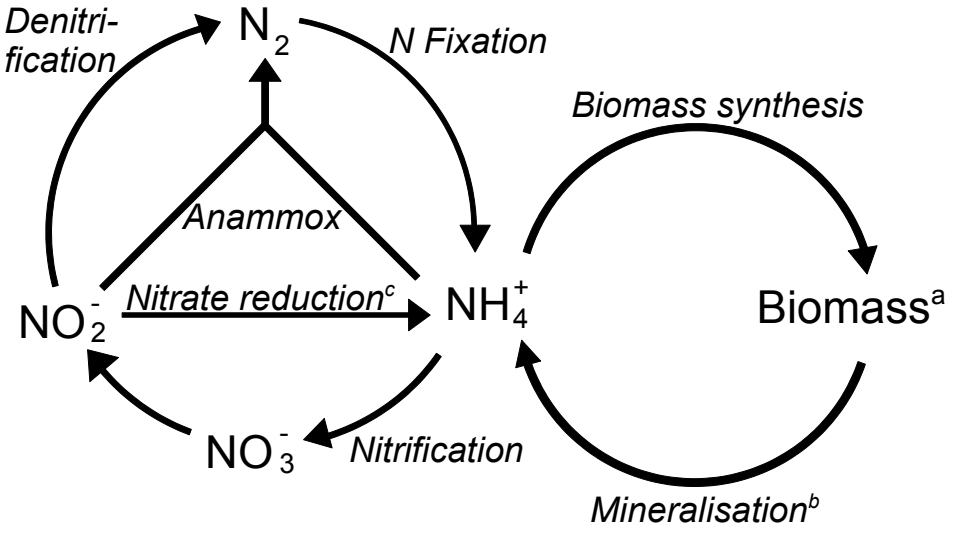

Figure 1.3: The microbial N cycle. Footnotes: [a]biomass stands for N in living organisms and soil organic matter (dead), e.g., proteins, peptidoglycan, and chitin. [b]mineralisation stands for the break down of complex organic matter into small molecules, e.g., *N*-Acetylglucosamine, amino acids, ammonium, which all contain reduced N.[c]assimilatory and respiratory nitrate reduction have be differentiated here.

which reduces nitrate to ammonium (Buresh and Patrick, 1978; Zumft, 1997). Finally, the processes **anaerobic ammonium oxidation** (**anammox**) and **denitrification** produce elemental N (Zumft, 1997; Francis et al., 2007).

Microorganisms drive the global N cycle. First, some processes of the N cycle can not be catalysed by higher organisms, therefore microorganisms control these processes. This is the case for N fixation, nitrification, anammox, and denitrification. Second, the microbial N turnover and microbial N pools are enormous in comparison to anthropogenic or plant N turnover. Biological N fixation, for example, reaches 285 Tg N per year (terrestrial and aquatic). In comparison, annual fertilizer production is 100 Tg N per year (Gruber and Galloway, 2008).

1.4 Thermodynamical considerations

Nitrogen occurs in different species, which vary by their oxidation states ranging from -3 in ammonia and amines to $+5$ in nitrate (Table 1.2). The biochemical processes outlined above oxidise or reduce these N species into one another. Considerations of the underlying thermodynamics provide valuable information about the energetics of the microbial metabolisms and hint at potential

Table 1.2: Oxidation states of important nitrogen species (after Reineke and Schlömann, 2007).

Species	Oxidation state of N	Occurrence
amine (R–NH_2)	-3	amino acids
ammonia (NH_3); ammonium (NH_3^+)	-3	gas; dissolved ion, salt
hydrazine (H_2N–NH_2)	-2	intermediate of anammox process
hydroxylamine (NH_4NO_2)	-1	intermediate of anammox process
molecular nitrogen (N_2)	0	gas, 78% (v/v) of the atmosphere
nitrous oxide (N_2O)	+1	gas
nitric oxide (NO)	+2	gas
nitrite (NO_2^-)	+3	dissolved ion, salt
nitrogen dioxide (NO_2)	+4	gas
nitrate (NO_3^-)	+5	dissolved ion, salt

bottlenecks. For example the process of N fixation is highly energy demanding ($\Delta G^{0\prime}$ =355 kJ per mol NH_4^+, Lewis, 1986). During the fixation of one molecule of elemental N_2, eight electrons are transferred and at least sixteen molecules ATP are hydrolysed, $N_2 + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2$ (Bothe, 2007). In addition, for the activation of the triple bond a very low redox potential is necessary. This causes the catalysing enzyme, nitrogenase, to be highy sensitive to oxygen that irreversibly inactivates the enzyme (Madigan, 2009).

Using the redox potential of the chemical reactions (Table 1.3) it is possible to calculate the free-energy yield $\Delta G^{0\prime}$ per reaction (Equation 1.1), where n is the number of transfered electrons, F is the Faraday constant (96.48 kJ V^{-1} mol^{-1}), and $\Delta E_0'$ is the difference in potentials. For example the denitrification process using formate as electron donor (CO_2/formate) and nitrate as electron acceptor (NO_3^-/NO_2^-), $\Delta E_0' = -0.42 - 0.43 = -0.85\,V$, yields a free-energy of $\Delta G^{0\prime} = -2 \times 96.48\,kJ\,V^{-1}\,mol^{-1} \times -0.84\,V = 164.0\,kJ$ per mol nitrate. In comparison, aerobically metabolising formate using oxygen as electron acceptor yields 239.3 kJ per mol $\frac{1}{2}O_2$.

$$\Delta G^{0\prime} = -n \times F \times \Delta E_0' \tag{1.1}$$

1.5 Microorganisms in the nitrogen cycle

The N cycle comprises diverse processes that in some cases run in parallel, but in some cases also reverse one another. Processes such as the oxidation of ammonium and the reduction of nitrate, reverse one another. At the same time, the three processes, nitrate reduction, denitrification, and anammox compete

Table 1.3: Reaction potentials E'_0(pH 7) of selected half reactions.

Redox couple	Number of transferred electrons	E'_0(pH 7) [V][a]
CO_2/formate	$4e^-$	-0.42
NO_2^-/NH_2OH	$4e^-$	+0.06
NH_4^+, NO/N_2H_4	$3e^-$	+0.34
NO_2^-/NO	$1e^-$	+0.34
NO_3^-/NH_4^+	$8e^-$	+0.36
NO_3^-/NO_2^-	$2e^-$	+0.43
N_2/NH_4^+	$8e^-$	+0.50[b]
$NO_3^-/\frac{1}{2}N_2$	$5e^-$	+0.74
N_2/N_2H_4	$4e^-$	+0.75
NO_2^-/NH_4^+	$6e^-$	+0.75
NH_2OH/NH_4^+	$2e^-$	+0.73
$\frac{1}{2}O_2/H_2O$	$2e^-$	+0.82
$NO/\frac{1}{2}N_2O$	$1e^-$	+1.17
$\frac{1}{2}N_2O/\frac{1}{2}N_2$	$1e^-$	+1.36

[a] Values taken from Jetten et al. (2009), except of half reactions CO_2/formate and $\frac{1}{2}O_2/H_2O$, which were taken from Madigan (2009).

[b] Calculated from$\Delta G^{0\prime}$ = 355 kJ per mol NH_4^+ (Lewis, 1986).

to reduce nitrite (Figure 1.3). This diversity of N cycling processes is reflected by the diversity of the microorganisms involved in the N cycle.

The diversity of the microorganisms is apparent from the diverse phylogeny of the organisms and the diverse ecological niches necessary to catalyse a certain process. While some of the N cycling processes are phylogenetically widespread, e.g., nitrate reduction, other processes appear to be linked to one narrow phylogenetic group, e.g., anammox (Table 1.4). Similarly, some processes appear to be catalysed by microorganisms of one ecological niche only, e.g., nitrification, while others are catalysed by microorganisms of diverse ecological niches, e.g., N fixation. This has important implications for the emergence of the N cycling processes during ecosystem development.

The emergence of a process in an ecosystem is linked to the establishment of the microbial species catalysing the process. Consequently, if a process is only catalysed by one narrow group of organisms, the specific niche for these organisms has to be present. In contrast, a process might occur more readily, if a process is catalysed by a diverse set of organisms, because functional redundancy allows different species to perform the same process. In this case, the occurrence of a process is less dependent on the availability of a specific niche.

Table 1.4: Microbial groups involved in the N turnover processes.

Process	Phylogenetic groups[a]	Prevalence[a]	Ecological niche
N fixation	Archaea, Bacteria	widespread	diverse[b]
Mineralisation	Archaea, Bacteria, Eukaryota	widespread	chemoorganoheterotrophs mainly
Nitrification	β- and γ-Proteobacteria, and Cren- and Thaumarchaeota	narrow	chemolithoautotrophs[c]
Nitrate reduction	Archaea, Bacteria, Eukaryota	widespread	diverse[b]
Anammox	Planctomyces (Bacteria)	narrow	chemolithoautotrophs, anaerobic
Denitrification	Archaea, Bacteria, Fungi	widespread	diverse[d]

[a]information from UniProt database (http:\\www.uniprot.org), January 2012.
[b]the process occurs under aerobic as well as anaerobic conditions. The organisms use different energy, reducing equivalent, and carbon sources.
[c]studies suggested that some archaea involved in nitrification might be mixotrophic (Hallam et al., 2006; Erguder et al., 2009), however, cultivated strains are chemolithoautotrophs. See Schmidt et al. (2002) for review.
[d]see Zumft (1997) for review.

1.6 Methods to study microbial nitrogen cycling

The microbial N cycle can be studied at different levels, ranging from subcellular to ecosystem scale (Bru et al., 2011). Four levels, which have frequently been used, will be presented here (Figure 1.4) and the methods to study them will be discussed. First, the **DNA level** characterises the genetic information that a microorganism harbours. If the information to perform a process is present, the organism potentially can catalyse this process. Second, the **mRNA level** is the intermediate level between DNA and protein level. In the microbial cell, DNA is transcribed into mRNA and the mRNA is then translated into the protein (Madigan, 2009). Third, most biological functions are catalysed on the **protein level**. Fourth and last, the **turnover level** characterises a chemical reaction by measuring reagent removal or product formation.

1.6.1 DNA based characterisation

The DNA of microorganisms contains the genetic information for proteins and enzymes. Therefore, it is possible to infer from the presence of genetic information to the potential enzymatic reactions that a microorganism or an entire

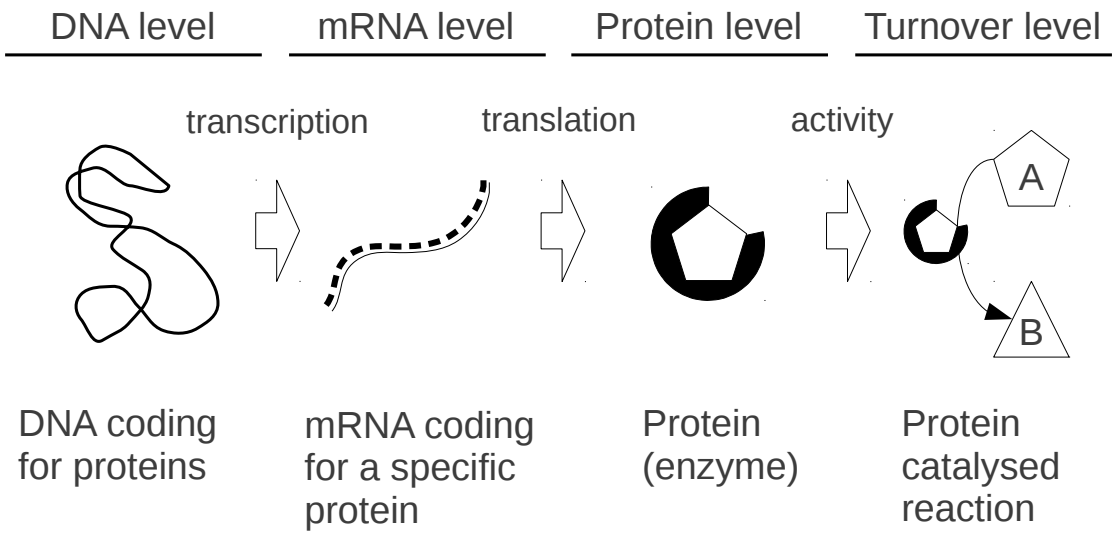

Figure 1.4: The four levels that can be used to characterise a microbial community regarding is contribution to N cycling processes.

microbial community can catalyse (Aneja et al., 2004; Sharma et al., 2007). Cultivation-independent approaches such as polymerase chain reaction (PCR) based techniques (Muyzer et al., 1993), fluorescent *in situ* hybridisation (FISH) (Amann et al., 1995), or metagenome analysis (Tyson et al., 2004) are used to characterise microbial communities by their genetic information on the DNA level.

Currently, a number of molecular probes for PCR and FISH techniques targeting functional genes coding for key enzymes of the N cycle are available. An overview is given in Table 1.5. Nitrogenase, for example, is the key enzyme for the N fixation process. The beta-subunit of the nitrogenase, coded by the *nifH* gene, was frequently used as marker gene to analyse the microbial community responsible for N fixation (for review see Zehr and Turner, 2001).

Using PCR based techniques two approaches have to be distinguished: qualitative and quantitative analysis of the microbial communities. The qualitative aspect—"who is it"— is addressed by clone libraries (Duc et al., 2009b), terminal restriction fragment length polymorphism (T-RFLP) (Lazzaro et al., 2009), denaturing gradient gel electrophoresis (DGGE) (Bürgmann et al., 2005), or micro-arrays (Duc et al., 2009a).

The quantitative analysis of microbial communities—"how many are there"— regularly employs real-time quantitative PCR (qPCR). It is a powerful technique, which is frequently used in different fields of science, such as medical

Table 1.5: Selection of molecular probes targeting functional genes involved in the N cycle.

Process, Enzymes	Marker genes	Primer or probes	References
N fixation			
Nitrogenase, EC 1.18.6.1	*nifH*	nifHF/nifR	Rösch et al. (2002)
		PolF/PolR	Poly et al. (2001)
		FISH probes	Pilhofer et al. (2009)
	nifD	FdB261/FdB260	Stoltzfus et al. (1997)
Mineralisation			
Chitinase, EC 3.2.1.14	*chiA*	chif1/chir	Xiao et al. (2005)
Metalloendopeptidases, EC 3.4.24.-	*aprA*[b]	FP aprI/RP aprII	Bach et al. (2001)
Nitrification			
Ammonia monooxygenase, EC 1.14.99.39	*amoA*	amoA-1f/amoA-2r	Rotthauwe et al. (1997)
		amoA19F	Leininger et al. (2006)
		CrenamoA616r48x	Schauss et al. (2009)
Nitrate reduction			
Assimilatory nitrate reductase, EC 1.7.1.1	*nasA*	nas22 /nasA1735	Allen et al. (2001)
Periplasmic nitrate reductase[a], EC 1.7.99.4	*napA*	napA V66 F/ napA V67 R	Flanagan et al. (1999)
Respiratory nitrate reductase, EC 1.7.99.4	*narG*	narG1960m2f/ narG2050m2r	López-Gutiérrez et al. (2004)
Anammox			
Hydrazine oxidoreductase, EC 1.7.99.8	*hzoAB*	hzoAB4F/ hzoAB4R	Hirsch et al. (2011)
Hydrazine synthase	*hzsA*	hzsA_526F/ hzsA_1857R	Harhangi et al. (2011)
Denitrification			
Nitrite reductase, EC 1.7.2.1	*nirK*	nirK1F/nirK5R	Braker et al. (1998)
		nirK876/nirK1040	Henry et al. (2005)
		FISH probes	Pratscher et al. (2009)
	nirS	nirS1F/nirS4R	Braker et al. (1998)
		F3nirS/3Rcd	Throback et al. (2004)
Nitrous-oxide reductase, EC 1.7.2.4	*nosZ*	nosZ2F/nosZ2R	Henry et al. (2006)
		Nos661F/Nos1773R	Scala and Kerkhof (1998)

[a]Periplasmatic nitrate reductases are involved in assimilatory as well as in respiratory nitrate reduction (Richardson et al., 2001).

[b]The gene name *aprA* is used for different proteases. Here it means aprA metallopeptidase (*Pseudomonas fluorescens*) of peptidase family M10, subfamily B, M10.060 (MEROPS database, http:\\merops.sanger.ac.uk, January 2012).

diagnostics (Espy et al., 2006; Mackay et al., 2002), quality assurance (Canales et al., 2006), and gene expression analysis (Wong and Medrano, 2005). In the field of microbial ecology, qPCR became an important tool because it enables cultivation-independent quantification of microorganisms by marker genes (Saleh-Lakha et al., 2005; Smith et al., 2006). Applications in this field are the quantification of specific phylogenetic groups by targeting the 16S rRNA gene (Bekele et al., 2011; De Gregoris et al., 2011) and the quantification of functional genes to quantify the genetic potential of a community (Wallenstein and Vilgalys, 2005; Schauss et al., 2009).

qPCR is based on the real-time monitoring of the amplicon formation by a reporter molecule (e.g. SYBR Green dye, hydrolysis probes) (for review see Bustin, 2004a). The fluorescence of the reporter molecule is measured after each temperature cycle and is proportional to the amount of synthesised amplicon (Figure 1.5 A). During the PCR, the amplicon concentration N_C grows exponentially and can be described by an exponential function of template starting concentration N_0, efficiency of the qPCR E and the number of qPCR cycles C (Equation 1.2).

$$N_C = N_0 \times E^C \tag{1.2}$$

The concept of the threshold cycle C_t is crucial to qPCR analysis. The C_t is the number of cycles that are necessary for one particular sample to reach a certain threshold fluorescence Y_t (Figure 1.5 A). Y_t is set the same for all samples of one experimental setup. Passing the C_t, all samples contain the same number of amplicon N_t, because fluorescence is a relative measure of DNA content ($N_t \propto Y_t$) (Ruijter et al., 2009).

To quantify the template starting concentration in a sample $N_{0\,\mathrm{sample}}$, the standard curve method is commonly used. The method employs a dilution series of known template concentrations N_0 in the qPCR assay. Then a linear regression of $\log(N_0)$ versus the C_t of the standard series gives the standard curve that is used to calculate $N_{0\,\mathrm{sample}}$ from $C_{t\,\mathrm{sample}}$ (Equation 1.3, Figure 1.5 B).

$$C_{t\,\mathrm{sample}} = a + b * \log(N_{0\,\mathrm{sample}}) \tag{1.3}$$

1.6.2 mRNA based characterisation

Metabolic activity is regulated by the genetic machinery. In order to activate a specific process, the specific gene has to be expressed as protein: the DNA

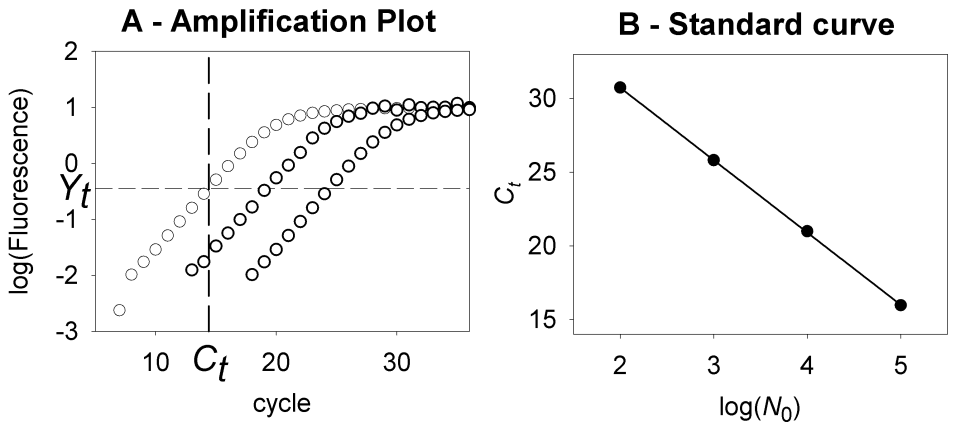

Figure 1.5: A - Amplification plot showing log fluorescence versus cycle number. Dashed lines indicate threshold fluorescence Y_t and threshold cycle C_t. B - Standard curve, plotting C_t over $\log(N_0)$.

is transcribed into mRNA that is then translated into the protein. Therefore, the detection of mRNA of a specific gene gives indication that the process is currently being activated (Aneja et al., 2004).

Techniques to study mRNA quality and quantity are similar to that on the DNA level. mRNA can be detected by PCR based techniques, FISH, or transcriptome analysis. To detect mRNA by PCR based techniques, the mRNA needs to be reverse transcribed to cDNA by a reverse transcription step (RT).

mRNA of microbial community samples is frequently quantified by RT-qPCR (Smith and Osborn, 2009). The quantification of mRNA, however, is challenged by three mechanisms. First, the biological half-life of mRNA in the cell is low. It was argued that mRNA analysis provide snapshots of the cell regulation only, while the enzymes are chemically active over a far longer period of time (Harrold et al., 1991). Second, the chemical half-life of mRNA is low. During mRNA extraction from the sample, mRNA is susceptible to degradation and might not represent the original amounts (Bürgmann et al., 2003). Last, the RT reaction performs with unknown efficiency, ranging from 2 to 100 %. For these reasons, quantification of mRNA by RT-qPCR poses significant challenges.

1.6.3 Protein based characterisation

The catalytic units of the N cycle are the active proteins. It is possible to identify and quantify the proteins from environmental samples by antibody staining, e.g., ELISA (Wright and Upadhyaya, 1996) or metaproteome approaches including protein sequencing (Benndorf et al 2007). The protein extraction from the sample matrix, however, is the crucial step (Ogunseitan, 2006; Chen

et al., 2009). Contamination of the protein extract or protein degradation, often hamper protein based analysis (Ogunseitan, 2006; Benndorf et al., 2007; Chen et al., 2009). In particular, co-extracted contaminants, such as humic substances, compromise downstream analysis. To date, quantitative analysis of proteins in soil samples requires further method development to be applied to specific questions in the N cycle.

1.6.4 Quantification on nitrogen turnover level

Actual N turnover can be quantified in soil samples in the lab (*in vitro*) or in the field (*in situ*). A wide set of assays is established to quantify N turnover. Direct methods measure the depletion of the reagent or the accumulation of the reaction product, e.g., the potential denitrification assay. Indirect methods measure the reaction of an alternative substrate that reacts in the same reaction mechanism but is more easily detectable, e.g., the acetylene reduction assay.

Moreover, two stabile isotopes of N, ^{14}N and ^{15}N, exist and can be used to calculate N transformation rates. Two approaches have to be differentiated. First, ^{15}N naturally occurs at a low frequency and is therefore used as tracer to follow N passage through systems (Mayland et al., 1966; Buckley et al., 2007, 2008). Second, the natural abundance of ^{15}N can be used to estimate N turnover rates (Hogberg, 1997). During incomplete reactions, a fractionation between the two isotopes occurs, which can be measured as δ^{15}N value. The natural abundance method has been applied to estimate different processes, such as N fixation, nitrification, denitrification, and mycorrhizal N translocation (Hogberg, 1997; Russow et al., 2005; Hobbie et al., 2005). However, within the N cycle a comparatively small number of nitrogen species is affected by a comparatively larger number of N turnover processes. This causes difficulties interpreting the δ^{15}N value if multiple processes affected the δ^{15}N value of the one N species.

Assay protocols for almost all N cycling processes exist. Some of the protocols are commonly used. These common protocols are established in the field and are well characterised; drawbacks and limitations are known. Table 1.6 gives an overview of common protocols used to quantify N turnover in soil, summarising methodology and limitations of each approach.

Table 1.6: Selection of protocols to quantify N turnover processes.

Process, Assays, References	Description	Limitation
N fixation		
Acetylene reduction assay, ARA, Jeffries et al. (1992)	nitrogenase reduces acetylene, detection of ethylene production	conversion factor varies considerably
^{15}N incorporation, Mayland et al. (1966)	detection of $\delta^{15}N$ shift after ^{15}N incorporation	destructive method
Mineralisation		
Litter bag, Marx et al. (2007)	monitoring the reduction of a defined litter mass in a bag *in situ*	artificial litter substrate
Incubation, van Gestel et al. (1991)	measuring N release from soil cores *in vitro* or *in situ*	difficult to exclude other N processes
Fluorescence assays, Hendel and Marxsen (2005)	*in vitro* hydrolysis of fluorescently labelled substrates	not representing field conditions
Nitrification		
Potential nitrification rates, PNR, Luo et al. (1996)	*in vitro* monitoring nitrite formation from ammonium	not representing field conditions
Nitrate reduction		
^{15}N incubation, Buresh and Patrick (1978)	monitoring $^{15}NO_3^-$ depletion and $^{15}NH_4^+$ formation	destructive method
Anammox		
Anammox potential, Strauss et al. (2011)	*in vitro* incubation of $^{15}NH_4^+$ and $^{15}NO_2^-$ to form $^{30}N_2$	not representing field conditions
Denitrification		
Potential denitrification assay, PDA, Luo et al. (1996)	*in vitro* monitoring N_2O formation from nitrate	not representing field conditions
Gas fluxes, Barger et al. (2005)	measuring NO or N_2O fluxes from soil in chambers	chamber might affect gas pressure

1.7 Background and research hypotheses of this dissertation

1.7.1 Research framework and study sites

Transregional Collaborative Research Centre 38

This dissertation is part of the Transregional Collaborative Research Centre (TRR) 38 framework, from 2006-2012. The aim of the TRR 38 is to identify relevant processes and structures of initial ecosystem development. Moreover, to study interactions of these processes. The project is subdivided into three project groups. Project group A focusses on abiotic structures and processes, e.g., substrate weathering and geochemical interactions of solid and liquid phases. Project group B studies biotic structures and processes, e.g., root penetration of initial soil substrate. Last, project group C integrates structures

and processes in models, such as hydrological modelling of water or solute transport.

The major study site of the TRR 38 is the artificial water catchment *Hühnerwasser*. It was constructed as post-mining landscape by the Vattenfall Europe Mining AG in the open-pit area at the lignite mine Welzow-Süd, Brandenburg, Germany (Figure 1.6). The Hühnerwasser catchment was constructed of a 2-3 m layer of clay that was covered with 2-3 m of quarternary sand. It covers an area of approximately 6 ha and its construction was finished in autumn 2005. As reference sites, inland dunes in Brandenburg, Germany and the forefield of the Damma glacier were investigated. The reference sites are initial ecosystems too, however they are characterised by different environmental conditions, such as geology, climate, or altitude. The reference sites are intended to allow separating site specific from universal processes and structures.

This dissertation was part of the project group B focussing on the development of the microbial N cycling in pioneer soils. As study sites the forefield of the Damma glacier and the Lieberose inland dune were chosen, as both field sites display different phases of soil development in their natural environment (Figure 1.6). This enables to study different phases of N cycle development at the same time.

The forefield of the Damma glacier

The forefield of the Damma glacier is located by the settlement Göschener-alp, Canton Uri, Switzerland (48°36'20"N, 8°28'00"E). It represents a typical soil chronosequence (Bernasconi and Biglink Project Members, 2008). Since 1850, the glacier has been retreating and the glacier length has constantly been monitored from 1921 (Duc et al., 2009b). This allowed to reconstruct a chronosequence covering 120 year of soil development. The forefield is situated at about 2000 m a.s.l. and has a NE exposition of the glacier front (Bernasconi and Biglink Project Members, 2008). The predominating bedrock is granite and the climate is characterised by short summers (5 months growing season), average air temperatures of 0 to 5 °C, and approximately 2400 mm rain (Duc et al., 2009b).

At the youngest sites of the forefield, scarce vegetation is observed. *Leucantemopsis alpina* is among the first colonising plants at the sites that are ice-free for 5 to 10 years. There, *L. alpina* is observed at a frequency of 0.1 individual per m^{-2} (Miniaci et al., 2007). Vegetation cover is closed at sites being ice-free for 70 years, and a dense vegetation of *Rhododendron ferrugineum, Salix* sp., *Agrostis gigantea, Festuca rubra, Poa alpina, Leucanthemopsis alpina*, *Rumex scutatus*, and *Lotus alpinus* can be observed (Bernasconi et al., 2011).

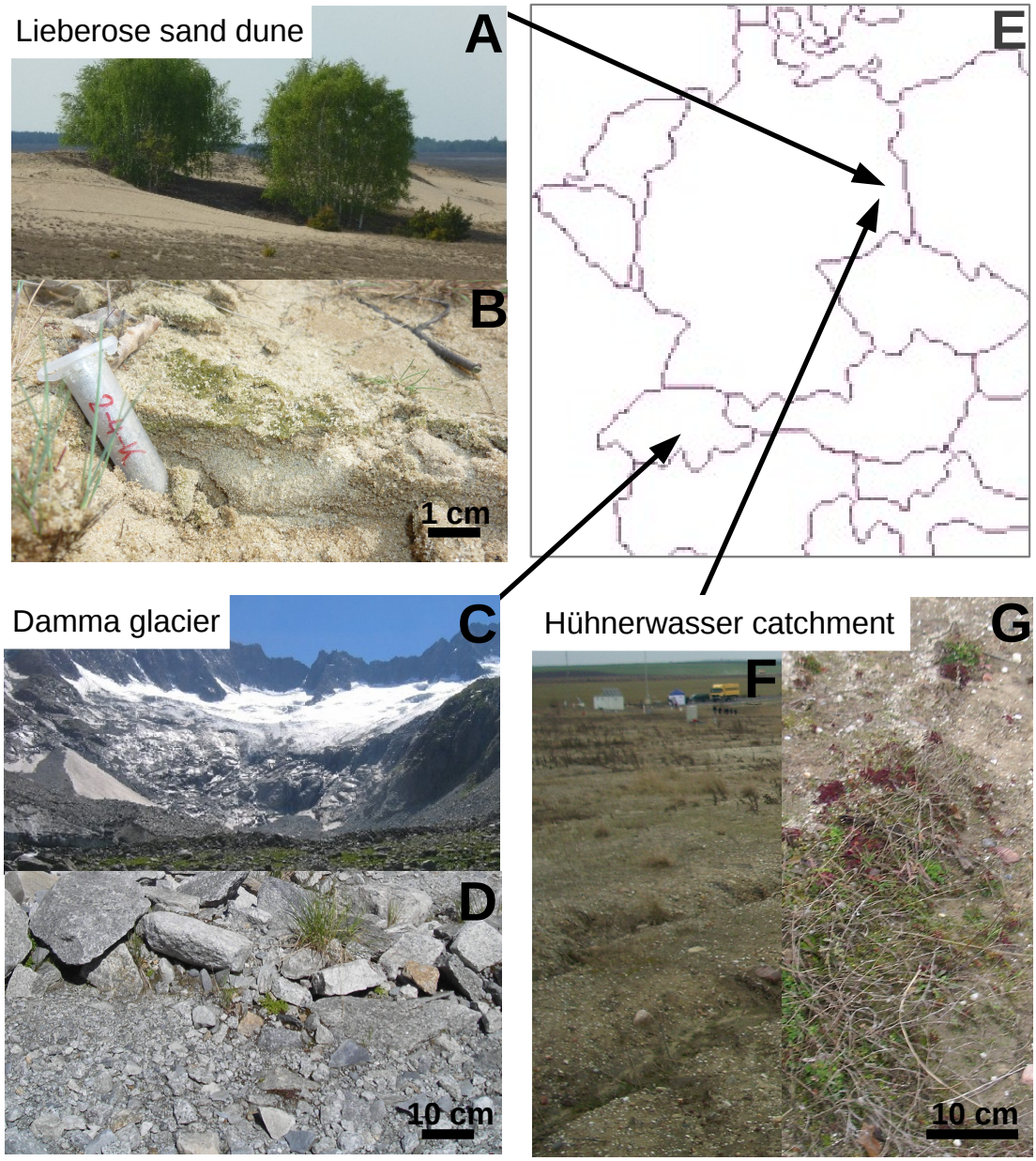

Figure 1.6: Pictures of the three study sites and their location in Switzerland and Germany. A, Lieberose sand dune; B, close-up of biological soil crust, picture taken May 2009 (bar equals ≈1 cm); C, glacier forefield and Damma glacier; D, close-up of forefield soil ice-free for 10 years, picture taken June 2008 (bar equals ≈10 cm); E, map of central Europe, arrows indicating the location of the sites; F, view across Hühnerwasser catchment with yellow truck in the background; G, close-up of the soil surface showing grasses and mosses, picture taken March 2009 (bar equals ≈10 cm).

The Lieberose sand dune

The Lieberose sand dunes are situated 100 km to the south-east of Berlin in Brandenburg, Germany (51°55'49"N, 14°22'22"E). The dunes originate from Pleistocene inland dunes that became deforested, as they were used as military training ground from 1954 to 1992 (Dieke, 2006). Deforestation led to aeolian sand transport and formation of new sand dunes. Today most sand dunes are stabilized with patches of the grass *Corynephorus canescens*, however some dunes are still mobile. The climate at the site is continental, with an annual temperature mean of 8.9 °C and 570 mm annual precipitation (Fischer et al., 2010). The site is situated 50 m a.s.l. and is characterised by a growing season of 7-8 months.

On the Lieberose sand dunes different developmental phases of biological soil crusts (BSC) are present. BSC are microbial communities of bacteria, fungi and algae, which colonise the soil top layer and stabilise it. At the Lieberose dune BSC appear along a gradient from the mobile part of sand dunes towards the parts that are stabilized by *C. canescens*. In the mobile part continuous disturbance by wind inhibits the development of BSC. Towards the foot of the dune (lee side), however, the degree of disturbance decreases and different phases of BSC are observed.

1.7.2 Hypotheses

Research gap and hypothesis one

Microorganisms control the N cycling in ecosystems. Therefore, they determine the development of the N cycle in pioneer sites and are key drivers of ecosystem development (West and Skujins, 1977; Evans and Ehleringer, 1993; Zaady, 1996; Crews et al., 2001; Veluci, 2006). Studies of the N cycle development focussed on individual processes such as N fixation (Schmidt et al., 2008; Duc et al., 2009b), nitrification (Deiglmayr et al., 2006) and mineralisation (Tscherko et al., 2003) and were conducted at different sites. Few studies only considered interactions of N cycling processes and the overall N cycle (Johnson et al., 2007). From the existing literature, it can be hypothesised that the N cycling processes develop over time. E.g., the mineralisation process was detected in initial soils (Tscherko et al., 2003), while N fixation and nitrification were only detected at a later phases of soil development (Deiglmayr et al., 2006; Duc et al., 2009b). However, a comprehensive study, analysing the development of the N cycle in one ecosystem or at one field site is missing.

Hypothesis one was that *at pioneer sites, the development of the N cycle follows a distinct pattern.* In this dissertation the development of the N cycle was analysed in two contrasting ecosystems, a glacier forefield (Chapter 2) and a sand dune (Chapter 4), both characterised by a gradient of soil development. The N cycling processes of different phases of soil development were characterised by enzyme assays and by measuring the abundance of functional marker genes.

Research gap and hypothesis two

Plants and microorganisms interact in a variety of relationships. In regard to the N cycle, N fixation (Duc et al., 2009b) and nitrification (Briones et al., 2003) are examples of mutual relationships. In addition, the majority of soil microorganisms exhibit heterotrophic energy metabolisms, relying on an external source of reduced carbon. Therefore, the fixation of carbon by primary producers and the subsequent carbon release into the soil, e.g., by root exudation (Jones, 1998) or leaching (Mager and Thomas, 2011), is crucial to a major part of the soil microorganisms.

Hypothesis two was that *the colonisation of the pioneer sites by primary producers will affect the N cycling microorganisms.* In Chapter 3 the effect of the presence of plants was tested. The abundance of functional marker genes was measured in bulk soil and rhizosphere soil, comparing the plant effect in initial and developed soil.

Research gap and hypothesis three

Pioneer sites are characterised by heterogeneity and steep environmental gradients. On the one hand, geological processes contribute to local heterogeneity, e.g., changes in lithology or small scale variations in bedrock composition. On the other hand, physical and chemical processes shape the environment causing medium to small scale heterogeneity, e.g., alluvial fens of silty material or preferential flow paths of water.

Variations of environmental parameters have a major impact on microorganisms. This is why, obtaining representative samples is one major concern in the field of microbial ecology (Ranjard et al., 2003). Different sampling approaches have been tested and applied in the field; and strategies differ from environment to environment. It is important to be aware of the gradients that exist in the system and to take them into account when sampling. For example, to account for small scale heterogeneity in soil, it is widely accepted to obtain composite samples (Dick et al., 1996).

Aim of this dissertation was to study the gradient of N cycle development by sampling different phases of soil development. Therefore, it was important to replicate the samples in a way that other gradients will not mask or overlay the effects caused by soil development.

Hypothesis three was that *replication of samples allows to capture the site heterogeneity and to detect changes specific to soil development.* In Chapters 2, 3 and 4 five biological replicates per sample were taken and analysed. In the field, each of the five replicates were composite samples taken 5 to 10 m apart.

Research gap and hypothesis four

In microbial ecology the standard curve method is commonly used as absolute quantification method for qPCR analysis. This method assumes that PCR kinetics of standard and sample are the same. However, for two reasons, this is not the case. First, in contrast to the standard that usually originates from a pure culture, in a natural sample, primer targets differing by only few bases might be present and co-amplified by the reaction (von Wintzingerode et al., 1997). Second, to account for sequence variability of the targeted group, degenerate primers are commonly used (Wallenstein and Vilgalys, 2005; Kandeler et al., 2006), each of the individual primer with different kinetics (Polz and Cavanaugh, 1998). Both mechanisms affect the kinetics of the qPCR reactions and compromise the accuracy of the quantification.

Hypothesis four was that *the efficiency of qPCR reactions depend on the reaction template, accounting for efficiency differences between templates will improve the accuracy of the quantification.* This hypothesis was tested in Chapter 5. A novel data analysis method for real-time quantitative PCR that is less susceptible to quantification errors, which are introduced by the reaction kinetics of different sample templates, was developed and tested.

1.7.3 Publications

Chapter two:

> **Abundances and Potential Activities of Nitrogen Cycling Microbial Communities Along a Chronosequence of a Glacier Forefield.** Robert Brankatschk[1], Stefanie Töwe[1], Kristina Kleineidam, Michael Schloter, Josef Zeyer. *The ISME Journal* (2011) 5, 1025–1037.

[1]Authors contributed equally to this work.

Chapter three:

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. Stefanie Töwe, Andreas Albert, Kristina Kleineidam, Robert Brankatschk, Alexander Dümig, Gerhard Welzl, Jean Charles Munch, Josef Zeyer, Michael Schloter. *Microbial Ecology* (2010) 60, 762–770.

Chapter four:

Succession of N Cycling Processes in Biological Soil Crusts on a Central European Inland Dune. Robert Brankatschk, Thomas Fischer, Maik Veste, Josef Zeyer. *FEMS Microbiology Ecology* (2012). doi: 10.1111/j.1574-6941.2012.01459.x

Chapter five:

Simple Absolute Quantification Method Correcting for Quantitative PCR Efficiency Variations for Microbial Community Samples. Robert Brankatschk, Natacha Bodenhausen, Josef Zeyer, Helmut Bürgmann. *Applied and Environmental Microbiology* (2012) 78, 4481–4489.

In addition to the four research articles that are part of this dissertation, Robert Brankatschk contributed to following publications:

Pioneer Communities in the Forefields of Retreating Glaciers: How Microbes Adapt to a Challenging Environment. Anna Lazzaro, Alessandro Guido Franchini, Robert Brankatschk, Josef Zeyer. In: *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology.* Méndez-Vilas (Ed.). 2012, Formatex, Badajoz, Spain.

Seasonal Dynamics of Nutrients and Bacterial Communities in Unvegetated Alpine Glacier Forefields. Anna Lazzaro, Robert Brankatschk, and Josef Zeyer. *Applied Soil Ecology* (2012) 5, 10-22.

Nitrate removal in a restored riparian groundwater system: functioning and importance of individual riparian zones. Simone Peter, Romana Rechsteiner, Moritz F Lehmann, Robert Brankatschk, Tobias Vogt, Samuel Diem, Bernhard Wehrli, Klement Tockner, and Edith Durisch-Kaiser. *Biogeosciences Discussions* (2012) 9, 6715-6750.

2 Abundances and Potential Activities of Nitrogen Cycling Microbial Communities Along a Chronosequence of a Glacier Forefield

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Published in: *The ISME Journal* (2011) 5, 1025–1037.

[1]Authors contributed equally to this work.

2.1 Abstract

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 stage of soil development. This clearly indicates that the genetic potential is not the driver for certain functional traits in the initial stage of soil formation but rather a well-balanced expression of the respective genes coding for selected functions.

2.2 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 covering different stages of soil development, 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; Hämmerli 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 microorganisms (Deiglmayr et al., 2006; Duc et al., 2009b; Schmalenberger and Noll, 2010). Moreover, initial sites are often dominated by microorganisms 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 et al., 2009b; Töwe 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 stages 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.

2.3 Materials and methods

2.3.1 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 2.1; 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 et al., 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 2.1). 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 et al., 2008).

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

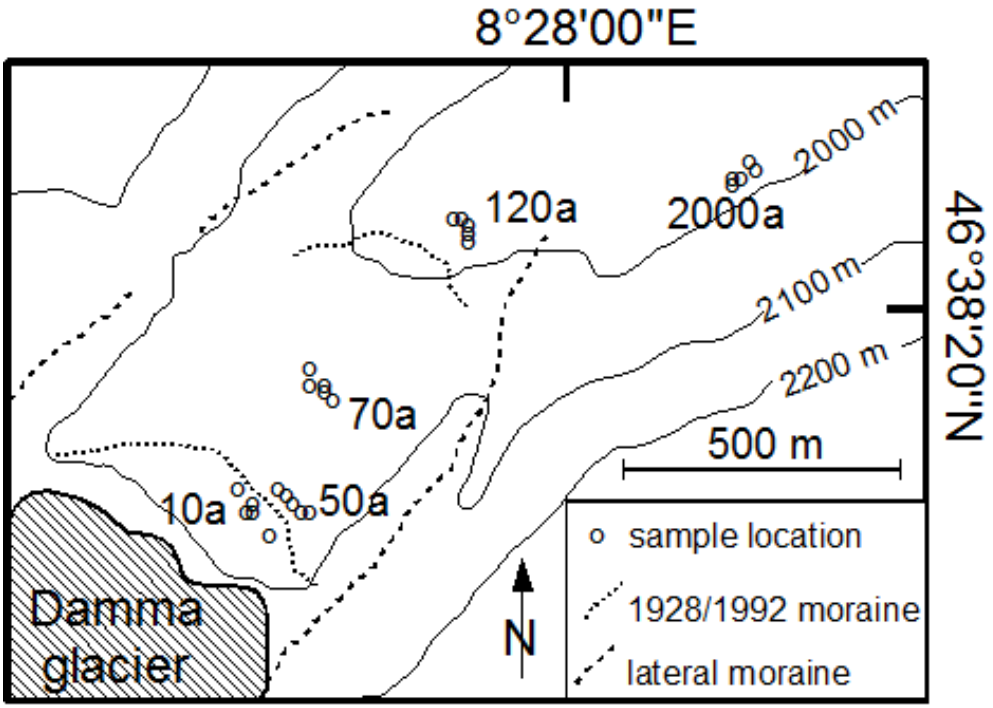

Figure 2.1: Site map illustrating the Damma glacier forefield. Circles mark the sampling locations. Five circles representing the soil samples of the same developmental stage are labeled with the corresponding sample code: 10, 50, 70, 120 and 2000a.

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 Göransson, ETH Zurich, personal communication). Dominant plant species were *Leucanthemopsis alpina*, *Agrostis gigantea*, *Rumex scutatus*, *Salix* sp. and *Lotus* alpinus (Hans Göransson, ETH Zurich, personal communication).

2.3.2 Nitrogen deposition

N depositions in alpine ecosystems of central Switzerland were modeled in different studies (Table 2.1). 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 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 2.1).

2.3.3 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., 2009b). 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 2.1). 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 sub-samples 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 (Schütte et al., 2009); soil for molecular analysis was stored on dry ice.

2.3.4 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 595½, 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,

Table 2.1: 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, BUWAL[a]	-	-	-	10 - 15
	central Switzerland, NABEL[b]	2.2	4.5	3.5	-
	central Switzerland, EMEP[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] Buwal (2005).
[b] Nabel (2008).
[c] Nyiri et al. (2009).
[d] Values indicate average of five replicates, s.e.m. in brackets.

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.

2.3.5 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).

2.3.6 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).

2.3.7 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 2.2. 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.

2.3.8 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.

2.4 Results

2.4.1 Soil parameters along the chronosequence

Measurements of the C and N pools indicate that soil-nutrient status changed substantially along the glacier chronosequence (Table 2.3). All C and N pools increased by one order of magnitude from 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.

2.4.2 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 2.2).

Table 2.2: Reaction composition, thermal profiles, sources of calibration standards and primer references of qPCR reactions.

Target gene	Reaction conditions							Source of calibration standard	Primer name and reference
	F- and R-primer [pmol μL^{-1}]	BSA, 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	25s at 53°C[c]	45	43	88-90 %	*Paenibacillus azotofixans* DSM 5976	nifH-F, nifH-R, Rösch et al. (2002)
chiA[b]	0.2	0.1, 0	10	90s at 65°C	30	40	95-96 %	*Streptomyces coelicolor* DSM 41189	chif2, chir, Xiao et al. (2005)
aprA[b]	0.2	0.2, 0	10	20s 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	30s 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	45s at 57°C	45	40	88-95 %	*Pseudomonas stutzeri*	nisS cd3af, Michotey et al. (2000), nirSR3cd, Throback et al. (2004)
nosZ[d]	0.2	0.6, 0	15	30s at 60°C[f]	30	45	92-95 %	*Pseudomonas fluorescens* C7R127[g]	nosZ2F, nosZ2R, Henry et al. (2006)
amoA[d] (AOA)	0.2	0.6, 0	45	45s at 55°C	45	40	85-87 %	*Fosmid* clone 54d98[h]	19F, Leininger et al. (2006), CrenamoA616r48x, Schauss et al. (2009)
amoA[d] (AOB)	0.3	0.6, 0	45	45s at 60°C	45	40	78-79 %	*Nitrosomonas* sp.	amoA 1F, amoa 2R, Rotthauwe et al. (1997)

[a] Efficiency calculated after Huić Babić 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).

Table 2.3: 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 [µg C g^{-1}]					
TOC [x 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 [µg N g^{-1}]					
TN [x 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 [µg 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 carbon.
[f] Microbial nitrogen.
[g] Ratio of TOC to TN.

Generally, potential enzyme activities related to dry soil increased along the chronosequence. N-fixation activity was below the detection limit in samples 10a, 50a, and 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 MUFh^{-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.

2.4.3 Abundance of functional genes

Gene copy numbers were related to gram soil as well as nanogram DNA (Figure 2.3). Abundance of *nifH* gene was lowest in sample 10a (2×10^6 copies per gram

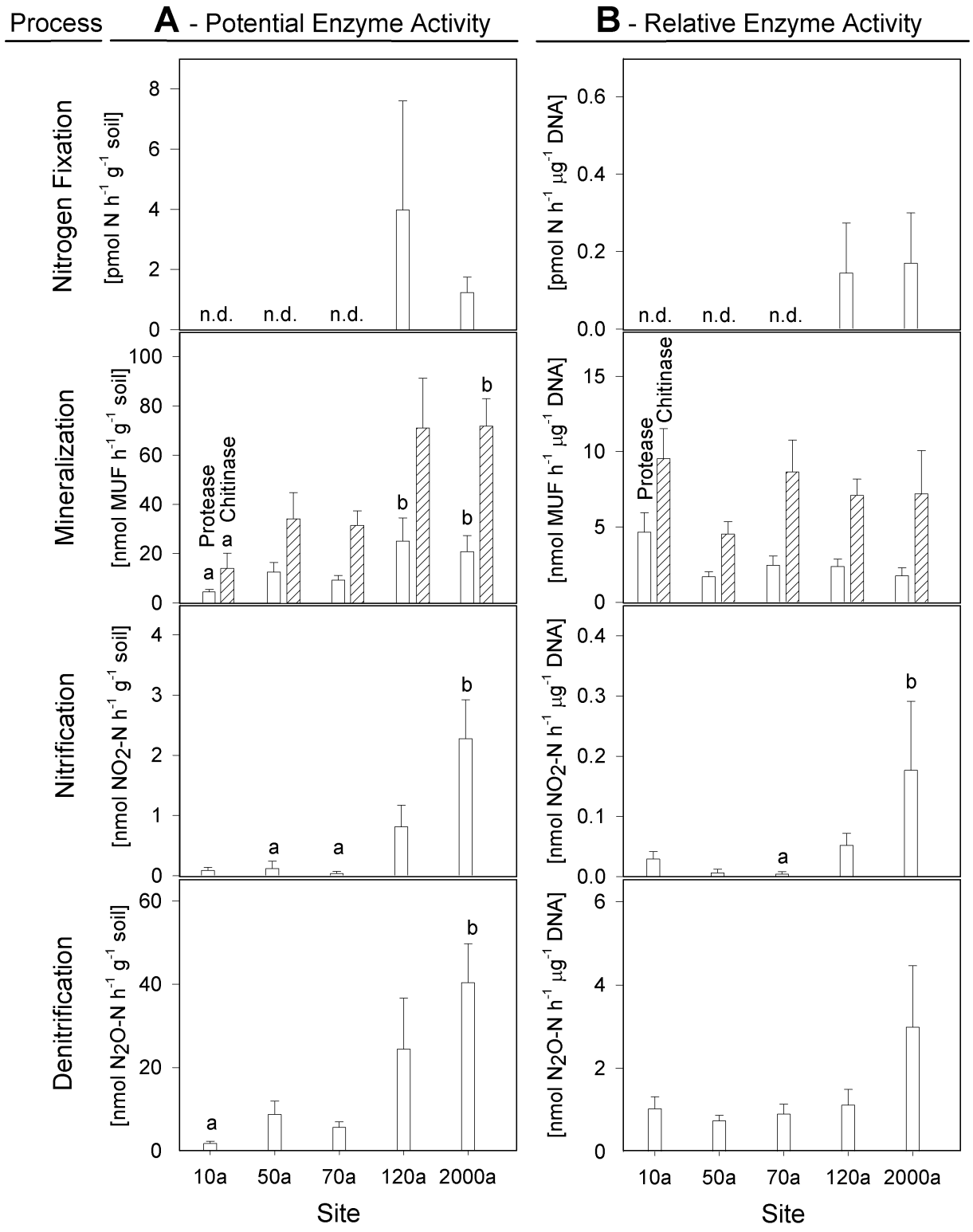

Figure 2.2: A, Potential; and B, relative enzyme activities for the processes nitrogen fixation, mineralization, nitrification and denitrification are displayed (n=5, error bars represent standard error of means). Only significant differences by one way ANOVA on ranks ($P<0.05$) are indicated by different 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.

soil) and increased up to 2×10^7 copies per 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 2.3): *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 2.3B) 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 9×10^6 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.

2.4.4 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 2.4). 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 (α=0.1).

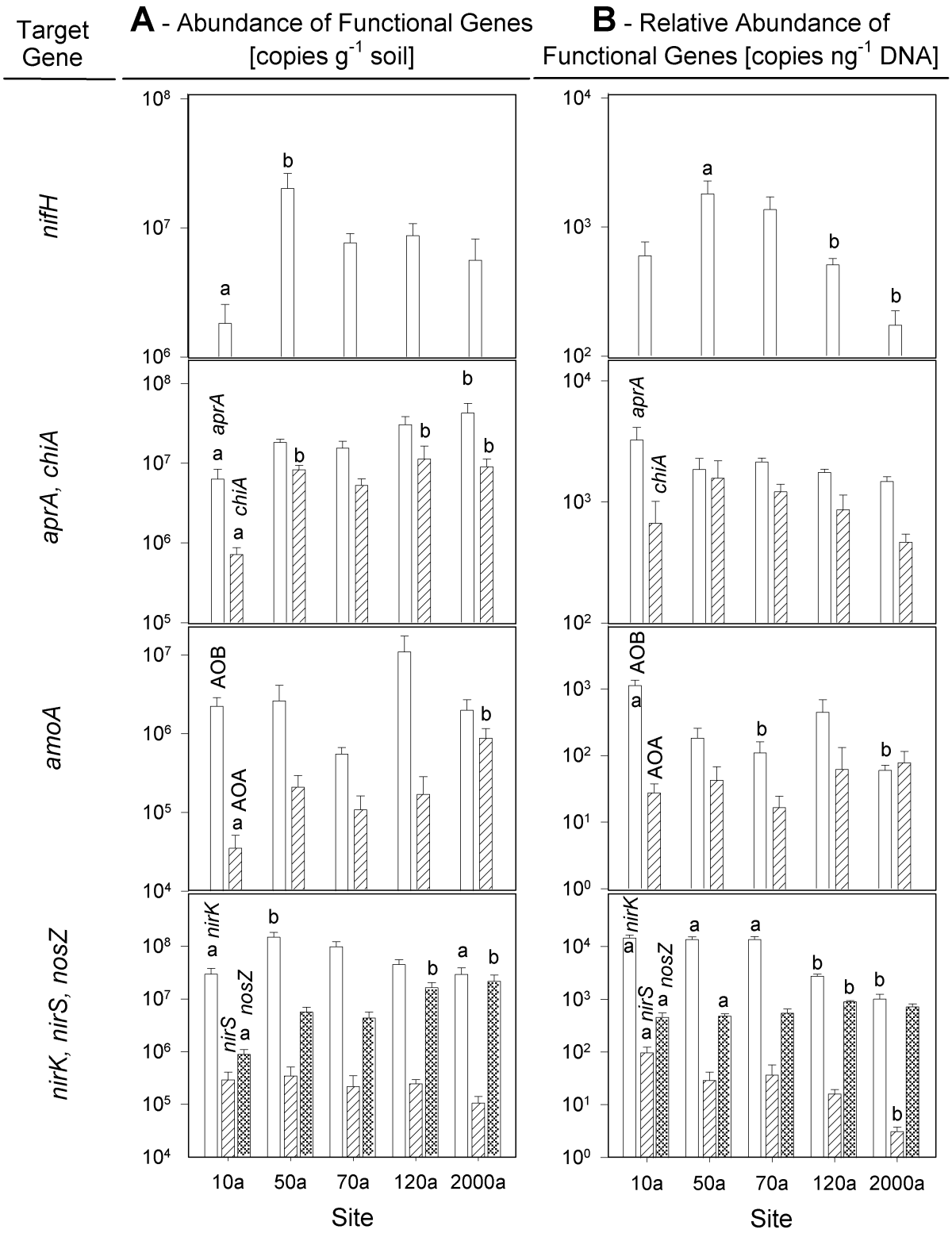

Figure 2.3: Gene copy numbers of functional genes involved in major steps of the nitrogen cycle (*nifH*, *aprA*, *chiA*, *amoA* AOB, *amoA* AOA, *nirK*, *nirS* and *nosZ*) are displayed A, related to gram soil; and B, related to nanogram DNA (n=5, error bars represent standard error of means). Only significant differences as revealed by one way ANOVA on ranks ($P<0.05$) are indicated by different letters.

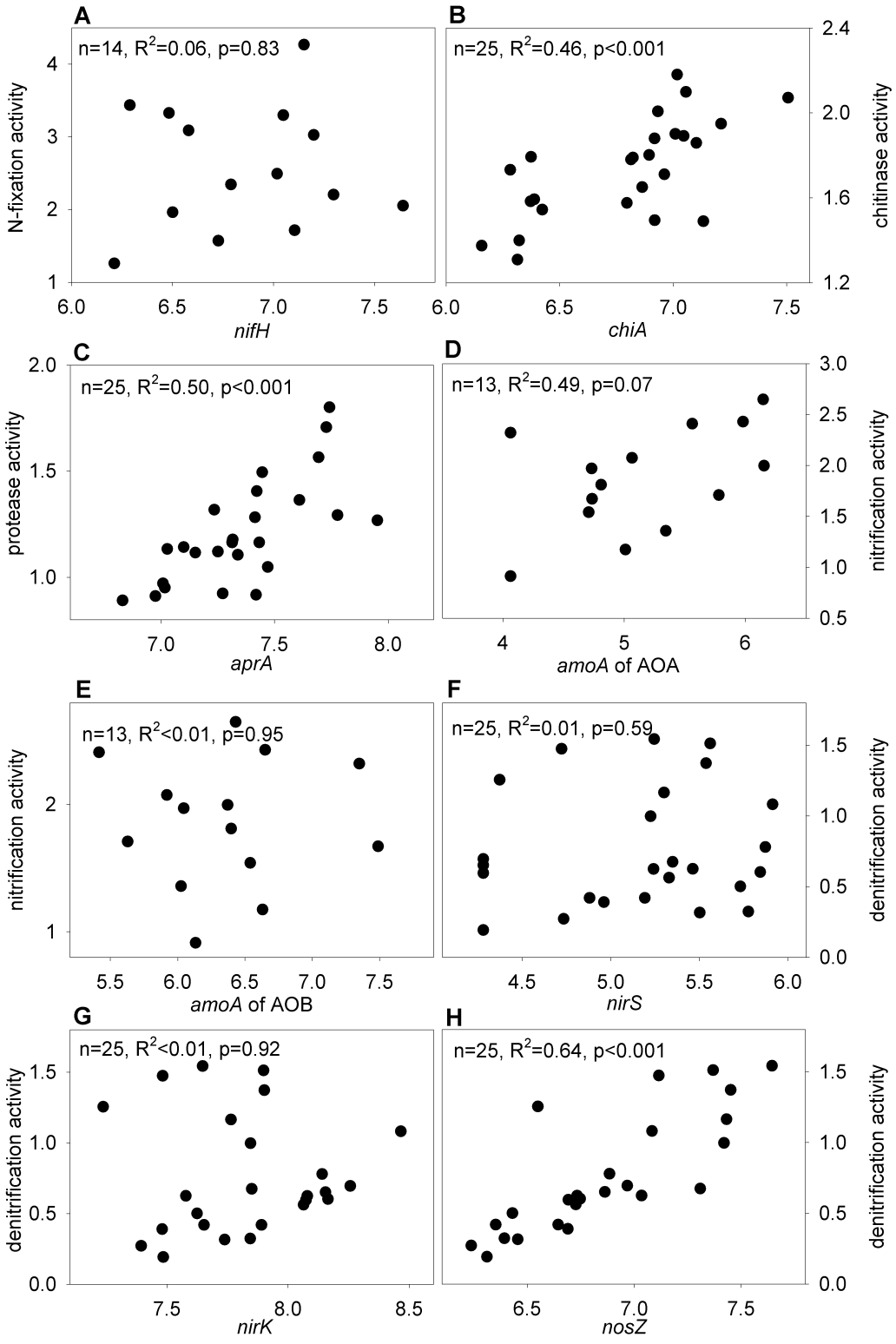

Figure 2.4: Correlations of the gene copy numbers and the corresponding potential enzyme activities are displayed for A, nitrogen fixation; B, C, mineralization; D, E, nitrification; and F–H, denitrification. The graphs plot log-transformed copy numbers vs. log-transformed enzyme activities. In the top left corner of each plot the parameters of the regression analysis, including P-values of t-tests, are provided.

2.5 Discussion

2.5.1 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 (Hämmerli 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 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. (2009b) 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; Nabel, 2008; Nyiri et al., 2009), (Table 2.1), *ii*) the cryoconite holes in the glacier ablation zone possibly inhabit small foodwebs of cyanobacteria and heterotrophs, which were relocated to the glacier forefield by the glacial stream after snowmelt (Xiang et al., 2009; Schmalenberger and Noll, 2010) and *iii*) microorganisms 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 2.1).

2.5.2 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 microorganisms 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; Hämmerli 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 (Chroňáková 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. (2009b) 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 microorganisms might have a role for N input at these sites.

Along with the increase in plant abundance and diversity (Hämmerli 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).

2.5.3 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 (von 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 (Töwe 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).

2.5.4 Linking potential enzymatic activities to gene abundance

We found correlations between gene abundance and enzymatic activities for chitinolytic and proteolytic microorganisms, 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 microorganisms that are able to mineralize N are restricted to a few specialized microorganisms 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 microorganisms, 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 microorganisms: In the first stage 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.

2.5.5 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., 2009b). 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.

2.6 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 become 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 microorganisms to the N budget.

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Authors contributions

RB and ST participated in the design of the study, took samples, performed enzyme and molecular analysis, did statistical analysis and wrote the manuscript. RB analysed the processes N fixation, mineralisation, and deposition. ST analysed the processes nitrification and denitrification. KK, MS and JZ participated in the design of the study and helped drafting the manuscript.

3 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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3.1 Abstract

Glacier forefields are an ideal system 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 complex 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.

3.2 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 is a mutual relationship. The plants provide up to 40 % of their photosynthetically fixed carbon to the microorganisms (Paterson and Sim, 1999) and enhance microbial biomass and activity compared to bulk soil, known as the rhizosphere effect (Butler et al., 2003; Raaijmakers et al., 2009). That occurs either due to uncontrolled leakage or controlled exudation of organic substances like malate, citrate or oxalate (Jones, 1998). In return, microorganisms 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 (Cocking, 2003) or ammonium oxidizers (Briones et al., 2003) to create an optimal environment for their growth.

In contrast, it has been postulated that in developing ecosystems like glacier forefields, plants and microorganisms compete for nutrients as, on the one hand, nutrient availability is low and, on the other hand, plant performance on exudation rates is reduced (Chapin et al., 1994; Hämmerli et al., 2007). However, recent studies have shown local patches of enhanced nutrient availability created by pioneering plants along different glacier forefields (Chapin et al., 1994; Tscherko et al., 2003, 2004, 2005; Deiglmayr et al., 2006; Duc et al., 2009b). In this respect, especially microbial nitrogen fixation may display a selective advantage for the pioneering plant (Kohls et al., 1994). Jacot et al. (2000) showed that 70–95% of nitrogen requirements of legumes in the Swiss Alps were provided by symbiotic nitrogen fixers. Furthermore, Duc et al. (2009b) 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 microorganisms via nitrogen fixation. In contrast, a balanced relationship concerning nitrogen exchange is expected in more developed soils with higher abundance of microorganisms involved in nitrification and denitrifi-

cation. 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 (Miniaci et al., 2007), 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.

3.3 Materials and methods

3.3.1 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 % (Hämmerli et al., 2007) 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 Dümig et al. (2008) 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 sieve.

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 (Torbert and Wood, 1992). 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 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 (Miniaci et al., 2007). 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 (Döhring et al., 1996; Thiel et al., 1996). 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 65 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 nightday 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) (Huić Babić et al., 2008) 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.

3.3.2 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 NANOCOLOR 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 shaken for 45 min with 4 and 20 mL of 0.01 M $CaCl_2$, respectively, on rotary shaker. Finally, the extracts were filtered through a Millex HV Millipore filter (pore size, 0.45 μm) and measured as described by the manufacturer.

3.3.3 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) (Marx et al., 2007).

3.3.4 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.

3.3.5 Real-time PCR assay

Quantitative real-time PCR was conducted on a 7300 Real-Time PCR System (Applied Biosystems, Germany) using SYBR Green as fluorescent dye. The 25 μL reaction mixture was composed as shown in Table 3.1. 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 SYBR

Green 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 3.1. 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 $\text{Eff} = [10^{\frac{-1}{\text{slope}}} - 1]$ and resulted in the following values: *nifH* 84–85%, *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 µL according to the manufacturers instructions. Accordingly, gene copy numbers below 10^4 copies g^{-1} dry weight of RRC and BS, respectively, were not detectable.

3.3.6 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 in order to detect influences of soil compartment and incubation time.

Table 3.1: 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 [µL]	DMSO [µL]
nifH	*Sinorhizobium meliloti*	95 °C, 45 s; 55 °C, 45 s; 72 °C, 45 s	40	nifHF, nifHR Rösch et al. (2002)	0.5	–
amoA AOA	Fosmid clone 54d9 Treusch et al. (2005)	95 °C, 45 s; 55 °C, 45 s; 72 °C, 45 s	40	amo19F, CrenamoA16r48x Leininger et al. (2006); Schauss et al. (2009)	0.5	–
amoA AOB	*Nitrosomonas* sp.	95 °C, 45 s; 60 °C, 45 s; 72 °C, 45 s	40	amoA1F amoA2R Rotthauwe et al. (1997)	0.75	–
nirS	*Pseudomonas stutzeri*	95 °C, 15 s; 57 °C, 30 s; 72 °C, 30 s	40	cd3aF, R3cd Michotey et al. (2000); Throback et al. (2004)	0.5	0.625
nirK	*Azospirillum irakense*	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	0.625
nosZ	*Pseudomonas fluorescens*	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	–
chiA	*Streptomyces griseus*	95 °C, 30 s; 60 °C, 30 s; 72 °C, 60 s	40	chif2 chir Xiao et al. (2005)	1	–
aprA	*Pseudomonas aeruginosa*	95 °C, 20 s; 53 °C, 30 s; 72 °C, 60 s	40	FR aprI RP aprII Bach et al. (2001)	2	–

PCR reaction mixture consisted of Power SYBR Green Master Mix (12.5 µl), BSA (0.5 µl), template (2 µl) as well as primer and DMSO as referred in the table (in µl), [a] touchdown: -1°C per cycle.

Table 3.2: 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). The significant impact of time of plant growth was checked by ANOVA ($P<0.05$).

	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	

3.4 Results

3.4.1 Nitrogen and carbon content of plant biomass

Mean values of plant nitrogen and carbon contents are presented in Table 3.2. 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 3.2). 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).

3.4.2 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 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 3.3.

Table 3.3: 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			
	T1		T2	
	BS	RRC	BS	RRC
NH_4^+–N	b. d. l.	0.7 (1.6)	0.02 (0.03)	b. d. l.
Statistics	no effect			
NO_3^-–N	0.07 (0.15)	49.3 (17.0)	b. d. l.	27.0 (11.8)
Statistics	effect of compartment			

	120 years			
	T1		T2	
	BS	RRC	BS	RRC
NH_4^+–N	5.9 (1.9)	8.1 (3.1)	2.3 (1.3)	0.9 (2.0)
Statistics	effect of incubation time			
NO_3^-–N	38.6 (23.5)	108.3 (74.3)	11.2 (9.9)	76.1 (17.6)
Statistics	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 ($P<0.05$).

3.4.3 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 (Figure 3.1 and Table 3.4).

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 ($p<0.001$) 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

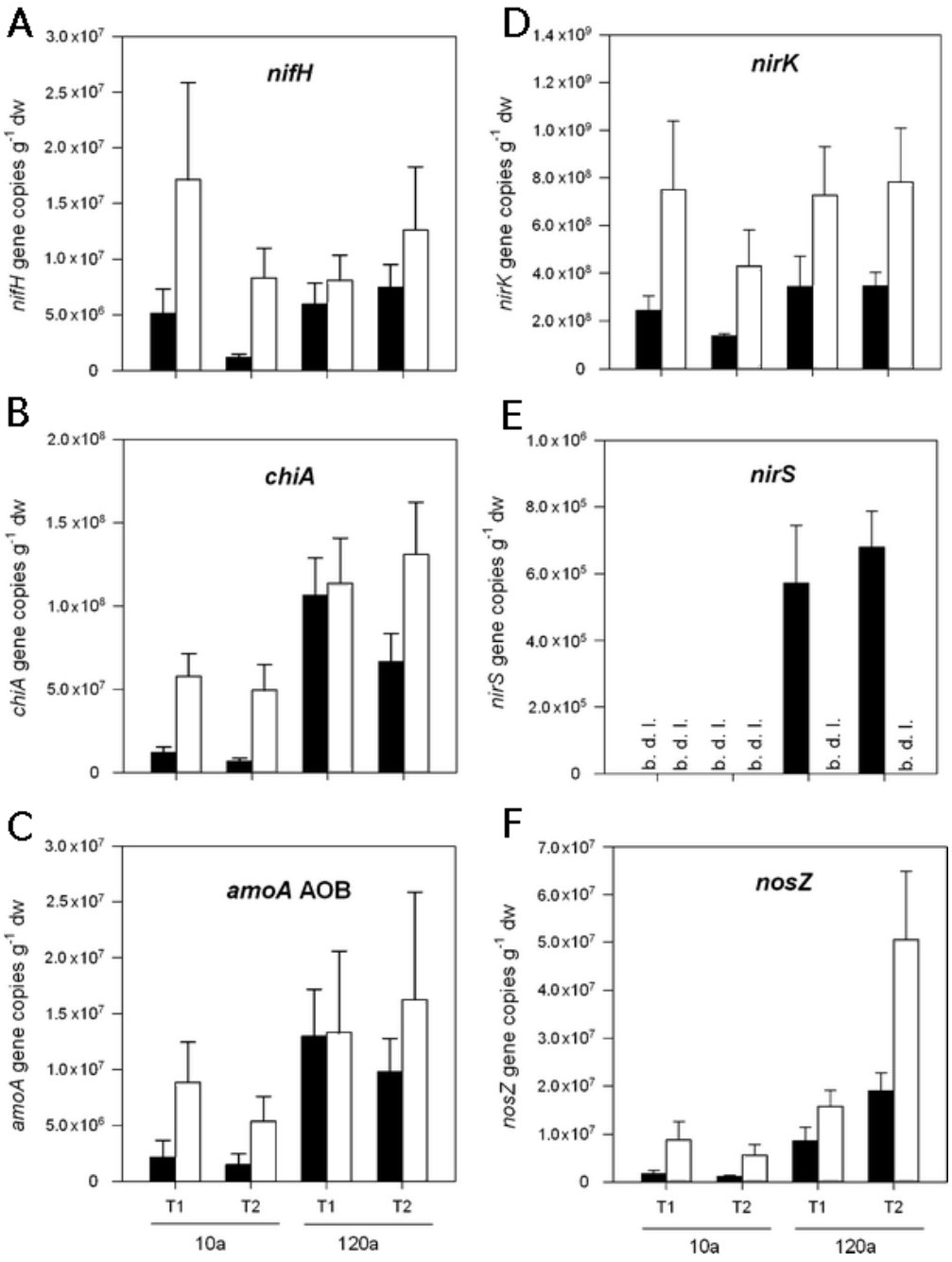

Figure 3.1: Copy numbers of functional genes involved in the nitrogen cycle (*nifH*, *chiA*, *amoA* AOB, *nirK*, *nirS* and *nosZ*) are displayed for RRC (empty bars) samples of *L. alpina* and the corresponding BS (solid bars) after 7 (T1) and 13 weeks (T2) of plant growth in the 10a and 120a soil (n=5, error bars represent standard deviations). b.d.l. indicates that samples were below detection limit of 10^4 gene copies g^{-1} dw.

Table 3.4: P values of the statistical evaluation of gene abundance data by multifactorial ANOVA.

Factor	*nifH*	*chiA*	*amoA* AOB	*nirK*	*nosZ*
Total					
Age (10a, 120a)	0.648	<0.001*	<0.001*	0.005*	<0.001*
Time (T1, T2)	0.207	0.141	0.489	0.088	<0.001*
Compartment (BS, RRC)	<0.001*	<0.001*	0.009*	<0.001*	<0.001*
Age×time	0.001*	0.704	0.53	0.027*	<0.001*
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)	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
Time×compartment	0.256	0.732	0.183	0.165	0.217
120a					
Time (T1, T2)	0.058	0.321	0.973	0.703	<0.001*
Compartment (BS, RRC)	0.027*	0.006*	0.26	<0.001*	<0.001*
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). Copynumbers of *aprA*, *amoA* AOA and *nirS* genes could not be statistically evaluated due to copy numbers below the detection limit.

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 *aprA* were below the detection limit in all cases ($<10^4$ *aprA* 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<0.001) 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.

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 ($p<0.001$) 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 ($p<0.001$) and soil compartment ($p<0.001$). 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.

3.5 Discussion

The aim of this study was to investigate the impact of two differently developed soils on the formation of plant-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 N fixing microorganisms in the 10a soil, as indeed their abundance was highest in the rhizosphere in this soil (T1). Secondly, recent studies showed (Lipson and Monson, 1998; Schimel and Bennett, 2004) 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 (Olander and Vitousek, 2000), 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 (Ohtonen et al., 1999; Bardgett and Walker, 2004). Also Tscherko et al. (2004) revealed a high relevance of N-acetyl-β-glucosaminidase in mature soils of the Rotmoosferner glacier, Austria. 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 (Sigler and Zeyer, 2002; Duc et al., 2009b). 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 (Hämmerli et al., 2007).

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 (2004) 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 microorganisms 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 (Leininger et al., 2006; Nicol et al., 2008; Shen et al., 2008; Schauss et al., 2009). Taking into account that AOB exhibit much higher turnover rates (32–86 fmol NO_2 per cell per hour) (Ward, 1987; Prosser, 1990; Okano et al., 2004) than AOA (0.3–0.14 fmol NO_2 per cell per hour) (Könneke et al., 2005; de De La Torre et al., 2008), it is possible that AOA might have been outcompeted by AOB. Accordingly, Sigler et al. (2002) 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. (2006) at the Rotmoosferner glacier, this phenomenon was already found in a previous study where only *nirK* was detected in the rhizosphere of three different grain legumes (Sharma et al., 2005). 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 (Otte et al., 1996; Morley et al., 2008), 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 (von Rheinbaben and Trolldenier, 1984). Additionally, the N_2O/N_2 ratio of denitrification products is positively related to nitrate concentrations (Dendooven et al., 1994). 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 plant-microbe interaction, the plant-mycorrhiza 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.

3.6 Acknowledgements

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Authors contributions

ST, AA, KK, RB and MS participated in the design of the study. ST analysed soil chemical parameters, measured gene abundance (*amoA*, *nirK*, *nirS*, and *nosZ*), did statistical analysis and wrote the manuscript. AA organised the experimental setup in the climate chambers. KK helped drafting the manuscript. RB researched climatic conditions and composition of rain at the forefield, measured gene abundance (*aprA*, *chiA*, and *nifH*), and helped drafting the manuscript. GW helped with statistical analysis and drafting the manuscript. AD, JCM, JZ and MS helped drafting the manuscript.

4 Succession of N Cycling Processes in Biological Soil Crusts on a Central European Inland Dune

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4.1 Abstract

Biological soil crusts (BSCs) are microbial assemblages that occur worldwide and facilitate ecosystem development by nitrogen (N) and carbon accumulation. N turnover within BSC ecosystems has been intensively studied in the past; however, shifts in the N cycle during BSC development have not been previously investigated. Our aim was to characterise N cycle development first by the abundance of the corresponding functional genes (in brackets) and second by potential enzyme activities; we focussed on the four processes: N fixation (*nifH*), mineralisation as proteolysis and chitinolysis (*chiA*), nitrification (*amoA*), and denitrification (*nosZ*). We sampled from four phases of BSC development, and from a reference located in the rooting zone of *Corynephorus canescens*, on an inland dune in Germany. BSC development was associated with increasing amounts of chlorophyll, organic carbon and N. Potential activities increased, and were highest in developed BSCs. Similarly, the abundance of functional genes increased. We propose and discuss three stages of N process succession. First, the heterotrophic stage (mobile sand without BSCs) is dominated by mineralisation activity. Second, during the transition stage (initial BSCs) N accumulates, and potential nitrification and denitrification activity increase. Third, the developed stage (established BSCs and reference) is characterised by the dominance of nitrification.

4.2 Introduction

Biological soil crusts (BSCs) are microbial communities generally composed of bacteria, archaea, cyanobacteria, fungi, algae, mosses, and lichens (Belnap and Lange, 2001), which colonise soil top layers. BSCs are highly stress-tolerant under extreme environmental conditions, and can therefore be found globally in a variety of ecosystems, including deserts and polar regions (Redfield et al., 2002; Nagy et al., 2005; Schmidt et al., 2008; Büdel and Veste, 2008; Ben-David et al., 2011). In ecosystems, such as volcanic areas, glacier forefields and mobile sand dunes, they form the pioneer system that initiates and accelerates further ecosystem development (Beyschlag et al., 2008; Cutler et al., 2008; Yoshitake et al., 2010). BSCs aggregate soil particles and stabilise the soil surface, thus reducing erosion by water (Eldridge and Greene, 1994) and wind (Belnap, 2001), and change soil hydrological properties (Yair, 2001; Belnap et al., 2005). Moreover, BSCs improve soil nutrient content, thus facilitating ecosystem productivity (West and Skujins, 1977; West and Klemmedson, 1978; Evans and Johansen, 1999; Smith et al., 2004).

Depending on the type of ecosystem, the expression *development* has to be interpreted with care. In systems affected by volcanic eruption, the soil surface may recover simultaneously at different locations (Vitousek et al., 1983; Crews et al., 2001). In the forefield of a receding glacier a chronosequence can be observed, and the phase of BSC development depends on the time span since deglaciation (Chapin et al., 1994). In a sand dune the development of BSCs depends on the shear stress by wind and sand. The shear stress is very high on the top of the sand dune and decreases along the lee slope (Kroy et al., 2002). Therefore, BSC development is not necessarily a function of time, but rather a function of distance from the top of the dune.

Nitrogen (N) is a limiting factor in initial ecosystems, because it is an essential macronutrient and initially absent from the soil. Therefore, N supply to the initial ecosystem is crucial for further development. One important input pathway is the biological N fixation that was shown to be the dominant process providing N to BSC (Rychert and Skujins, 1974; West and Skujins, 1977; Zaady, 2005). Within the BSC, three microbially mediated processes dominate the N cycle: mineralisation, nitrification, and denitrification. All N turnover processes depend on the availability of N, which is closely linked to soil water content and soil hydrology. Moreover, atmospheric N deposition by precipitation and N leaching from the soil are important input and output pathways that are closely linked to soil hydrology. Therefore, soil hydrology needs to be taken into account when assessing the N cycle.

N turnover processes in BSCs have been assayed by a variety of methods (for an overview see West and Skujins, 1978 and Belnap and Lange, 2001) and most methods are well established with standardised protocols (Jeffries et al., 1992; Luo et al., 1996; Hendel and Marxsen, 2005; Hoffmann et al., 2007). Within the last few years, however, molecular tools have become increasingly reliable. Using quantitative real-time PCR (qPCR) the abundance of functional genes in soil can be measured (Henry et al., 2004; Yeager et al., 2004) to assess the genetic potential of a microbial community catalysing the process of interest. The qPCR assay quantifies functional genes, encoding for key enzymes of the process of interest. Correlation between gene abundance and process activity was found for different processes, e.g., hydrocarbon degradation (Salminen et al., 2008) or denitrification (Hallin et al., 2009) and qPCR assays for many genes involved in the N cycle are established (Brankatschk et al., 2011), namely nitrogenase (*nifH*), chitinase (*chiA*), protease (*aprA*), ammonia monooxygenase (*amoA*) of AOA (ammonia oxidising archaea), *amoA* of AOB (ammonia oxidising bacteria), and nitrous oxide reductase (*nosZ*).

To date, numerous studies have focussed on individual N processes (Mayland et al., 1966; Johnson et al., 2005; Barger et al., 2005), N redistribution (West and Klemmedson, 1978; Zaady, 1998; Johnson et al., 2007) and N balance of BSC systems (West and Skujins, 1977; Zaady, 2005). Other studies have compared the N cycle in different types of BSCs (Evans and Belnap, 1999; Zaady et al., 2010; Strauss et al., 2011). Efforts to study the development of the N cycle have concentrated on systems such as volcanic areas (Vitousek et al., 1983; Crews et al., 2001) and glacier forefields (Chapin et al., 1994; Brankatschk et al., 2011), but BSC development has been neglected.

The aim of this study was to investigate the N cycle during BSC development and the Lieberose inland dune (Germany) was chosen as study site (Fischer et al., 2010). Our first hypothesis was that the Lieberose dune displays the full spectrum of BSC development from mobile sand to established BSC. Second, we hypothesised that the development of the N cycle in the studied BSC system proceeds in stages. To test the hypotheses we analysed the major N cycling processes: N fixation, mineralisation (proteolysis and chitinolysis), nitrification, and denitrification, employing standard enzyme assays and novel qPCR techniques.

4.3 Materials and methods

4.3.1 Study site

The study site is located on a Central European inland dune on Pleistocene sand (Fischer et al., 2010) near Lieberose, Brandenburg, Germany (N 51°55'49", E 14°22'22"). In this area an initial mobile sand dune occurs as a result of human disturbance of the natural vegetation by former military activities. The climate of the region is characterised by an annual rainfall of 570 mm and an annual average temperature of 8.9 °C (Fischer et al., 2010). A private meteorological station (WS 2500, ELV Elektronik AG, Germany) is maintained in the settlement of Hollbrunn, located 2 km northwest of the study site (http://-www.hollbrunn-wetter.de/). Daily precipitation and average daily temperature for 2010 are shown in Figure 4.1A. The annual atmospheric N deposition is 12 to 15 kg ha^{-1} a^{-1} (Gauger et al., 2008, http://gis.uba.de/website/depo_gk3/-index.htm).

4.3.2 Hydrology

Using the Hydrus-1D modelling environment (Radcliffe and Šimůnek, 2010), we calculated evaporation from the soil surface and the water flux in the soil. Evaporation from the dune was estimated using the Penman-Monteith equation, as implemented in Hydrus-1D (Radcliffe and Šimůnek, 2010). The calculation was based on air temperature, wind speed, relative humidity, and sun hours (data not shown). Water flux on the soil surface and at 25 cm depth was calculated by solving the Richard's equation in the one-dimensional finite element model implemented in Hydrus-1D (Radcliffe and Šimůnek, 2010).

4.3.3 BSCs at the study site

At the Lieberose dune, different phases of BSC development can be observed (Fischer et al., 2010, 2012). On the top of the dune, wind keeps the sand mobile and no BSC develops because of frequent disturbances due to shear stress. However, on the lee slope of the dune, disturbance by wind gradually decreases. This disturbance gradient down the lee slope causes the persistence of different phases of BSC development, which lie 5 to 10 m to one another. At the foot of the dune the lowest level of disturbance is observed. We sampled four phases of BSC development, i.e. mobile sand, phase 1, phase 2, and phase 3, which were previously characterised (Fischer et al., 2010, 2012). The mobile sand is

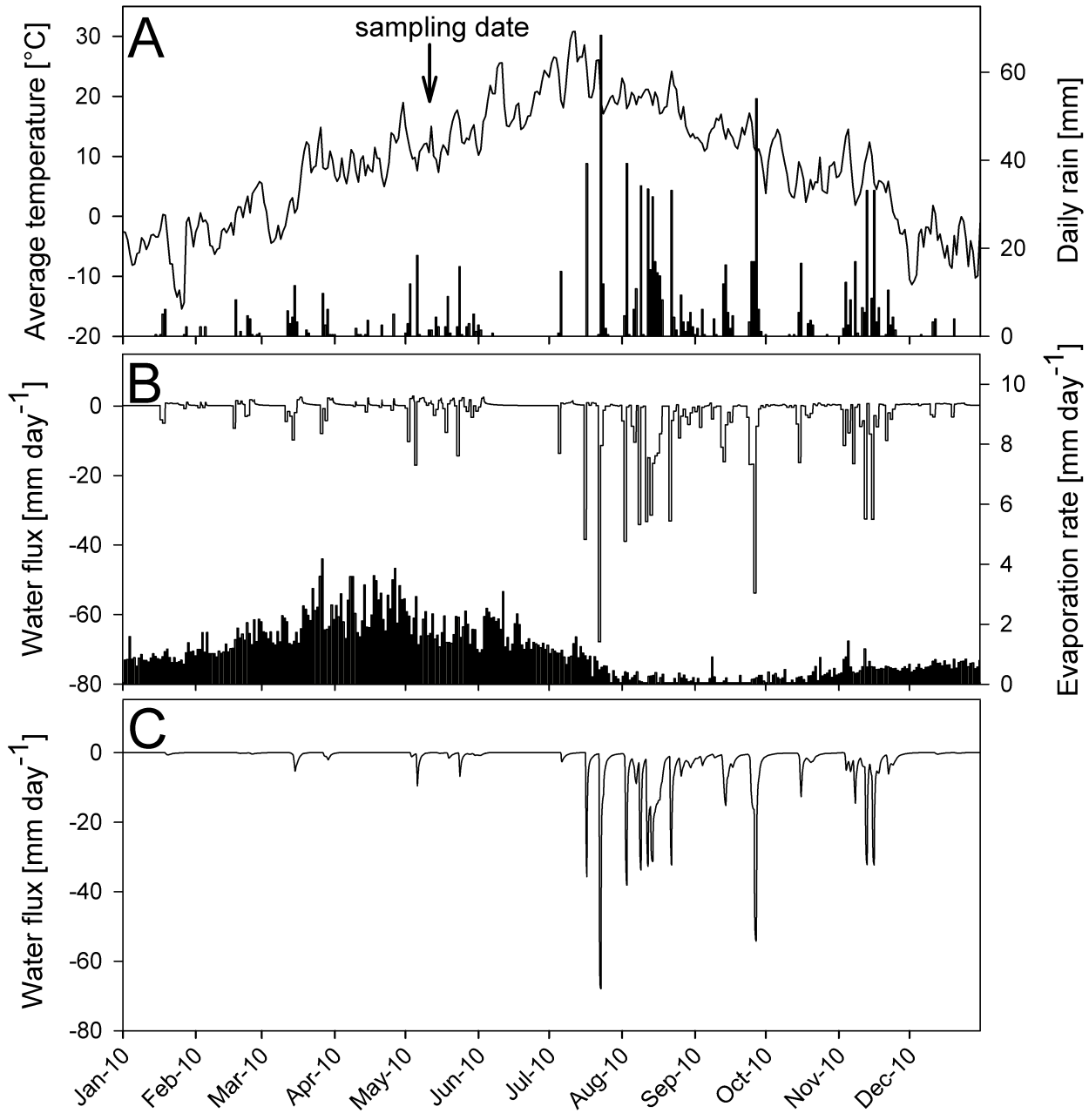


Figure 4.1: Temperature, precipitation, water fluxes and evaporation for the year 2010. A. Average air temperature (solid line) and daily rain (bars) measured at the private meteostation in Hollbrunn (http://-www.hollbrunn-wetter.de/). B. Modelled water flux (solid line) and evaporation (bars) on soil surface. C. Modelled water flux in 25 cm depth.

the bare substrate with no BSC development. BSC of phase 1 consists of sand grains that are stabilised at their contact zones by filamentous cyanobacteria and green algae. BSC of phase 2 is characterised by cyanobacteria and green algae, which partially fill matrix pores and enmesh with sand grains. BSC of phase 3 shows intense growth of filamentous and coccoid green algae, and a few cyanobacteria, fungal hyphae and mosses (Fischer et al., 2010). The flora of the BSCs at the Lieberose dune was described earlier (Fischer et al., 2012). BSC depth increased from about 1 mm in phase 1 to up to 4 mm in phase 3. Together with the increasing surface stability and the development of the BSCs the grass *Corynephorus canescens* establishes, which is characteristic for early successional stages of dry grassland ecosystems on Central European sand dunes. The highest grass cover (17 %) can be found at the dune base together with crust 3.

4.3.4 Sampling procedure

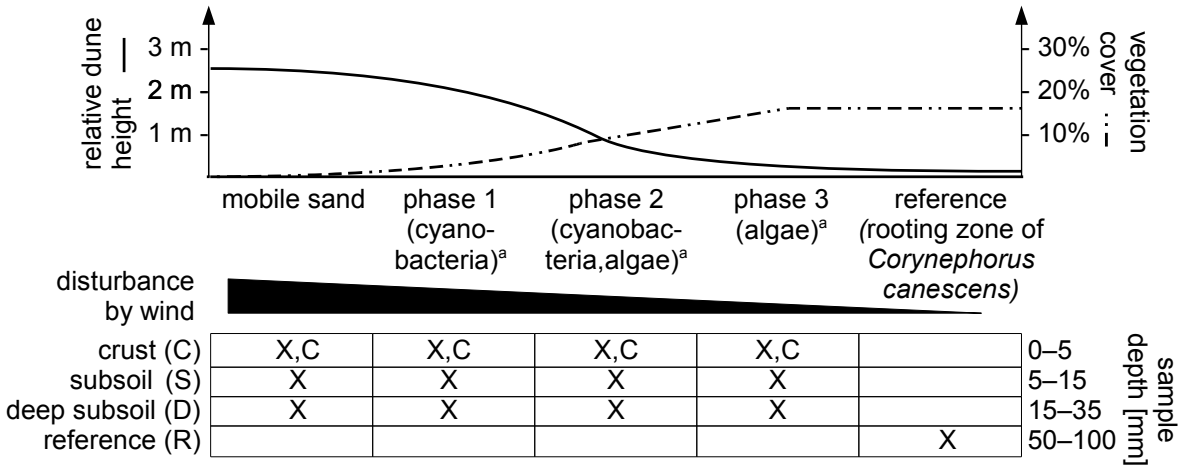
All four phases of BSC development (mobile sand, phase 1, phase 2, phase 3) were sampled on 12 May 2010. The crusts (C) of each phase were sampled by pressing Petri dishes into the topsoil. The subsoils (5-15 mm, S) and the deeper subsoils (15-35 mm, D) of each phase were sampled using shovels. Five independent samples of each phase of BSC development were sampled 1 to 2 m apart. Each sample was a composite sample of 5 Petri dishes (8.7 cm diameter) or shovels taken within 0.5 m^2. In addition, a reference soil (R) from the rooting zone (50-100 mm depth) of *Corynephorus canescens* was sampled in five replicates at the base of the dune, near phase 3. The sampling scheme is summarised in Figure 4.2.

In addition to the samples mentioned above, cored samples (1 cm depth) of the crusts were taken in triplicates with a corer (Fischer et al., 2010) close the other samples. These cored samples were used to measure chlorophyll content and repellency index (see below).

Samples for molecular analysis were aliquoted, kept on ice in the field, and stored at -80 °C upon arrival in the lab. All other samples were air dried in the lab, and kept dry and in the dark until analysis.

4.3.5 Soil chemical and physical analysis

Total organic carbon (TOC) was determined as total carbon, as the samples contained no carbonates. Total nitrogen (TN) and total carbon were measured on an elemental analyser (vario EL III, Elementar Analysensysteme GmbH,



[a] Fischer *et al.* (2010)

Figure 4.2: Sampling scheme. The upper panel shows relative dune height (solid line) and vegetation cover (dashed line) of the different phases of BSC development (centre). Distance between the phases was 5 to 15 metres, but is drawn without scale. The lower panel lists the sample types taken of each phase of BSC development, where "X" stands for five replicates of soil samples taken with Petri dish or shovel. "C" stands for triplicates of cored samples that were used for chlorophyll and repellency analysis only.

Hanau, Germany). Soil nitrate, ammonium, and pH were analysed in water extracts 1:2.5 (w/w). Nitrate was measured on a Dionex ICS 90, ammonium on a Dionex DX 100 (both Dionex, Sunnyvale, CA, USA). Chlorophyll extracts were prepared with 80% acetone, as previously described (Fischer et al., 2010). Chlorophyll *a* and *b* content was calculated from the absorption spectra of the acetone extracts (Ziegler and Egle, 1965). A laser Diffraction Particle Size Analyser LS 13 320 (Beckman Coulter, Miami, FL, USA) fitted with an Aqueous Liquid Module and an Auto Prep Station was used to determine the particle size distribution.

4.3.6 Potential enzyme activities

We choose to employ potential enzyme activity assays, as they are a robust indication for the microbial functional capacity, reflecting activity rates under ideal conditions, e.g., as was shown for denitrification assay in BSCs (Johnson et al., 2007). Potential N fixation activity was measured by acetylene reduction assays (Jeffries et al., 1992) (Table 4.1). As a proxy for mineralisation activity (Schimel and Bennett, 2004), potential chitinase and protease activity were measured in fluorescence assays (Hendel and Marxsen, 2005). Potential nitrification activity was measured as previously described (Hoffmann et al., 2007). Potential denitrification assays were performed as reported by Luo et al. (1996). All enzyme assays were performed in triplicates. The limit of detection (LOD) was calculated as the mean of six blank reactions plus 3 times standard deviation of the blanks. The limit of quantification (LOQ) was calculated as the mean of six blank reactions plus 10 times standard deviation of the blanks.

4.3.7 DNA extraction and real-time PCR

DNA was extracted from 0.5 g thawed BSC or soil material using the Fast DNA Spin Kit for Soil (MP Biomedicals, Illkirch, France) and eluted in 60 µL TE buffer. DNA yields were quantified using the SYBR Green I assay as described by Matsui et al. (2004).

SYBR Green based real-time PCR assays were run on a 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA). Reaction volumes were 20 µL and contained 1-fold concentrated Kapa SYBR Fast PCR master mix (Kapa Biosystems, LabGene, Chatel-St-Denis, Switzerland), 200 nM of each of the respective forward and reverse primers, and 1 µL DNA template (1:10 diluted DNA extract). PCR primers, thermal cycles, efficiencies, LOQs and calibration standards used are summarised in Table 4.2. Each PCR run included

Table 4.1: Conditions and references of the performed potential enzyme assays.

Process (Assay)	Amount of sample	Incubation time and temperature	LOD; LOQ[a]	Reference
N fixation(Acetylene reduction assay)	2 g	20 h at 23 °C[b]	1; 3 pmol C2H4 g^{-1} h^{-1}	Jeffries et al., 1992
Mineralisation (Chitinase assay)	0.5 g	6 h at 23 °C[c]	6; 24 pmol MUF g^{-1} h^{-1}	Hendel and Marxsen, 2005
Mineralisation (Protease assay)	0.5 g	6 h at 23 °C[c]	8; 28 pmol MUF g^{-1} h^{-1}	Hendel and Marxsen, 2005
Nitrification (Potential nitrification assay)	5 g	5 h at 25 °C	0.6; 0.9 nmol NO2-N g^{-1} h^{-1}	Hoffmann et al., 2007
Denitrification (Potential denitrification assay)	5 g	16 h at 20 °C[d]	10; 20 pmol N2O-N g^{-1} h^{-1}	Luo et al., 1996

[a] LOD, limit of detection; LOQ, limit of quantification.

[b] light intensity (30 µmol photons m^{-2} s^{-1}), constant rate for 20 to 72h of incubation.

[c] 50 mM acetate buffer, 10 µM fluorescence substrate.

[d] 0.1 mM glucose, 0.1 mM potassium nitrate, and 0.1 g L^{-1} chloramphenicol, constant rate from 14 to 48h.

triplicate sample templates, calibration standard series and no template control. Copynumbers were calculated by the delta Ct method using LinRegPCR program version 11.4 (Ruijter et al., 2009). Any outliers detected in LinRegPCR analysis (no amplification, outside 5 % of group mean efficiency) were excluded. The presence of desired amplicons was verified by agarose gel electrophoresis. If a desired amplicon was not detected, samples were reported as not detected. Assuming one PCR template will produce the desired band, the theoretical LOD of our setup was $1.2{\cdot}10^3$ copies g^{-1}. The LOQ was calculated as mean of five or more reactions without desired amplicon (amplicon negative samples or blanks) plus 10 times the standard deviation.

4.4 Results

4.4.1 Soil physical and chemical characterisation

Water flux at the surface was variable (Figure 4.1B). During precipitation events, water flux was negative as water infiltrated into the soil. When no precipitation occurred, the water flux was positive and up to 2.9 mm day^{-1} evaporated from the soil surface to the atmosphere. Water flux at 25 cm depth was less variable then at the soil surface (Figures 4.1B, 4.1C), and not every precipitation event caused a negative water flux at this depth.

Soil particle size distribution was constant throughout the sand dune, with a homogeneous bell shaped particle size distribution and a mean particle size

Table 4.2: Temperature cycles, mean efficiencies, sources of calibration standards, and primers of qPCR reactions.

Target gene	Temperature cycles[a]			Efficiency[b]	LOQ[c] (copies g^{-1})	Source of calibration standard	Primer name and reference
	Denaturation time at 95°C [s]	Annealing time and temperature	Elongation time at 72°C [s]				
nifH	15	25 s at 53 °C[d]	45	75-79 %	$7 \cdot 10^5$	*Paenibacillus durus* DSM 5976	nifH-F, nifH-R, Rösch et al., 2002
chiA	10	90 s at 65 °C	30	79-82 %	$5 \cdot 10^7$	*Streptomyces coelicolor* DSM 41189	chif2, chir, Xiao et al., 2005
aprA	10	20 s at 54 °C	30	70-72 %	$7 \cdot 10^5$	*Pseudomonas fluorescens* DSM 50090	FR apr I, RP apr II, Bach et al., 2001
nosZ	15	30 s at 58 °C[e]	30	77-79 %	$2 \cdot 10^3$	*Pseudomonas fluorescens* C7R12[f]	nosZ2F, nosZ2R, Henry et al., 2006
amoA (AOA)	10	45 s at 50 °C	30	77-78 %	$2 \cdot 10^4$	Fosmid clone 54d9[g]	19F, Leininger et al., 2006; CrenamoA616r48x, Schauss et al., 2009
amoA (AOB)	10	60 s at 60 °C	30	81-83 %	$4.5 \cdot 10^5$	*Nitrosomonas europaea*	amoA 1F, amoa 2R, Rotthauwe et al., 1997

[a] 40 cycles started with 5 min initial denaturation and activiation of polymerase at 95 °C.
[b] Efficiency was calculated using LinRegPCR (Ruijter et al., 2009).
[c] LOQ - limit of quantification.
[d] touch down starting at 63 °C temperature decrease of 2 °C per cycle.
[e] touch down starting at 63 °C temperature decrease of 1 °C per cycle.
[f] Eparvier et al., 1991.
[g] Treusch et al., 2005.

between 340 and 600 µm in diameter (Figure 4.3). Sand was classified as medium sand with 10-30% coarse sand (630-2000 µm), 55-62% medium sand (200-630 µm), 15-20% fine sand (63-200 µm), and less than 4% of particles below 63 µm in diameter. Soil pH was around 4.8 in all samples and only slightly increased in crust 2 and crust 3, which had a pH of 5.2 (data not shown).

Soil chemistry was distinctly different for the BSC phases. TOC, TN, ammonium and DNA content in the crusts generally increased from mobile sand to phase 3 (Figure 4.3). The same pattern was observed in the subsoils: from mobile sand towards phase 3, TOC, TN, and DNA content increased (Figure 4.3). Content of TOC, TN and DNA in the reference (182 µmol C g^{-1}, 9.9 µmol N g^{-1}, 1.9 µg DNA g^{-1}) was comparable with the subsoil of phase 3 (139 µmol C g^{-1}, 9.3 µmol N g^{-1}, 1.2 µg DNA g^{-1}). In general, TOC and TN were decreasing in each of the phases from the crust to the deeper soils. Nitrate concentrations were highest in crust of phase 1 (0.24 µmol NO_3^--N g^{-1}) and did not follow any trend observed for the other chemical parameters (Figure 4.3).

N pool sizes of TN, nitrate and ammonium were calculated for the total sampling depth of 35 mm (Figure 4.4). The TN pool increased from 173 mmol N m^{-2} in mobile sand to 470 mmol N m^{-2} in phase 3. In the reference, the TN pool was 525 mmol N m^{-2}. The nitrate pool was highest in phase 1 (5.1 mmol NO_3^--N m^{-2}) and lowest in phase 3 and reference. The ammonium pool was highest in phases 1, 2, and 3 (1.7 to 2.1 mmol NH_4^+-N m^{-2}) and slightly lower in mobile sand and the reference.

4.4.2 Development of BSCs

Apart from increasing nutrient content (see above), crust repellency developed and chlorophyll content increased during BSC development (Figure 4.5). Repellency index increased from 0.9 in the mobile sand to 2.0 in phase 3. Chlorophyll content increased by one order of magnitude from phase 1 to phase 3, with the latter containing 35 mg chlorophyll *a* per m^2 and 12 mg chlorophyll *b* per m^2. The ratio between chlorophyll *a* and *b* decreased from 5.0 in phase 1 to 2.7 in phase 3, indicating a proportionally higher increase of chlorophyll *b*.

4.4.3 Potential enzyme activity

Within each different BSC phase, potential chitinase and denitrification activity decreased from the crust down to the subsoils, where activities were generally 2 to 10 times lower compared to the crusts (Figure 4.6). When comparing the

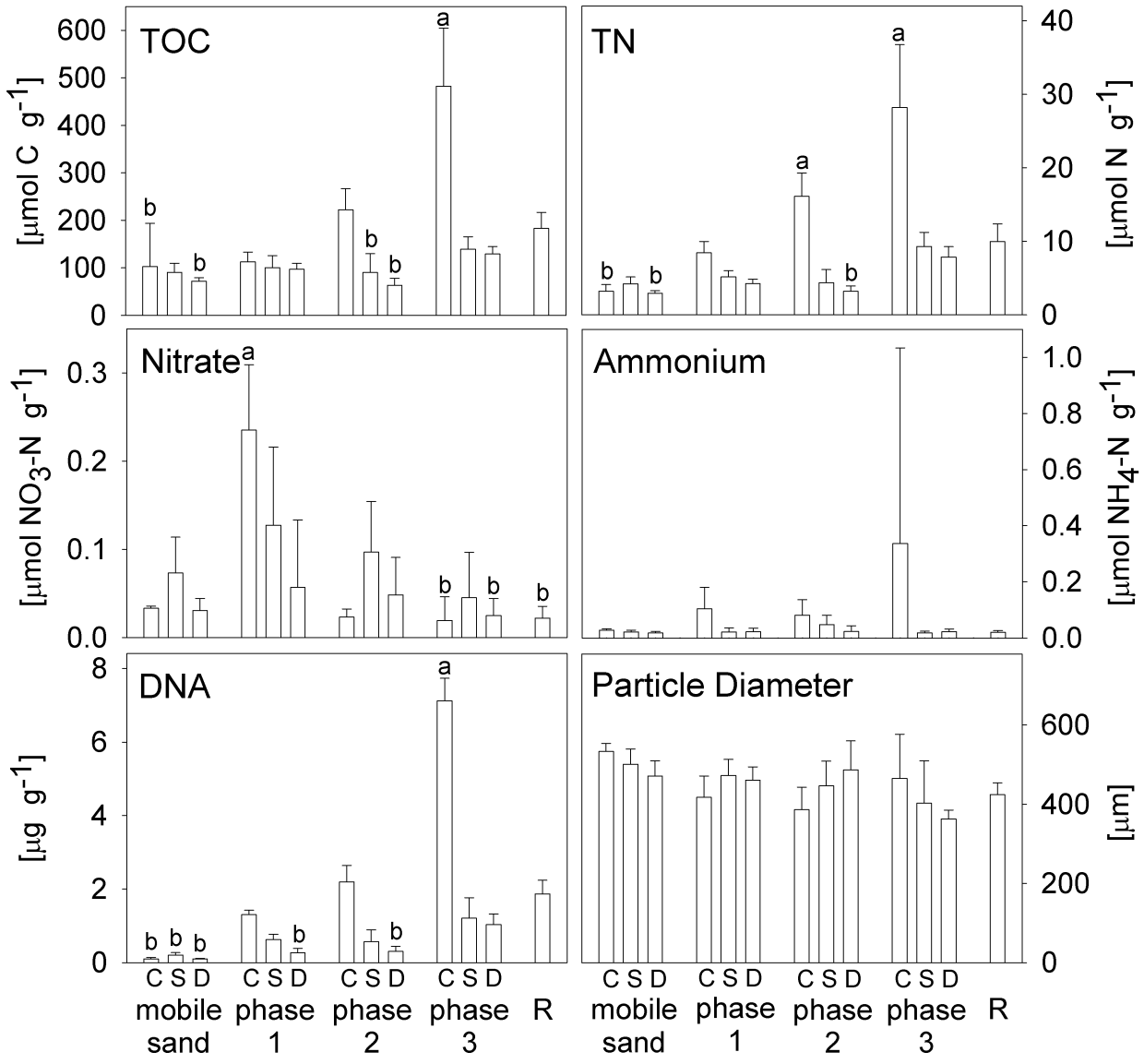


Figure 4.3: Total organic carbon, total nitrogen, ammonium, nitrate, amount of extracted DNA, and mean particle diameter. Bars are average of five replicates, error bars indicate standard deviation. Significant differences tested by one-way ANOVA on ranks ($P < 0.05$) are indicated by different letters. Letters C, S, and D indicate sampling depth; C crust (0-5 mm depth), S subsoil (5-15 mm depth), and D deeper subsoil (15-35 mm depth).

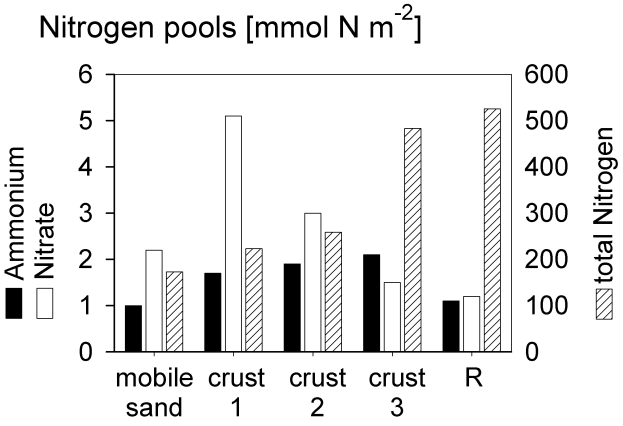


Figure 4.4: Total nitrogen, nitrate and ammonium pools for each phase of BSC development given in mmol N m^{-2}. For calculations a soil depth of 35 mm was considered and a bulk soil density of 1.5 $g \cdot cm^{-3}$ was assumed. For mobile sand and phase 1 to 3, the 35 mm included crust (5 mm), subsoil (10 mm) and deeper subsoil (20 mm) samples.

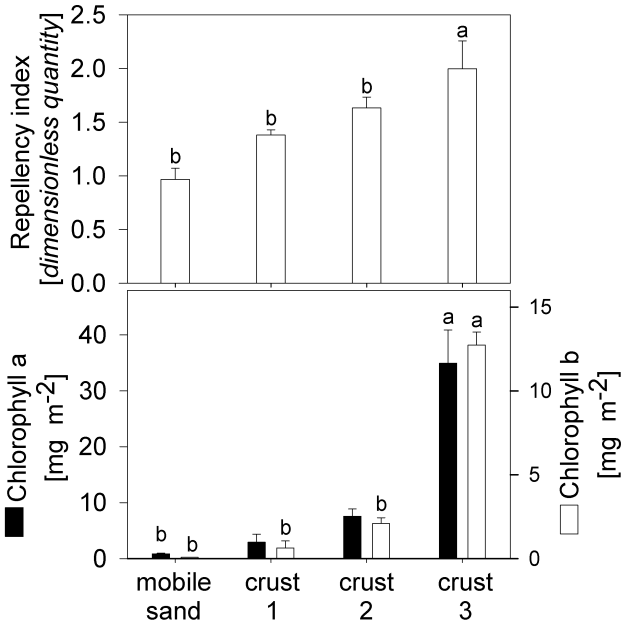


Figure 4.5: Repellency index and chlorophyll contents of the BSCs. Significant differences tested by one-way ANOVA ($P < 0.05$) are indicated by different letters.

different phases with each other, a clear increase of potential enzyme activities was observed from phase 1 to phase 3. An increase of protease, nitrification, and denitrification activity could first be observed in the subsoil and then in the deeper subsoil (Figure 4.6). Activities in mobile sand and the deeper subsoil were often close to or below the LOD. In reference, potential chitinase, protease and denitrification activities were similar to the activities observed in the subsoils of phase 3 and ranged between 0.1 and 0.3 nmol g^{-1} h^{-1}. In contrast, levels of potential nitrification activity in the reference sample (2.7 nmol NO_2^--N g^{-1} h^{-1}) were higher than in the subsoils, and comparable to the crust of phase 3 (3.4 nmol NO_2^--N g^{-1} h^{-1}).

4.4.4 Gene abundance

All quantified functional genes were of lowest abundance in mobile sand and of highest abundance in phase 3 (*nifH, chiA, amoA* AOA) or the reference (*nosZ*) (Figure 4.6). Two functional genes could not be quantified. *AprA* gene was detected in all samples, but below the LOQ of $7{\cdot}10^5$ copies g^{-1}. *AmoA* gene of AOB was not detected; PCR on sample templates did not produce amplicons of the right size, but a number of unspecific bands and smear (gels not shown). For all functional genes quantified, the abundance was always higher in the crusts than in the subsoils. In contrast to enzyme activity, gene abundances did not show a steady increase from phase to phase 3. While *nifH* and *chiA* abundance remained constant from phase 1 to phase 2, *amoA* (AOA) and *nosZ* abundance decreased in crust and subsoil of phase 2 and increased in phase 3 again. Abundance of *NosZ* was high in the crust of phase 1 ($3.8{\cdot}10^4$copies g^{-1}) and of similar range as in crust of phase 3 ($3.5{\cdot}10^4$copies g^{-1}).

4.5 Discussion

4.5.1 Identification of BSC development

A prerequisite of our study was the presence of different phases of BSC development, and our measurements confirmed our first hypothesis that this was in fact the case. BSC development was evident from the distinct changes in chemical, physical, and biological parameters (Belnap and Eldridge, 2001; Fischer et al., 2010). As expected, the development proceeded from mobile sand, via BSC of phase 1 and 2, to the established BSC in phase 3. In this order, TOC, TN, and chlorophyll content increased, and measurements in phase 2 and 3 are comparable to previous characterisations of BSCs in arid (Johnson et al., 2005; Barger

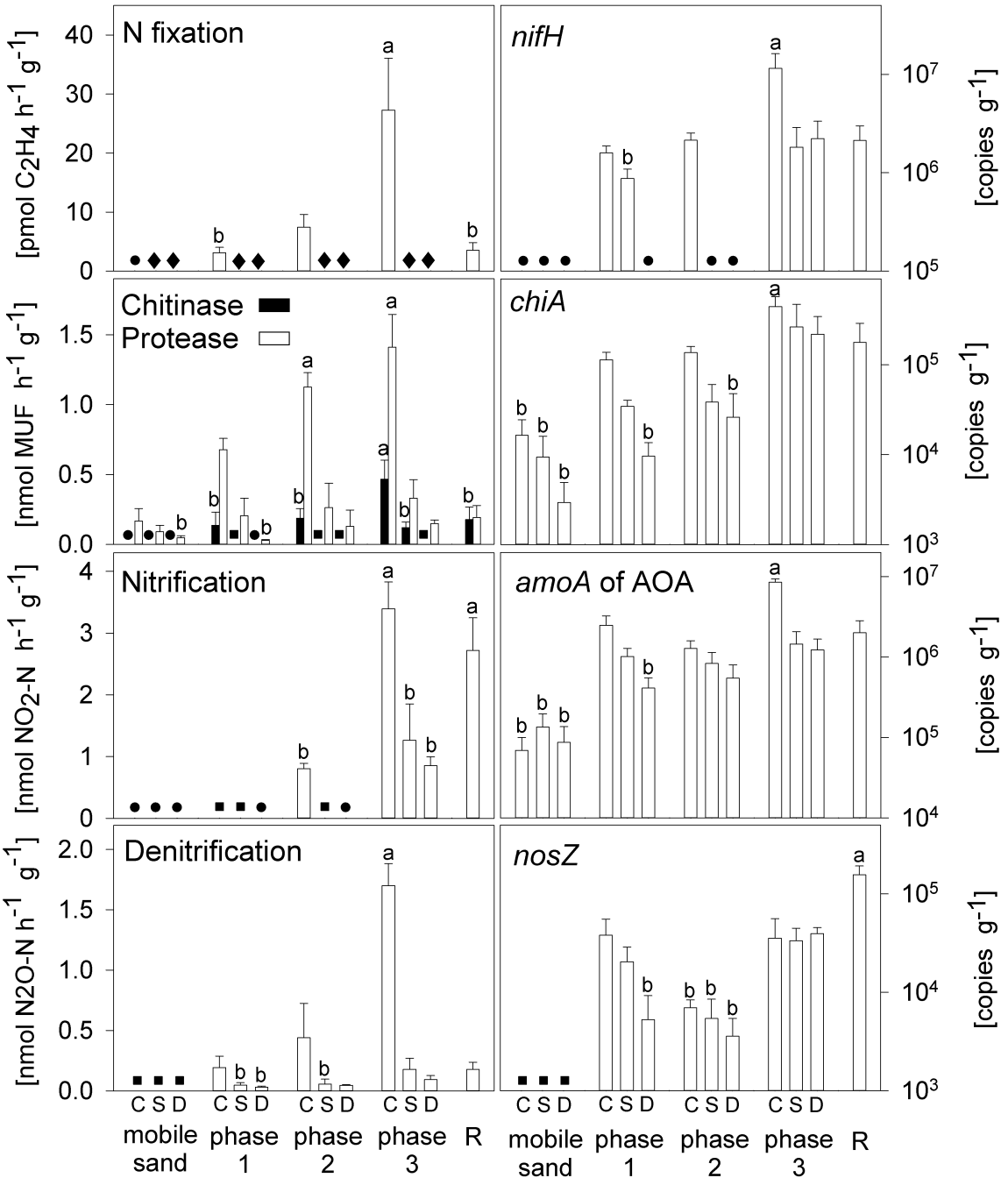


Figure 4.6: Potential enzyme activities (left panel) and abundance of functional genes (right panel). Each bar represents mean of 5 replicates, error bars indicating the standard deviation. Significant differences tested by one-way ANOVA on ranks ($P < 0.05$) are indicated by different letters on top of the error bars. Letters C, S, and D indicate sampling depth; C crust (0-5 mm depth), S subsoil (5-15 mm depth), and D deeper subsoil (15-35 mm depth).

et al., 2005; Fischer et al., 2010) and temperate climate (Langhans et al., 2009; Fischer et al., 2010). The proportional decrease of chlorophyll *a* compared to chlorophyll *b* indicates a shift from cyanobacterial BSCs to BSCs dominated by algae and mosses (Belnap and Eldridge, 2001; Zaady et al., 2010), confirming that our sampling approach represents typical BSC development. Langhans et al. (2009)found significant differences in floral composition between initial and developed BSC in temperate climate, however, TN content did not differ. In contrast, we identified changes in TN content and other parameters, underlining the presence of a gradient of BSC development at the Lieberose dune.

4.5.2 Development of nitrogen pools

Beyond the changes in chemical, physical and biological parameters that occur during BSC development, we were interested in the development of the N cycling processes which depend on N availability. While TN concentrations increased in the crusts and in the subsoils, they were always higher in the subsoils right under the crust compared to the deeper subsoil, indicating that a trans-location from the crusts to the underlying soil took place (West and Klemmedson, 1978; Johnson et al., 2005). The pool size of TN showed a strong increase from mobile sand to phase 3. The TN pool in the reference was comparable to that of crust 3. This might indicate that TN reaches a steady state in the BSC system (Wallace et al., 1978). Nitrate and ammonium content did not follow the general pattern of nutient increase. The highest nitrate concentrations and nitrate pools were found in phase 1, while the highest ammonium concentrations and ammonium pools were found in phase 3. During phase 1 the BSC is thin and oxygen penetration is high, this might favour the process of nitrification while denitrification rate is low, causing an accumulation of nitrate. In contrast, in the developed crust of phase 3 TOC content is high and anaerobic conditions may form after rewetting events, facilitating denitrification (Johnson et al., 2007) and causing depletion in nitrate. This is supported by the high potential denitrification rates measured in phase 3. The ammonium might originate from N fixation, as the development of N fixation parallels ammonium concentrations and has been reported to increase soil ammonium content (Klubek et al., 1978; Evans and Belnap, 1999).

4.5.3 Hydrological effects on nitrogen pools

In addition to the N pool sizes, the redistribution of N in the soil will impact N availability, thus affecing N cycling processes. The soil hydrology, in particular, affects N redistribution in sand dunes, as was previously shown for arid

ecosystems (Evans and Lange, 2001; Yair, 2001; Johnson et al., 2007). At the studied dune the slope is gentle and BSC repellency is low in the upper part of the slope. In addition, the soil texture is sand with a low proportion of silt and clay. Therefore, we expect infiltration and vertical N transport rather than surface runoff and lateral N allocation. The evaporation length in this soil was estimated to be 25 cm depth (Dani Or, pers. comm.), i.e. the depth water can be drawn back to the surface by capillary force. For this reason, all dissolved nutrients leaching below 25 cm depth become inaccessible for the BSCs. Heavy rains are expected to leach dissolved nutrients. However, mild to moderate rain may not move nutrients beyond the 25 cm depth. This means that during moderate rains N would not be lost via leaching, but would continue to accumulate in the dune. For this reason, we modelled the water flux at the dune surface and at a depth of 25 cm depth for the year 2010. We could identify a number of rain events that caused a water flux at the surface, but did not lead to water flux at 25 cm depth (Figure 4.1). If we assume that the rain event, which occurred 10 days prior to our sampling (18 mm), leached all nitrate and ammonium (due to the low ion exchange capacity of sand), then 2.9 mmol N m^{-2} would have been deposited (atmospheric N deposition rate of 12 µmol m^{-2} h^{-1}) at our site within the 10-days period between the rain event and our sampling. This is in the same order of magnitude as the nitrate and ammonium pools we could measure.

4.5.4 Succession of nitrogen cycling processes

To assess N turnover of each phase of BSC development, we calculated area based enzyme activities (Figure 4.7). We are aware that these calculations are tentative, as they rely on potential enzyme activities only. Additionally, the different enzyme assays vary in methodology and are not comparable. Nevertheless, we find this synopsis useful and stimulating for interpreting our results.

The development of the N cycling processes proceeded in a distinct order. Therefore, we propose three stages of N cycle succession. First, *the heterotrophic stage*, represented by the mobile sand; second, *the transition stage* which is represented by phase 1 and phase 2; third, *the developed stage*, represented by phase 3 and reference.

The heterotrophic stage (mobile sand) is characterised by mineralisation as the prevailing process and the dominance of opportunistic heterotrophic bacteria. The heterotrophic stage was described earlier in glacier forefields (Sigler et al., 2002; Brankatschk et al., 2011); and Hodkinson et al. (2002) propose an initial heterotrophic stage as general rule in primary succession. In mobile sand, we

found *aprA* and *chiA* genes, and protease activity was present in the same order of magnitude as previously reported mineralisation rates in the Negev Desert (Zaady, 1996). In the heterotrophic stage, cyanobacteria and algae are virtually absent. Therefore, the carbon sources of the microbial community may be allochthonous (Hodkinson et al., 2002; Brankatschk et al., 2011) and/or recalcitrant (Bardgett et al., 2007) organic matter. Quantifying the macroscopic litter (>2 mm) in mobile sand, we found 0.2 g m^{-2} of litter material, such as birch leaves, birch seeds, and tree bark (data not shown). This demonstrates that approximately 3 % of TOC in mobile sand is allochthonous and readily available carbon. By metabolising the organic matter, the heterotrophic microorganisms release inorganic N to the soil. This N, together with the inorganic N from atmospheric deposition, is easily leached and N does not accumulate during that stage.

In *the transition stage* (phase 1 and phase 2), cyanobacteria and algae establish in the BSC, and—via photosynthesis—build up organic matter, which requires a steady N supply. Although N fixation activity and *nifH* gene abundance increase, the contribution of N fixation to soil N supply appears to be low. Atmospheric deposition rates are two orders of magnitude higher than N fixation activity (0.1 µmol C_2H_4 m^{-2} h^{-1}), ranging from 9.8 to 12.2 µmol N m^{-2} h^{-1} (12-15 kg N ha^{-1} a^{-1}) (Gauger et al., 2008). Previously reported N fixation rates in BSCs ranged from 50-100 µmol C_2H_4 m^{-2} h^{-1} (MacGregor and Johnson, 1971; Jeffries et al., 1992; Abed et al., 2010) down to 0.02 µmol C_2H_4 m^{-2} h^{-1} (Evans and Belnap, 1999; Strauss et al., 2011). Despite the fact that *nifH* gene abundance in the Lieberose sand dune is the same as in the Colorado Plateau, USA and the Chihuahuan Desert, USA (Yeager et al., 2004, 2007), we found a comparatively low N fixation activity. This difference is likely due to the early developing phase (Barger et al., 2005) and high N deposition rates (Vitousek et al., 2002) that are typical for Central Europe. The discrepancy between gene abundance and potential enzyme activity might be due to different levels of cell activity (Roling, 2007). Furthermore, a given process may be catalysed by a broader variety of enzymes, whereas the primers used in the study may only capture a selected number of microorganisms. In the transition stage, the potential denitrification activity increases, ranging from 2.8 to 4.3 µmol N m^{-2} h^{-1}, which is comparable to previous studies (West and Skujins, 1977; Peterjohn and Schlesinger, 1991). Conditions for denitrification might be ideal during the transition stage, as both important substrates for denitrification, i.e. nitrate and carbon (Zumft, 1997), are available. The atmospheric deposition rate of nitrate is high, and cyanobacteria excrete extracellular polymeric substances that are readily available carbon sources (Mazor et al., 1996; Mager and Thomas, 2011).

In *the developed stage* (phase 3 and reference) the potential enzyme activities are comparable to developed ecosystems (West and Skujins, 1977; Peterjohn and Schlesinger, 1991; Johnson et al., 2007; Brankatschk et al., 2011), and might advance towards a steady state. The N cycle is dominated by the nitrification process (Figure 4.7), and marks the shift towards internal N cycling. Nitrification rates in the crust of phase 3 were comparable to rates measured in the Great Basin, USA (Skujins and Trujillo Y Fulgham, 1978). The nitrification activity pattern is matched by gene abundance, showing highest amoA (AOA) gene abundance in crusts. Interestingly, amoA gene abundance of AOB was below 1,200 copies per g soil, i.e. two to three orders of magnitude lower than AOA abundance. This is supported by previous studies reporting that AOA outnumber AOB up to 200-fold (Leininger et al., 2006; He et al., 2007; Wankel et al., 2011), and suggests that AOA show a greater relative activity compared to AOB, as was previously reported from other acidic environments (Nicol et al., 2008).

4.5.5 Conclusions

We studied the development of the N cycle along a gradient of BSC development. In the traditional view, the BSC development is characterised by increase of nutrients, carbon, chlorophyll, BSC thickness, as well as a distinct succession of colonising species (Belnap and Eldridge, 2001). We characterised the development of BSCs by the occurrence and intensity of N processes and identified three distinguished successional stages: *i*) the heterotrophic stage dominated by mineralisation activity, *ii*) the transition characterised by an increase of biomass and TN, and *iii*) the developed stage dominated by nitrification activity and high internal N turnover.

4.6 Acknowledgements

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Figure 4.7: Potential process activities for each phase of BSC development. Each diagram gives input, output and internal turnover terms as determined for each phase of BSC development. Processes were calculated for considering 35 mm soil depth and assuming a bulk soil density of 1.5 g · cm−3 . For mobile sand and crust 1 to 3, the 35 mm include crust (5 mm), subsoil (10 mm) and deeper subsoil (20 mm) samples.

Authors contributions

RB participated in the design of the study, performed enzyme and molecular analysis, and wrote the manuscript. TF participated in the design of the study, performed chemical analysis of soil and BSC, and helped draft the manuscript. MV participated in the design of the study and helped draft the manuscript. JZ participated in the design of the study and drafted the manuscript.

5 Simple Absolute Quantification Method Correcting for Quantitative PCR Efficiency Variations for Microbial Community Samples

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5.1 Abstract

Real-time quantitative PCR (qPCR) is a widely used technique in microbial community analysis, allowing the quantification of the number of target genes in a community sample. Currently, the standard curve (SC) method of absolute quantification is widely employed for these kinds of analysis. However, the SC method assumes that amplification efficiency (E) is the same for both the standard and the sample target template.

We analysed nineteen bacterial strains and nine environmental samples in qPCR assays, targeting the *nifH* and 16S rRNA genes. The E of the qPCR reactions differed significantly, depending on the template. This has major implications for the quantification. If the sample and standard differ by their E, quantification errors of up to orders of magnitude are possible.

To address this problem, we propose and test the One-Point-Calibration (OPC) method for absolute quantification. The OPC method corrects for differences in E and was derived from the $\Delta\Delta C_t$ method with correction for E, which is commonly used for relative quantification in gene expression studies. The SC and OPC methods were compared by quantifying artificial template mixes from *Geobacter sulfurreducens* (DSM 12127) and *Nostoc commune* (CCAP 1453/33), which differ in their E. While the SC method deviated from the expected *nifH* gene copy number by 3 to 5-fold, the OPC method quantified the template mixes with high accuracy. Moreover, analysing environmental samples, we show that even small differences of E between the standard and the sample can cause significant differences between the copy numbers calculated with the SC as compared to the OPC method.

5.2 Introduction

In microbial ecology, real-time quantitative PCR (qPCR) is used to measure the number of copies of a gene of interest in a community or an environmental sample (Schubert et al., 2006; Smith et al., 2006; Liebner et al., 2009, 2011). The presence of specific phylogenetic groups can be quantified by targeting the 16S rRNA gene (Bekele et al., 2011; De Gregoris et al., 2011). Moreover, functional genes have been targeted to quantify the genetic potential of a community to catalyse certain processes (Wallenstein and Vilgalys, 2005; Leininger et al., 2006; Zehr et al., 2008; Smith and Osborn, 2009).

Originally, the qPCR technique was developed as a tool in medical diagnostics targeting non-variable regions (Mackay et al., 2002; Espy et al., 2006). However, for two reasons the application of qPCR in microbial ecology needs to take into account genetic variations of the targeted region. First, although primers are designed to target a certain phylogenetic group, highly similar sequences, in which primer target sites differ only in a few bases, might be present and co-amplified from environmental samples (von Wintzingerode et al., 1997). Second, to account for sequence variability within the target phylogenetic group, degenerate primers have been frequently used in qPCR assays (Wallenstein and Vilgalys, 2005; Kandeler et al., 2006; Leininger et al., 2006; Brankatschk et al., 2011). Primer mismatches (Bru et al., 2008) as well as the use of degenerate primers differing in GC-content (Polz and Cavanaugh, 1998) affect primer annealing kinetics and therefore might affect accuracy of the qPCR assay.

The qPCR technique is based on real-time monitoring of amplicon formation by a reporter molecule (e.g. SYBR Green dye) (Bustin, 2004b). Fluorescence (Y) is measured after each temperature cycle and is proportional to the amount of synthesised amplicon N, $N \propto Y$ (Figure 5.1A). The exponential growth of the amplicon concentration in the reaction N_C can be described as an exponential function of template starting concentration N_0, efficiency of the qPCR E and the number of qPCR cycles C, $N_C = N_0 \times E^C$ (Bustin, 2004b). Two parameters are essential for quantification: threshold cycle C_t and qPCR E. The C_t is the number of cycles necessary to reach a certain threshold fluorescence Y_t. In one experimental setup, Y_t is the same for all samples. Since fluorescence is a relative measure of DNA content, all samples contain the same number of amplicons N_t when passing the C_t. E is a measure of amplification quality and depends on factors such as primer GC-content (Polz and Cavanaugh, 1998), primer mismatch (Bru et al., 2008), and the presence of PCR inhibitors (Kontanis and Reed, 2006). If $E = 2$, the number of amplicons doubles per cycle, i.e. the efficiency is 100 %. Two distinct methods can be used to estimate E. First, E_{fi} can be estimated from the fluorescence increase using linear (Peirson,

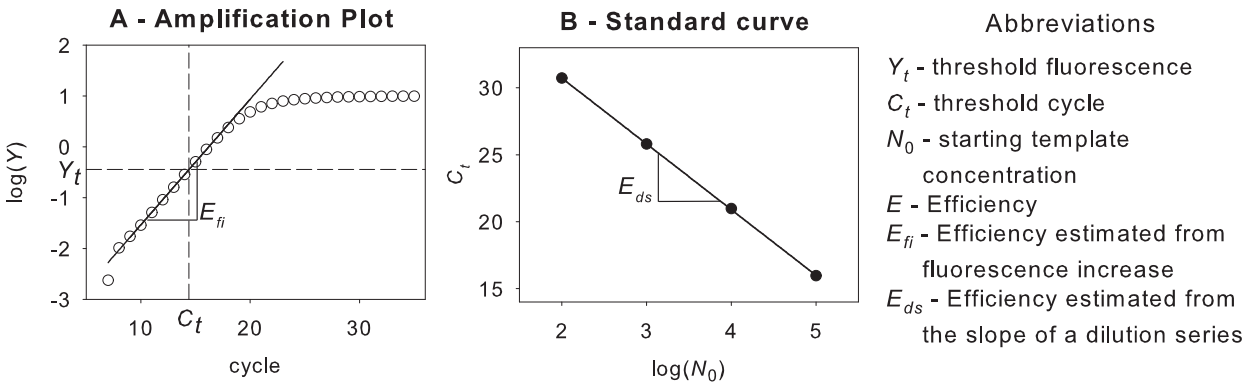

Figure 5.1: A. Schematic amplification plot of log fluorescence increase over qPCR cycles, indicating the slope to estimate E_{fi} by linear regression. B. Schematic standard curve of a dilution series, plotting C_t values over log template concentrations. The slope is used to estimate E_{ds}.

2003; Ruijter et al., 2009) or non-linear regression models (Liu and Saint, 2002; Tichopad et al., 2003) (Figure 5.1A). Second, E_{ds} can be estimated from the slope of a dilution series (Higuchi et al., 1993; Rasmussen, 2001) (Figure 5.1B).

Two major quantification methods have been developed and are widely used, relative quantification and absolute quantification. Relative quantification using the $\Delta\Delta C_t$ method is the most common method used in gene expression analysis (VanGuilder et al., 2008), but has also been applied in environmental microbiology (Walsh et al., 2011). The method determines the gene expression ratio (or abundance ratio) of a target gene in a sample compared to a control, normalising with the expression ratio of a reference gene. Target and reference genes often differ by their E, thus the $\Delta\Delta C_t$ method has been improved in order to account for differences in E (Pfaffl, 2001).

Absolute quantification by the standard curve (SC) method is frequently used in environmental microbiology. The SC method employs a dilution series of known template concentrations N_0 in the qPCR assay. Linear regression of $\log(N_0)$ versus C_t gives the standard curve and this is then used to calculate template concentrations N_0 of the sample. This method assumes that E of the sample is the same as E of the standard.

In environmental microbiology, the SC method has been used to quantify the concentration of target genes in diverse samples, e.g., microbial communities of biofilms (De Gregoris et al., 2011), the rumen (Bekele et al., 2011), and alpine soils (Brankatschk et al., 2011). In most cases, the sample template is different from the standard template used to prepare the SC. The standards usually originate from pure cultures, while the sample is composed of a mixture of different species. Previous studies demonstrated that E estimates significantly affect

quantification accuracy. It is therefore recommended that accurate E estimates be employed in quantification analysis (Liu and Saint, 2002; Ramakers et al., 2003; Ritz and Spiess, 2008; Ruijter et al., 2009; Tichopad et al., 2010). In spite of these recommendations, the SC method assumes a constant E for standard and sample. Although this introduces the possibility of increased quantification errors, it is still the method of choice in environmental microbiology.

Our first aim was to demonstrate the variability of qPCR E with the template source. As a model functional gene we selected the *nifH* gene coding for the nitrogenase iron protein (NifH, EC 1.18.6.1) because it is the focus of numerous studies in ecology and therefore well characterised. In addition, the 16S rRNA gene was used to demonstrate the universality of the problem studied. Universal 16S rRNA gene targeting approaches are frequently used as a community reference in qPCR based studies (Geets et al., 2007; Bekele et al., 2011; De Gregoris et al., 2011). We estimated E_{fi} from the fluorescence increase of nineteen bacterial strains covering Actinobacteria, Cyanobacteria, Firmicutes, and Proteobacteria, as well as nine environmental samples representing typical terrestrial and aquatic habitats. Moreover, we propose a novel absolute quantification method that accounts for differences in E. We demonstrate that the new method is less susceptible to quantification errors than the SC method by analysing an artificial mix of two templates that differ in E.

5.3 Methods

5.3.1 Bacterial cultures

Strains and cultivation conditions

Nineteen bacterial strains containing the *nifH* gene were used in this study. The strains were selected to cover different phyla and to be genome-sequenced, if possible. The strains listed in Table 5.1 were grown at room temperature in the media specified by the Culture Collection of Algae and Protozoa (CCAP), the Pasteur Culture Collection (PCC), and the German Collection of Microorganisms and Cell Cultures (DSMZ) for the respective strain, except *Methylosinus trichosporium* and *Methylococcus capsulatus*, which were grown in nitrate mineral salts (NMS) medium (DSMZ medium 632, Whittenbury et al., 1970) with 10% methane in air (v/v) in the headspace. 16S rRNA and *nifH* genes were sequenced for strains that were taken from our laboratory collections (glycerol stocks) in order to verify the identity of the strains. Sequencing was performed at the Genetic Diversity Centre of ETH Zurich using BigDye Terminator v1.1

Table 5.1: Bacterial strains used in this study.

Name	Strain	Phylogenetic group
Frankia sp.	DSM 43829	Actinobacteria
Cyanothece sp.	PCC 7425	Cyanobacteria
Nostoc commune	CCAP 1453/33	Cyanobacteria
Nostoc sp.	PCC 7120	Cyanobacteria
Nostoc punctiforme	PCC 73102	Cyanobacteria
Paenibacillus sabinae	DSM 17841	Firmicutes
Azospirillum brasilense	DSM 1690	α-proteobacteria
Bradyrhizobium japonicum	USDA 110	α-proteobacteria
Methylosinus trichosporium	OB3b[a]	α-proteobacteria
Rhizobium leguminosarum	DSM 30132	α-proteobacteria
Sinorhizobium meliloti	1021[b]	α-proteobacteria
Sphingomonas azotifigens	DSM 18530	α-proteobacteria
Xanthobacter autotrophicus	DSM 432	α-proteobacteria
Acidithiobacillus ferrooxidans	DSM 14882	β-proteobacteria
Burkholderia xenovorans	DSM 17367	β-proteobacteria
Azotobacter vinelandii	DSM 85	β-proteobacteria
Methylococcus capsulatus	Bath[c]	β-proteobacteria
Pseudomonas stutzeri	DSM 4166	β-proteobacteria
Geobacter sulfurreducens	DSM 12127	δ-proteobacteria

[a, c] Whittenbury et al. (1970).
[b] Capela et al. (2001).

chemistry (Applied Biosystems, Foster City, CA, USA) on an 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

DNA extraction and quantification

Bacterial colonies from agar plates or bacterial pellets from liquid cultures were washed three times in phosphate buffered saline (pH 7.4) prior to DNA extraction with a Wizard Genomic DNA kit (Promega, Madison, USA). Extraction was performed according to manufacturer's instructions and DNA was eluted with 100 µL DEPC treated water (Carl Roth, Karlsruhe, Germany). DNA purity was checked on a nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, USA), and quantified with SYBR Green I (Matsui et al., 2004).

5.3.2 Environmental samples

DNA extracts from three natural habitats, soil, plant, and lake, were used in this study to represent a broad range of samples typically studied in environmental microbiology. The DNA extracts were obtained in three previously conducted studies (Brankatschk et al., 2011; Knief et al., 2011; Köllner et al.,

2011) and stored at -80 °C until analysis. Samples *bulk soil, young* and *bulk soil, old,* were taken from the forefield of the Damma glacier in Switzerland and represent more than 50 years of soil development in the former case and over 2000 years in the latter (Brankatschk et al., 2011). Soil samples were taken with shovels and frozen in the field on dry-ice. Upon arrival in the laboratory the soil samples were stored at -20 °C. Samples *rhizosphere* and *phyllosphere* were obtained from rice plants grown at the International Rice Research Institute, Los Baños, Philippines (Knief et al., 2011). Samples from the *eutrophic lake* were taken at 10, 80 and 170 m depth at Lake Zug, Switzerland (Köllner et al., 2011). The lake is meromicitc and permanently anoxic below 160 m (Köllner et al., 2011). Samples from the *oligotrophic lake* were taken at 5 and 200 m depth at Lake Brienz, Switzerland, which is fully oxic throughout the year (Köllner et al., 2011). Lake water samples were stored in the dark on ice. Upon arrival in the laboratory, the samples were filtrered onto 0.2 μm polycarbonate filters following 5 μm prefiltration and the filters were frozen in liquid nitrogen and stored at -80 °C prior to DNA extraction (Köllner et al., 2011). Details on sampling and DNA extraction are given in the respective reference (Brankatschk et al., 2011; Knief et al., 2011; Köllner et al., 2011).

5.3.3 Real-time PCR assays

All qPCR assays were performed on an ABI 7300 system (Applied Biosystems, Foster City, CA, USA). Reaction volumes were 20 μL and contained 1-fold Kapa SYBR Fast PCR master mix (Kapa Biosystems, Cape Town, South Africa), DEPC treated water (Carl Roth, Karlsruhe, Germany), and 200 nM of both forward and reverse primer. Templates were added in 1 μL volume per reaction. Triplicates of no-template controls, containing DEPC treated water, were included in each run. To amplify the *nifH* gene, the primer pairs nifHF/nifHR, PolF/PolR, and ForA/Rev were used. The 16S rRNA gene was amplified using the 27F/518R primer pair. All primers employed in this study were published previously and have been extensively used before. Details on primer sequences and thermal cycles are given in Table 5.2. In preliminary experiments, the annealing temperatures of all reactions were optimised for high specificity (crisp band, no smear) and high yield when amplifying the environmental samples. All qPCR templates were tested for amplification inhibition by dilution series. After each qPCR run, melting curve analysis was performed to verify the presence of the desired amplicon.

Table 5.2: Primers and thermal qPCR profiles used.

Primer pair	Target gene	Annealing time and temperature[a]	Primer sequences[d] (5'-3')	T_m^e [°C]	Primer references
PolF/ PolR	*nifH*	25 s at 53 °C[b]	TGCGAYCCSAARGCBGACTC/ ATSGCCATCATYTCRCCGGA	60-64/ 62-68	Poly et al., 2001
nifHF/ nifHR	*nifH*	25 s at 53 °C[b]	AAAGGYGGWATCGGYAARTCCACCAC/ TTGTTSGCSGCRTACATSGCCATCAT	76-82/ 78-80	Rösch et al., 2002
ForA/ Rev	*nifH*	60 s at 50 °C	GCIWTITAYGGNAARGGNGG/ GCRTAIABNGCCATCATYTC	56-68/ 54-64	Widmer et al., 1999
27F/ 518R	16S rRNA	30 s at 56 °C[c]	GAGTTTGATCMTGGCTCAG/ ATTACCGCGGCTGCTGG	56-58/ 60	Janssen et al., 2002/Muyzer et al., 1993

[a] all reactions started with 300 s initial denaturation at 95 °C, then 40 cycles of 15 s denaturation at 95 °C, annealing step, and 45 s elongation at 72 °C.
[b] touchdown cycle starting at 63 °C, temperature decrease of 2 °C per cycle.
[c] touchdown cycle starting at 63 °C, then 61, 59, 57, and 56 °C annealing temperature.
[d] primer sequences using IUPAC ambiguity code, except I stands for inosine.
[e] ranges of melting temperatures of degenerate primers calculated by "The Wallace rule": Tm=2*(A+T)+4*(G+C) (Sambrook and Russell, 2001).

5.3.4 Determination of C_t and E_{fi}

Raw data were exported from the ABI system and imported into the LinRegPCR program (ver 11.4) (Ruijter et al., 2009). In the program settings, all samples of one qPCR run were treated as one amplicon group in order to set one common window-of-linearity. Then the programm automatically determined the fluorescence threshold for all samples and calculated the individual C_t and E_{fi} values (Ruijter et al., 2009). Results were exported and the mean E_{fi} of each sample was calculated as the arithmetic mean of all replicates, excluding as outliers any replicates that were 5% above or below median efficiency of all replicates.

5.3.5 Quantification methods

Standard curve (SC) method

The SC method is widely used in environmental microbiology and employs a dilution series of a defined target concentration. The target concentration, c_{target} [copies μL^{-1}], was calculated from the DNA concentration c_{DNA} [ng μL^{-1}],

the length of the DNA fragments l_{DNA} [bp] (e.g. genome size), the number of targets per DNA fragment n_{target} [copies], the Avogadro constant N_A ($6.022 \cdot 10^{23}$ bp mol^{-1}), and the average weight of a double-stranded base pair M_{bp}(660 g mol^{-1} = $6.6 \cdot 10^{11}$ ng mol^{-1}) (Equation 5.1).

$$c_{\mathrm{target}} = n_{\mathrm{target}} \times \frac{c_{\mathrm{DNA}} \times N_A}{l_{\mathrm{DNA}} \times M_{\mathrm{bp}}} \tag{5.1}$$

The linear regression of $\log(N_{0\,\mathrm{standard}})$ versus C_t gives the constants intercept a and slope b of the standard curve (Equation 5.2). The amount of copies in the sample $N_{0\,\mathrm{sample}}$ can be calculated based on the regression. Slope b of the linear regression is used to estimate E_{ds} (Equation 5.3). For the individual experiments (see below) dilution series of *Bradyrhizobium japonicum, Geobacter sulfurreducens,* and *Nostoc commune* genomic DNA were used to prepare standard curves.

$$C_{t\,\mathrm{sample}} = a + b * \log(N_{0\,\mathrm{sample}}) \tag{5.2}$$

$$E_{ds} = 10^{(-1/b)} \tag{5.3}$$

One-Point-Calibration (OPC) method

We propose the One-Point-Calibration (OPC) method as an alternative method for absolute quantification from qPCR data. The method accounts for template-related variability of E by correcting for differences in E between sample and standard.

OPC is performed by defining one standard containing a known number of template copies $N_{0\,\mathrm{standard}}$. Each standard point reaction contained approximately 0.05 to 0.5 ng genomic DNA, equivalent to $10^4 - 10^6$ copies (Equation 5.1), and was replicated three to four times. The template concentration of a sample $N_{0\,\mathrm{sample}}$ was estimated from the cycle thresholds $C_{t\,\mathrm{sample}}$ and $C_{t\,\mathrm{standard}}$, and the efficiencies E_{sample} and E_{standard} (Equation 5.4).

$$N_{0\,\mathrm{sample}} = N_{0\,\mathrm{standard}} \times \frac{E_{\mathrm{standard}}^{C_{t\,\mathrm{standard}}}}{E_{\mathrm{sample}}^{C_{t\,\mathrm{sample}}}} \tag{5.4}$$

We calculated E_{sample} and E_{standard} as the mean E_{fi} from individual replicates because previous studies recommend using the means of E_{fi} (Peirson, 2003; Schefe et al., 2006; Čikos et al., 2007). Alternatively, E_{sample} and E_{standard}

could be estimated from dilution series, E_{ds} (Equation 5.3) (Pfaffl, 2001). In addition, it is necessary to verify the linear range of quantification, for example, by analysing a dilution series of a standard. Information about the coefficient of variation of the C_t values of replicated standard dilutions can also be helpful in assessing the limit of detection (Burns and Valdivia, 2008; Bustin et al., 2009).

5.3.6 E_{fi} of bacterial strains and environmental samples

Amplification efficiencies of the bacterial strains and environmental samples were determined in qPCR assays and calculated from the fluorescence increase as decribed above (Ruijter et al., 2009); 0.1 to 0.01 ng of DNA per reaction was used. Each sample was run in four replicates. The number of samples prohibited the simultaneous amplification of all bacterial strains and environmental samples in a single run. Therefore, one qPCR run was performed to compare all bacterial strains, and one qPCR run was performed to compare all environmental samples. One-way ANOVA was performed in SigmaPlot (ver 11.0, Systat Software) to test for significant differences ($p < 0.01$) between the bacterial strains and between the environmental samples.

5.3.7 Analysis of template mixtures from two species

Serial dilutions of *G. sulfurreducens* and *N. commune* DNA templates were prepared. From the different dilution steps, template mixtures of *G. sulfurreducens* and *N. commune* were prepared. The template mixtures were prepared to have template ratios of approximately 100:1, 10:1, 1:1, 1:10, and 1:100 of *G. sulfurreducens* to *N. commune*. The dilution series and the template mixtures were run as triplicates in one qPCR assay, using nifHF/nifHR primers. Standard curves were calculated from *G. sulfurreducens* and *N. commune* dilution series. All samples were quantified using the SC and the OPC method. Each of the two methods was calibrated once with *G. sulfurreducens* and once with *N. commune* as the standard.

5.3.8 Analysis of environmental samples

Four replicates of each environmental sample and triplicates of a *B. japonicum* dilution series were amplified by qPCR using the nifHF/nifHR primer pair. This was the same run in which the E_{fi} of the environmental samples was measured. All samples were quantified using the SC and the OPC method, using *B. japonicum* as the standard. The Mann-Whitney rank sum test ($p <$

0.01) was performed to test for significant differences between SC and OPC quantifications. *B. japonicum* was used for calibration as it is heterotrophic and simple to maintain, and therefore likely to be used for calibration in other laboratories.

5.4 Results and discussion

5.4.1 Efficiency of qPCR reactions depends on the template

The qPCR efficiency of nineteen bacterial strains covering four bacterial phyla were compared. The qPCR assays targeted the *nifH* gene using three independently developed and previously published primer pairs, and targeted the 16S rRNA gene using one likewise well established primer pair (Table 5.2). In all assays, the strains differed significantly by their efficiency E_{fi} (Figure 5.2). In the assay employing the nifHF/nifHR primer pair, E_{fi} ranged from 1.67 in *G. sulfurreducencs* to 1.77 in *N. commune.* In the assay targeting the 16S rRNA gene (27F/518R), E_{fi} ranged from 1.84 in *P. stutzeri* to 1.98 in *Frankia* sp. Some bacterial strains did not amplify when targeting the *nifH* gene with nifHF/nifHR and ForA/Rev primer pairs. In contrast, qPCR on *nifH* with PolF/PolR primers as well as qPCR on the 16S rRNA gene (27F/518R) amplified DNA from all strains. Variation of E_{fi} appeared to be independent from the phylogeny of the strains; however, we analysed only a small subset of bacterial phyla and did not include Archaea or Eukarya in the analysis.

Similar to the observations made for bacterial strains, the E_{fi} determined for environmental samples differed significantly (Figure 5.2). E_{fi} ranged from 1.72 for the oligotrophic lake (5 m) to 1.80 in the rhizosphere sample with the nifHF/nifHR primer pair. In the assay primed with PolF/PolR, E_{fi} ranged from 1.77 in the eutrophic lake (10 m) to 1.99 in the bulk soil (old). Targeting the 16S rRNA gene, E_{fi} ranged from 1.85 in the bulk soil (old) to 1.95 in the eutrophic lake (170 m). Interestingly, E_{fi} was also variable for samples originating from within the same ecosystems. When amplified with the ForA/Rev primers, E_{fi} in the oligotrophic lake sample ranged from 1.58 in 5 m depth to 1.83 in 200 m depth.

These results are supported by previous studies showing that E varies with the PCR template. Using the LinRegPCR method to calculate E_{fi}, Čikos et al. (2007) found the interleukin 6 gene to amplify with an E_{fi} of 1.88, while the beta actin gene amplified with an E_{fi} of 1.99. Moreover, they found that these differences between the genes remained regardless of the method used to determine E_{fi} (Čikos et al., 2007). Similarly, other studies found that E

determined for the same PCR template is more constant (Liu and Saint, 2002; Bustin, 2004b; Čikos et al., 2007). For example Bustin (Bustin, 2004b) reports a constant E_{fi} of around 1.8 and low variation among the replicates for the amplification of the metalloproteinase inhibitor 1 gene. Furthermore, we could reproduce findings reported by Töwe and coworkers (Töwe et al., 2010b); we analysed bulk soil from the Damma glacier and found that E_{fi} of bulk soil (E_{fi} =1.75-1.76) was very similar to E_{fi} of *S. meliloti* (E_{fi} = 1.75) when using the nifHF/nifHR primer pair. Similarly, Töwe et al. (2010b) report the same E_{fi} for both the sample from the Damma glacier and the standard from *S. meliloti* (E_{fi} = 1.9). The difference in E_{fi}, 1.75 compared to 1.9, might be due to differing reaction conditions, i.e. assay chemistry, use of BSA, and thermocycler program. In summary, we demonstrated that over a range of bacterial strains and environmental samples tested, E_{fi} differed significantly.

The differences in E can be caused by a number of mechanisms that have been previously addressed. The first studies on the problem emerged in environmental microbiology, as PCR was used to simultaneously amplify different templates (Suzuki and Giovannoni, 1996; Polz and Cavanaugh, 1998). The term *PCR bias* was coined to describe the preferential amplification of some templates over others, and the phenomenon has been studied extensively. Mechanisms identified to affect PCR amplification and bias are: GC-content of the primer binding site (Polz and Cavanaugh, 1998), primer mismatches (Bru et al., 2008), reannealing of amplicon to templates (Suzuki and Giovannoni, 1996), homoduplex formation during temperature decrease (Kurata et al., 2004), annealing temperature (Ishii and Fukui, 2001), PCR inhibitors (Wiedbrauk et al., 1995; Kontanis and Reed, 2006), and steric hindrance (Hansen et al., 1998).

In our analysis, two mechanisms might have emphasised the significant differences in E: *i)* the use of degenerate primers and *ii)* the occurrence of primer mismatches. The use of degenerate primers has become widespread in qPCR analysis in order to target a broad set of organisms (Widmer et al., 1999; Wallenstein and Vilgalys, 2005; Geets et al., 2007; Töwe et al., 2010b). Degenerate primers are mixtures of highly similar primers that differ in at least one base, causing differences in the melting temperature (Chen and Zhu, 1997). In the qPCR assay, we used primers of different degrees of degeneracy (2- to 128-fold), exhibiting wide ranges of melting temperatures (Table 5.2) that might have affected kinetics of the qPCR reactions (Polz and Cavanaugh, 1998; Ishii and Fukui, 2001) and so might have affected the E of the individual assays. Similarly, primer mismatches significantly reduce E (Ishii and Fukui, 2001; Bru et al., 2008). Despite the fact that we used degenerate primers (nifHF/nifHR), commonly used in the literature, several of the bacterial strains exhibited one or more mismatches (Table 5.3). Although the number of mismatches was not

correlated with E (n=17, $R^2 = 5 \times 10^{-5}$, $p = 0.979$), they might have accentuated the E differences. Nevertheless, the aim of our study is to demonstrate the effect of differences in E on the accuracy of the quantification, not to provide a detailed analysis for underlying mechanisms of primer bias.

5.4.2 Limitation of the SC method

As demonstrated here, significant differences in qPCR E exist among bacterial strains and environmental samples. The SC quantification method does not account for the differences in E between the sample and the standard. Errors of up to orders of magnitude were observed in studies where E of the standard and the sample differed (Bru et al., 2008; Ruijter et al., 2009). Bru et al. (2008) observed that primer mismatches of a single base decrease E and can cause underestimation of actual the copy numbers by up to a factor of 1000. A solution to this problem was found in gene expression analysis: The $\Delta\Delta C_t$ method with correction for efficiency, also called the Pfaffl method or the comparative C_t method with correction was established (Pfaffl, 2001; VanGuilder et al., 2008). It considers the differences in E of the analysed genes to calculate the gene expression ratio (Pfaffl, 2001). However, the $\Delta\Delta C_t$ method is a relative quantification method only, and cannot be used for absolute quantification.

Here we propose the One-Point-Calibration (OPC) method, which is based on the $\Delta\Delta C_t$ method (Pfaffl, 2001). The OPC method is an absolute quantification method. In contrast to the SC method, the OPC method accounts for template-related variability of E. This major advantage is achieved by considering the individual E of the sample and the standard. One further advantage is that the OPC method does not necessarily rely on E_{ds} estimates. Previous studies described that E_{ds} shows higher variability than E_{fi} and demonstrated that E_{fi} is less biased than E_{ds} (Peirson, 2003; Schefe et al., 2006; Karlen et al., 2007; Ruijter et al., 2009). For example Karlen et al. (2007) found that estimates of E_{fi} are more robust than E_{ds} estimates from dilution series. Similarly, in our analysis of five independently prepared dilution series, the overall variability was significantly higher for E_{ds} than for E_{fi} (Figure 5.3, Table 5.4).

5.4.3 Hypothetical comparison of SC and OPC method

Based on the efficiencies we detected (Figure 5.2), we illustrate in a hypothetical example the errors that occur by not correcting for individual efficiency (Figure 5.4). We quantify the *nifH* gene (PolF/PolR primers) in a hypothetical rhizosphere sample of $C_{t\,\mathrm{sample}} = 21$ and $E_{\mathrm{sample}} = 1.99$ using *M. trichosporium* as

Table 5.3: Alignment of the primer sites targeted by the nifHF/nifHR primer pair.

Organism	Accession	Forward priming site (5'-3')		Reverse priming site (5'-3')[a]		Total	E_{fi}
		primer nifHF	Mis-	primer nifHR	Mis-	mis-	
		AAAGGYGGWATCGGYAARTCCACCAC	matches	ATGATGGCSATGTAYGCSGCSAACAA[b]	matches	matches	
Frankia sp.	NC_008278[c]	AAGGGTGGTATCGGCAAGTCCACCAC	1	ATGATGGCGATGTACGCGGCGAACAA	0	1	1.73
Cyanothece sp.	NC_011884	AAAGGCGGGATTGGTAAATCCACCAC	2	ATGATGGCCATGTACGCTGCCAACAA	1	3	1.76
N. commune	L23514[c]	AAAGGCGGTATCGGTAAATCTACCAC	1	ATGATGGCGATGTATGCTGCTAACAA	2	3	1.77
N. punctiforme	NC_010628	AAGGGCGGTATCGGTAAATCTACCAC	2	ATGATGGCGATGTTTGCAGCTAACAA	2	4	1.76
Nostoc sp.	NC_003272	AAAGGCGGTATCGGTAAATCTACCAC	1	ATGATGGCGATGTATGCTGCTAACAA	2	3	1.77
P. sabinae	HM583800	AAAGGAGGAATCGGCAAATCCACCAC	0	ATGATGGCCATGTACGCAGCCAACAA	1	1	1.76
A. brasilense	X51500[c]	AAGGGCGGTATCGGCAAGTCCACCAC	1	ATGATGGCGCTCTACGCCGCCAACAA	1	2	1.77
B. japonicum	NC_004463	AAGGGCGGAATCGGCAAGTCCACCAC	1	ATGATGGCAATGTATGCCGCAAACAA	2	3	1.76
M. trichosporium	NZ_ADVE01000002	AAGGGCGGTATTGGCAAGTCGACGAC	4	ATGATGGCCATGTATGCCGGCAACAA	0	4	n.d.
R. leguminosarum	NC_008381	AAGGGCGGCATTGGCAAGTCCACTAC	4	ATGATGGCGCTCTATGCCGCCAACAA	1	5	1.75
S. meliloti	NC_003037	AAGGGGGGTATCGGCAAGTCCACGAC	3	ATGATGGCGCTCTATGCCGCCAACAA	1	4	1.75
S. azotifigens	AB217474[c]	~~~~~TGGTATTGGTAAGTGGACGAC[d]	4	ATGATGGCGATGTATGCCGCCAACAA	0	4	n.d.
X. autotrophicus	NC_009720	AAGGGTGGCATCGGCAAGTCCACCAC	2	ATGATGGCCATGTATGCGGCCAACAA	0	2	1.73
A. ferrooxidans	NC_011761	AAGGGGGGCATTGGCAAGTCTACCAC	4	ATGATGGCCATGTATGCCGCCAACAA	0	4	1.75
B. xenovorans	NC_007952	AAAGGTGGCATCGGCAAGTCGACCAC	2	ATGATGGCCATGTACGCGGCCAACAA	0	2	1.74
G. sulfurreducens	NC_002939	AAAGGCGGCATCGGCAAATCGACCAC	2	ATGATGGCCATGTACGCTGCCAACAA	1	3	1.67
A. vinelandii	M20568[c]	AAAGGTGGTATCGGTAAGTCCACCAC	0	ATGATGGCCATGTACGCCGCCAACAA	0	0	1.77
M. capsulatus	NC_002977	AAAGGCGGCATCGGCAAGTCCACCAC	1	ATGATGGCCATGTATGCGGCCAACAA	0	1	1.77
P. stutzeri	NC_009434	AAGGGTGGAATCGGCAAATCCACCAC	1	ATGATGGCGATGTATGCCGCCAACAA	0	1	1.71

[a] DNA sequences in the reverse priming site are given in the same sense as in the forward priming site.

[b] Reverse complement to primer nifHR, in order to match the sequence alignment.

[c] Sequences of strains that are closely related to the strains used in the analysis.

[d] Available sequence information on *nifH* of *S. azotifigens* was limited and did not cover the entire priming site.

n.d. - no amplification was detected.

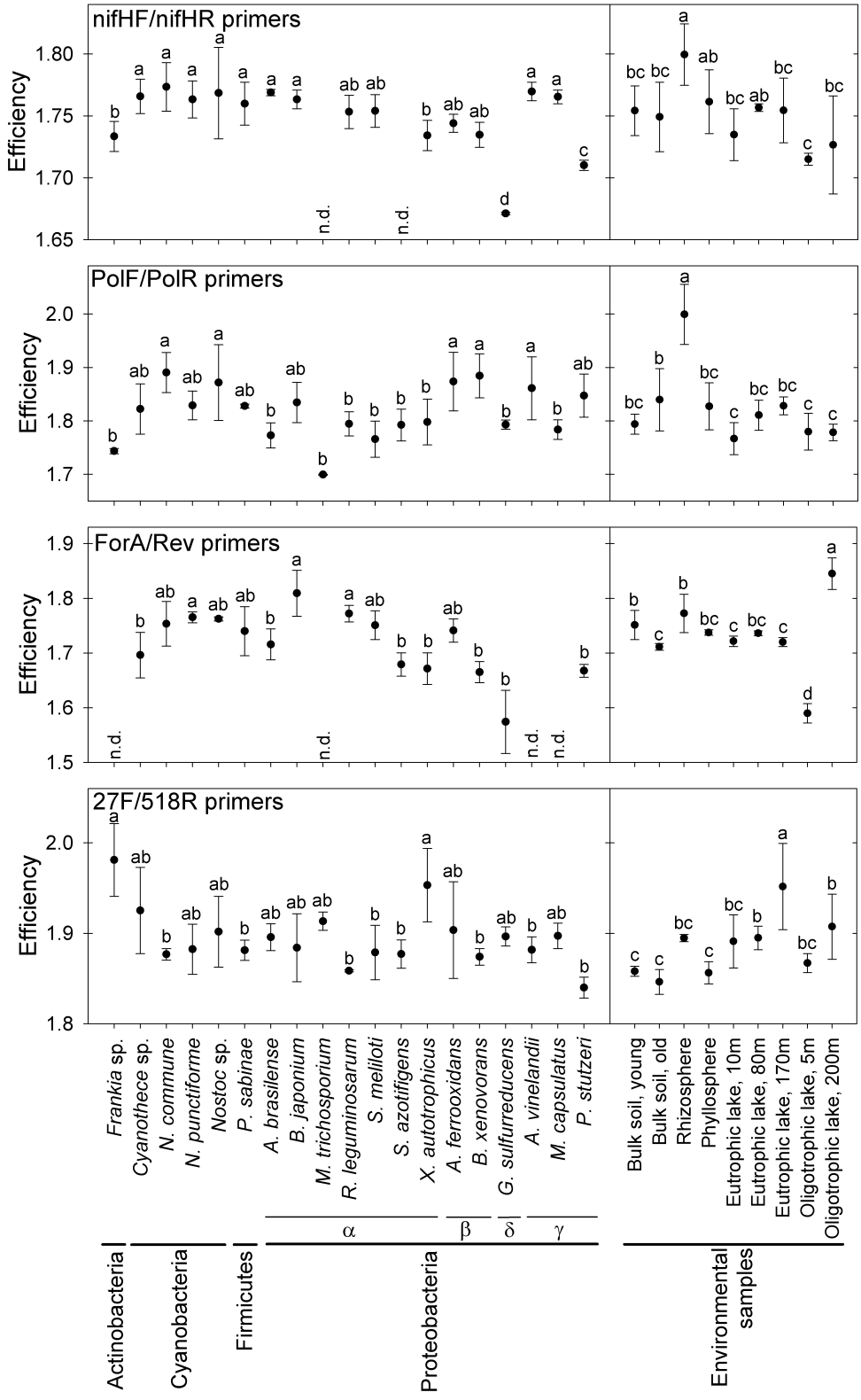

Figure 5.2: Variability of qPCR efficiency E_{fi} of bacterial strains (left panels) and environmental samples (right panels) targeting *nifH* and 16S rRNA gene, estimated from fluorescence increase. Error bars give the standard deviation of four replicates. Letters indicate significance groups by ANOVA ($p<0.01$) tested for each panel individually. n.d. – no amplification detected.

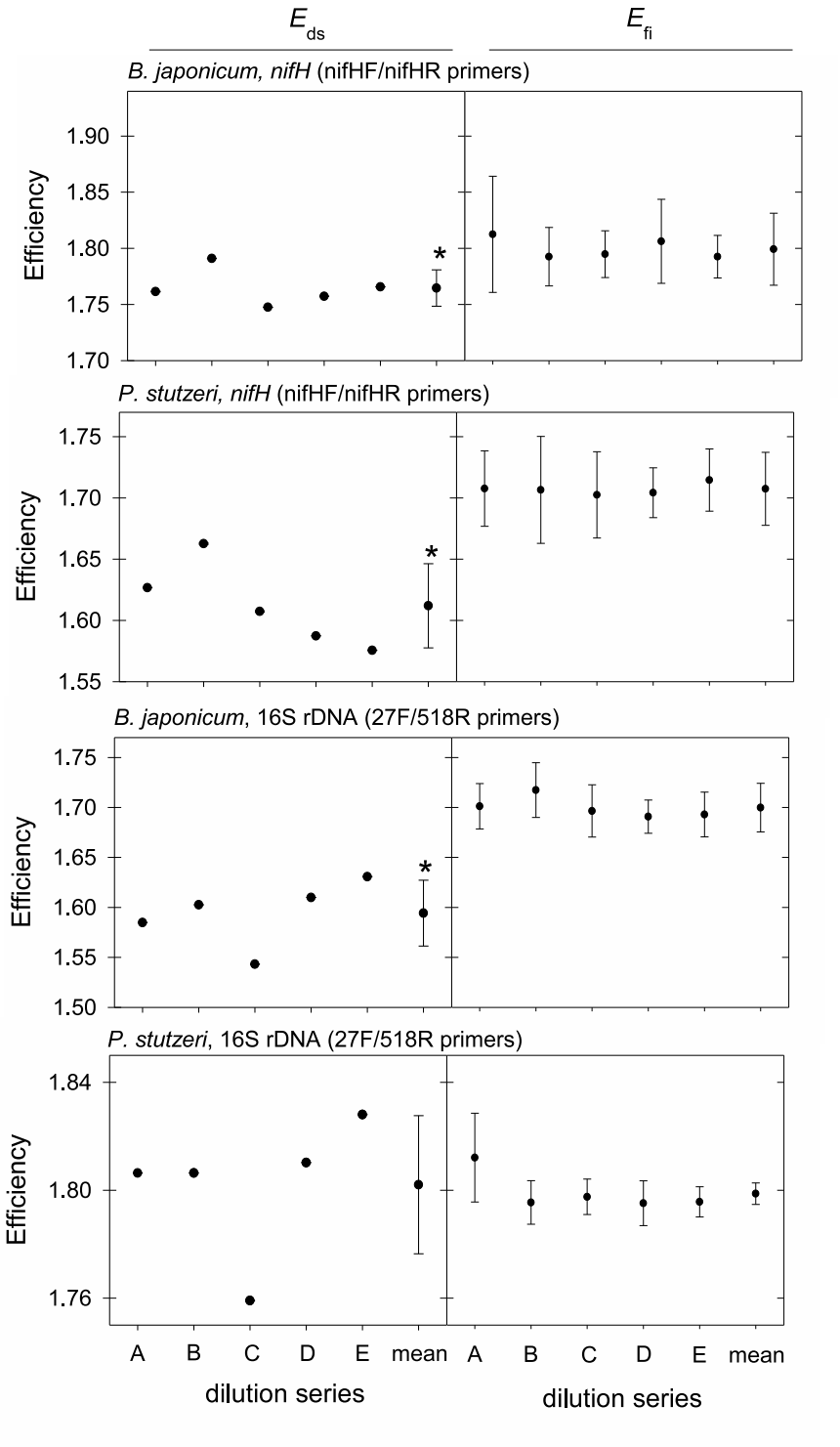

Figure 5.3: Variability of qPCR efficiency estimations by dilution series (E_{ds}) and fluorescence increase (E_{fi}). E_{ds} and E_{fi}, and means of E_{ds} and E_{ds} determined on dilution series independently prepared by five researchers (A–E) using the same DNA stock and lab equipment. Error bars indicate the standard deviation. Asterisk indicates significant differences between mean E_{ds} and mean E_{fi} by the Mann-Whitney rank sum test ($p<0.01$).

Table 5.4: Variation of the SC method parameters for dilution series prepared by five researchers (A–E) and run by one researcher in one qPCR assay per primer pair.

Template	Target gene primer pair	Dilution series[a]	Slope	Intercept	R^2	n[b]	E_{ds}
B. japonicum	*nifH*	A	-3.946	36.33	0.987	11	1.76
	nifHF/nifHR	B	-4.106	36.81	0.988	11	1.79
		C	-4.356	37.95	0.985	14	1.75
		D	-4.188	37.25	0.991	13	1.76
		E	-3.970	36.22	0.981	15	1.77
P. stutzeri	*nifH*	A	-4.732	39.71	0.981	11	1.63
	nifHF/nifHR	B	-4.529	39.69	0.964	8	1.66
		C	-4.852	41.06	0.940	9	1.61
		D	-4.983	40.97	0.989	12	1.59
		E	-5.065	40.88	0.984	12	1.58
B. japonicum	16S rRNA	A	-5.000	34.98	0.987	8	1.58
	27F/518R	B	-4.882	43.29	0.986	9	1.60
		C	-5.307	45.18	0.996	9	1.54
		D	-4.835	43.28	0.996	9	1.61
		E	-4.708	42.62	0.995	8	1.63
P. stutzeri	16S rRNA	A	-3.909	32.55	0.994	9	1.80
	27F/518R	B	-3.894	32.40	0.992	10	1.81
		C	-4.077	33.28	0.987	11	1.76
		D	-3.880	32.46	0.995	11	1.81
		E	-3.817	32.19	0.997	12	1.83

[a] five researchers (A-E) independently prepared dilution series of each template.

[b] number of points in calibration.

Table 5.5: Parameters of SC and OPC method used for sample quantification.

		SC method					OPC method		
Template	Primer pair	Slope	Intercept	R^2	n[c]	E_{ds}	C_t	N_0	E_{fi}
G. sulfurreducens[a]	nifHF/nifHR	-4.249	40.86	0.993	8	1.72	22.2	22160	1.69
N. commune[a]	nifHF/nifHR	-3.552	36.79	0.991	8	1.91	24.8	2331	1.77
B. japonicum[b]	nifHF/nifHR	-4.008	35.82	0.991	15	1.78	15.9	74945	1.77

[a] used to quantify two species mixes of *G. sulfurreducens* and *N. commune*.

[b] used to quantify environmental samples.

[c] number of points in calibration.

standard $E_{\text{standard}} = 1.70$. Using the SC method ($C_t = 33.382 - 4.34 \times \log(N_0)$), the estimate of the starting template concentration is $\log(N_{0\ \text{sample}}) = 2.85$, i.e. $N_{0\ \text{sample}} = 711$ copies (Figure 5.4). If we quantify the same sample using the OPC method ($C_{t\,\text{standard}} = 3$, $N_{0\,\text{standard}} = 1 \times 10^7$, $E_{\text{standard}} = 1.70$), we get $N_{0\ \text{sample}} = N_{0\,\text{standard}} \times E_{\text{standard}}^{C_{t\,\text{standard}}} / E_{\text{sample}}^{C_{t\,\text{sample}}} = 10^7 \times 1.70^3 / 1.99^{21} = 26$. The estimate using the OPC method is one order of magnitude below the estimate by SC method. This demonstrates the importance of correcting for the individual efficiencies.

The bias due to ignoring the efficiency can be calculated as a ratio between OPC and SC quantification (Equation 5.5). It depends on the ratio of E_{standard} to E_{sample} and the C_t value of the sample. The more the ratio of E_{standard} to E_{sample} deviates from one, and the higher the C_t value, the larger the bias will be. Therefore, the potential for error is particularly large when applying the SC method to samples with low template concentration.

$$\text{ratio of } \frac{\text{OPC quantification}}{\text{SC quantification}} = \frac{N_{0\,\text{standard}} \times \frac{E_{\text{standard}}^{C_{t\,\text{standard}}}}{E_{\text{sample}}^{C_{t\,\text{sample}}}}}{N_{0\,\text{standard}} \times \frac{E_{\text{standard}}^{C_{t\,\text{standard}}}}{E_{\text{standard}}^{C_{t\,\text{sample}}}}} = \left(\frac{E_{\text{standard}}}{E_{\text{sample}}}\right)^{C_{t\ \text{sample}}} \tag{5.5}$$

5.4.4 Experimental comparison of the SC and OPC method

Defined amounts of templates from two species were mixed in different ratios. The species were selected for their significant differences in E. *Geobacter sulfurreducens* showed a lower E_{fi} of 1.69 compared to *N. commune*, which had an E_{fi} of 1.77. We found that samples with a relatively higher number of *G. sulfurreducens* templates had lower E_{fi} than samples with a relatively higher number of *N. commune* templates (Figure 5.5).

Each of the defined mixtures was quantified with both the SC and OPC methods. Both methods were calibrated once with dilutions of *G. sulfurreducens*

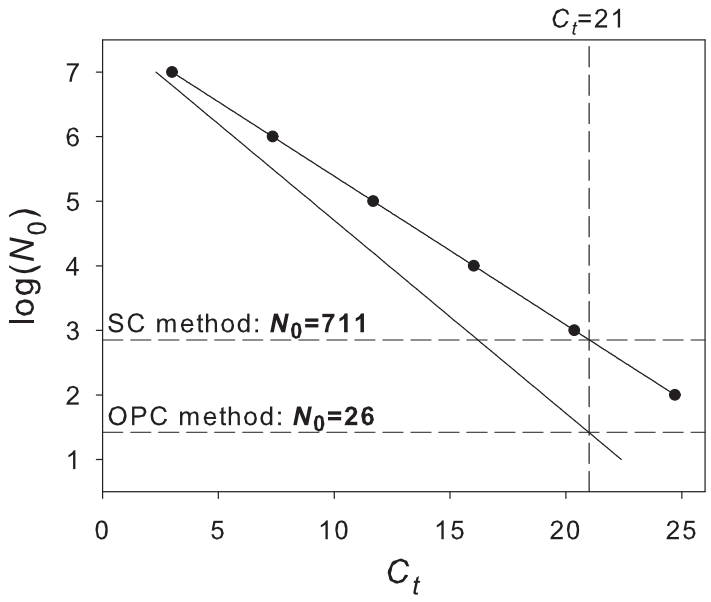

Figure 5.4: Quantification of a hypothetical sample (E_{sample}=1.99, C_t=21) with SC and OPC method using M. trichosporium (E_{standard}=1.70) as a standard.

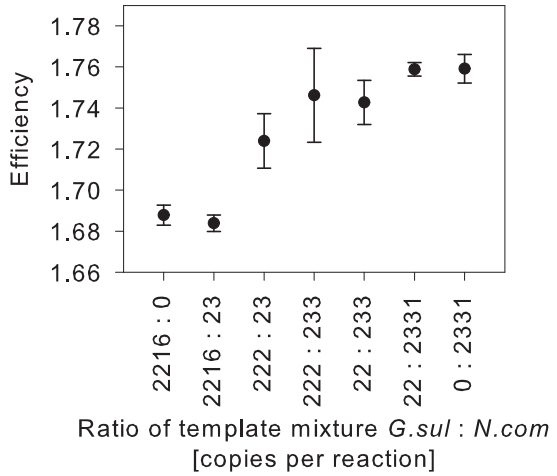

Figure 5.5: qPCR efficiency E_{fi} of defined mixtures of *G. sulfurreducens* and *N. commune* templates amplified with nifHF/nifHR primers. The values are the means of three replicates, error bars indicate the standard deviation.

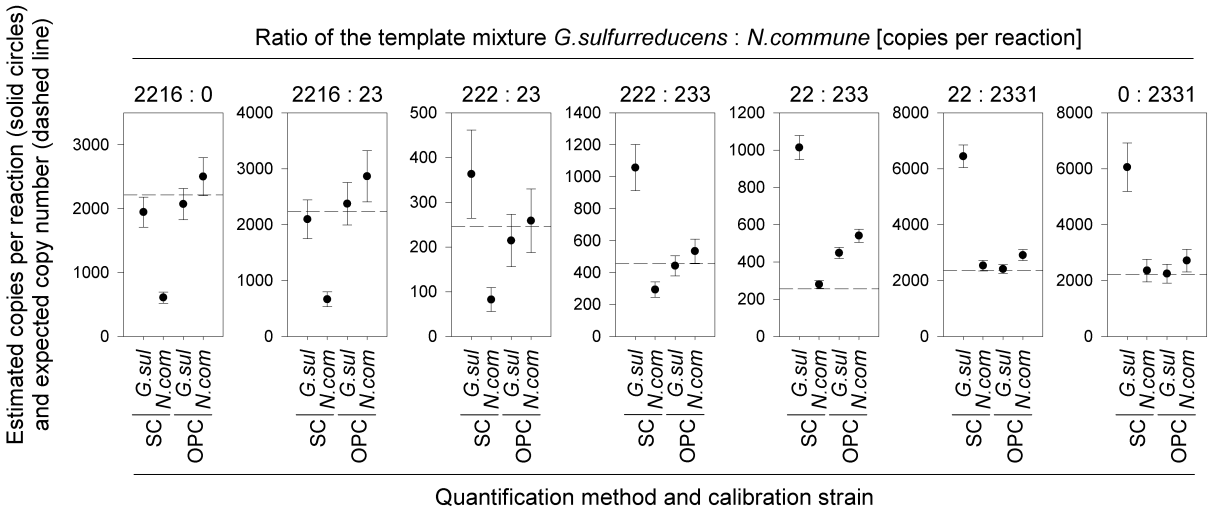

Figure 5.6: Copy numbers of defined mixtures of *G. sulfurreducens* and *N. commune* templates estimated using SC and OPC method that were calibrated using both strains. The assays used nifHF/nifHR primers. The dashed line gives the expected copy number. Error bars give the standard deviation.

DNA and once with dilutions of *N. commune* DNA (Table 5.5). We considered a quantification to be accurate when the deviation of the estimate was within ±30 % of the expected value. Using the SC method, the quantification of samples with a relatively higher number of *G. sulfurreducens* templates was accurate when *G. sulfurreducens* was used as the standard (Figure 5.6). Conversely, samples with a relatively higher number of *N. commune* templates were accurately quantified when *N. commune* was used as the standard. SC method quantifications using the less suited standard were 3- to 5-fold above or below the expected copy numbers. In contrast, the OPC method accurately quantified samples, independent of the ratio between G. *sulfurreducens* and *N. commune,* and independent of the standard used. Only one mixture (22:233) was quantified up to 110 % above the expected copy number.

This experiment demonstrates that correcting for the individual E of each sample improves the accuracy of absolute quantification. The two bacterial strains amplified with different E. Detecting the differences in E and accounting for them in the OPC method improved quantification accuracy independent of the calibration standard used and across a wide range of template mixtures amplifying with different E. Therefore, the OPC method allows for differences in the qPCR reaction kinetics to be corrected, which might have been introduced, e.g., by primer mismatches (Bru et al., 2008) or primer degeneracy (Chen and Zhu, 1997).

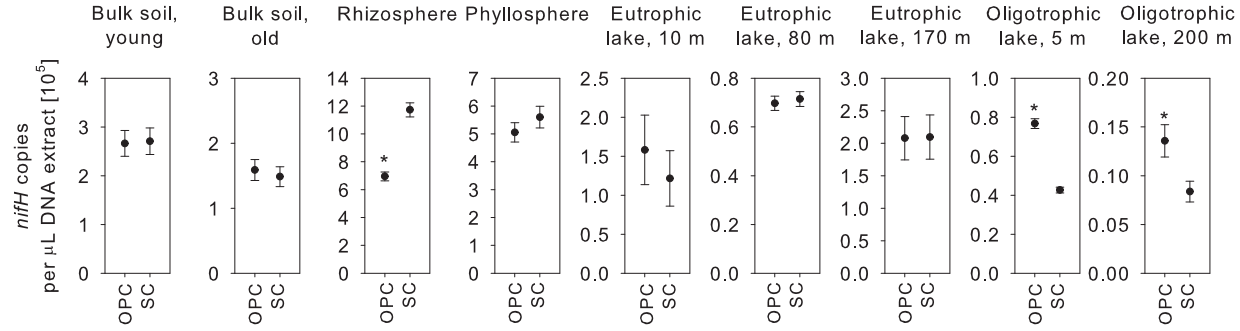

Figure 5.7: Comparison of the OPC and the SC methods for quantifying *nifH* gene copy numbers in environmental samples using nifHF/nifHR primers. Error bars give the standard deviation. Asterisks mark significant differences between the two methods by the Mann-Whitney rank sum test ($p<0.01$).

5.4.5 Quantification of environmental samples by SC and OPC methods

Nine environmental samples were quantified by SC and OPC methods using *B. japonicum* as the standard (Figure 5.7). For the samples that had a similar E_{fi} (Figure 5.2) as the E_{ds} of the standard (Table 5.5), differences between SC and OPC quantification were not significant according to the Mann-Whitney rank sum test ($p < 0.01$). However, for the rhizosphere sample, copy numbers calculated by SC and OPC methods were significantly different. This is because E_{fi} of rhizosphere (1.80) was higher than E_{ds} of the SC (1.78). In this case the SC method, therefore, overestimated the copy number in the rhizosphere sample compared to the OPC method. On the other hand, the samples of the oligotrophic lake had an E_{fi} lower than E_{ds} of the SC, therefore the SC method significantly underestimated the copy numbers compared to the OPC method (Figure 5.7). Quantification of the 16S rRNA gene in the environmental samples showed significant differences between SC and OPC method too (data not shown). These results demonstrate the necessity to consider sample and standard E, also in comparative qPCR analysis of microbial communities, i.e. even if the absolute copy number is not of primary concern.

5.4.6 Limitations of the OPC method

The OPC method is an absolute quantification method that is simple to use. It corrects copy number estimates for the E of the individual sample and is compatible with any method to determine E, i.e. E_{ds} (Higuchi et al., 1993; Rasmussen, 2001) or E_{fi} (Liu and Saint, 2002; Peirson, 2003; Ruijter et al., 2009) can be used.

Although the OPC method corrects for differences of E, it can not overcome principle biases or limitations of the underlying PCR reaction. For example, primer bias might introduce significant errors in the analysis of samples of unknown composition (Suzuki and Giovannoni, 1996). Abundant templates and templates with the optimal primer kinetics might be preferentially amplified compared to templates with low abundance and suboptimal primer kinetics (Suzuki and Giovannoni, 1996; Polz and Cavanaugh, 1998). To some extent these differences are accounted for because the OPC method corrects for differences in E. However, using degenerate primers, one specific template that matches one of the primers in the primer mixture might be preferentially amplified with the PCR program because of differences in GC-content (Polz and Cavanaugh, 1998). Lowering annealing temperature has been suggested to solve this problem (Ishii and Fukui, 2001). However, this may in turn increase unspecific amplification which is undesirable, specifically in SYBR Green based qPCR assays. Solid testing of the specificity and universality of the primers and the PCR protocol is therefore of high importance. Assays based on TaqMan are intrinsically less prone to reporting unspecific amplification. In principle the OPC method could be applied to TaqMan qPCR and the LinReg program can be used to derive correct E_{fi} estimates from TaqMan data, but this has not been tested.

The OPC method requires reliable E estimates, which might depend on the template concentration. Results by Bustin (2004b) and our own results (data not shown) do not support this hypothesis. However, other studies found indications that high template concentrations ($> 10^6$ copies per reaction) had an inhibitory effect (Töwe et al., 2010b; Tichopad et al., 2010). Furthermore, according to the model presented by Suzuki and Giovannoni (1996), an inhibitory effect of high template concentrations on E would be expected. This problem should be adressed in future studies.

Since quantification with the OPC method relies on a single standard, care should be taken that C_t and E for the standard are determined correctly, e.g., by using a larger number of replicates. The extent to which the OPC method can reliably correct for variation in E has not been determined. Samples with very low E values, or irregularly shaped amplification plots should be carefully considered. The method can also not address cases where low template concentration, inhibition, or poorly optimized PCR conditions lead to negative results. In such cases, additional tests might be required.

5.4.7 Conclusion

Currently, the gold standard for qPCR analysis in environmental microbiology is based on the SC method. This method assumes that the calibration standard and the sample have a similar E, although the SC method itself does not allow this assumption to be validated through testing. While the E of the standard is routinely assessed and considered a quality criterion, few studies have tested if the E of the sample and standard are similar (Schubert et al., 2006; Töwe et al., 2010b); however, even small differences can cause considerable errors (Bru et al., 2008).

Here we demonstrate that significant differences in E can ocurr between bacterial strains and environmental samples, specifically when amplifying broad ranges of templates using, e.g., degenerate primers, which is a common practice in microbial ecology. Not considering those differences has the potential to severely diminish the accuracy of the quantification.

To solve the problem, we propose the OPC method, which corrects for E of individual samples. Moreover, we demonstrated experimentally that by correcting for the E of a sample with this method, the accuracy of the quantification increases.

Based on the results and considerations presented in this paper, E needs to be considered in qPCR analysis whenever working with samples of unknown template composition. The E of each sample can be calculated from the increase in fluorescence units using freely available tools (Liu and Saint, 2002; Peirson, 2003; Ruijter et al., 2009). If the sample and standard have the same E, the SC method can be used, although we see no principle advantage over the OPC method.

5.5 Acknowledgements

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Authors contributions

RB designed the study, performed the experiments and wrote the manuscript. NB, JZ and HB participated in the design of the study and helped writing the manuscript.

6 General discussion

6.1 Evidence for microbial succession in soil

The term *succession* has been used in different disciplines. In biology *succession* is most often used to refer to ecological succession, i.e. the predictable change of plant communities during initial colonisation or after a disturbance event (Kuttler, 1995).

The concept of succession has also been applied to microbial communities. One of the earliest reports of microbial succession might be the microbial colonisation of glass slides submerged in the Pacific Ocean and experiments on microbial attachment using pure cultures (Zobell and Allen, 1935). It was discovered that some bacteria possess the ability to colonise the sterile glass slide, while others required an already established biofilm to colonise the slides. This simple observation points out two driving factors of microbial succession: *i)* the presence and availability of niches and *ii)* the bacteria shape their environment. Since that time different habitats have been analysed for microbial succession (Fierer et al., 2010), ranging from biofilms on macroalgae (Burke et al., 2011) and garbage composting facilities (Ishii et al., 2000) to water pipes carrying drinking water (Kerr et al., 1998).

The colonisation of bare soil by microorganisms at glacier forefields has previously been addressed (Ohtonen et al., 1999; Kastovska et al., 2005; Nemergut et al., 2007). Studying microbial succession at Puca glacier in Peru, using clone libraries of the 16S rRNA gene, a distinct change of the phylogenetic groups over time was observed (Nemergut et al., 2007). In the youngest soil, 0-1 years ice-free, β-proteobacteria were dominating (≈60 %) the clone libraries. In the soils that were ice-free for 20 years a more complex community dominated by cyanobacteria (≈25 %) was observed and β-proteobacteria were rare (1-3 %). The analysis was performed with two biological replicates and the independent clone libraries showed the same trends, supporting the notion of a directed change and providing evidence of microbial succession on phylogenetic level.

In this dissertation evidence for functional succession of microbial communities based on the abundance of functional genes and potential enzyme activities

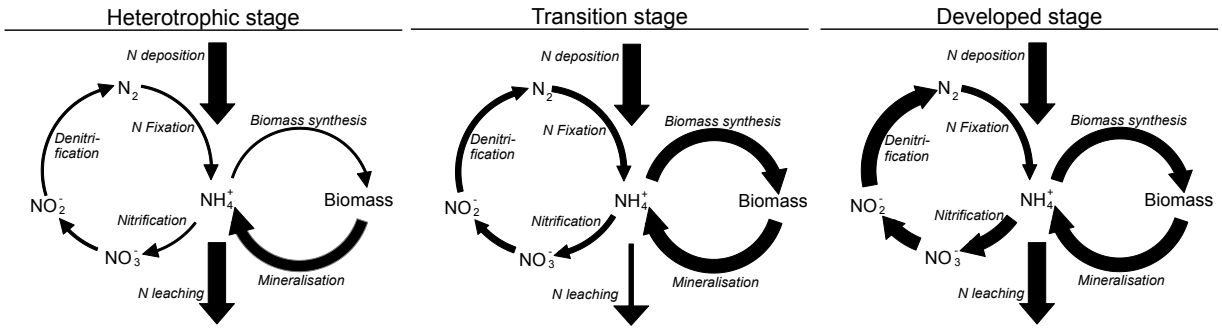

Figure 6.1: General pattern of the development of the N cycle observed at the Damma glacier and at the Lieberose dune. Modified after Ollivier et al. (2011).

(Chapters 2 and 4) is presented. As hypothesised, a pattern of N cycle development was identified. It was found that microbial succession can be subdivided into three stages: *i)* the heterotrophic stage, *ii)* the transition stage and *iii)* the developed stage (Figure 6.1). During the heterotrophic stage allochtonous and recalcitrant organic matter serves as C and N source (Hodkinson et al., 2002; Bardgett et al., 2007) and the N cycle can be described as open. That is, the N entering the soil is not accumulating nor is it transformed, but most N will be leached from the initial soil (Vitousek and Reiners, 1975; Gülland, 2012). Then in the transition stage, N begins to accumulate in the soil as primary producers, such as cyanobacteria and algae in BSCs (Lieberose dune) or plants (Damma glacier) colonise the soil, physically stabilise the soil surface, and build up biomass. In the developed stage, the processes nitrification and denitrification increase significantly and the N cycle can be described as closed, as all processes participate in N turnover. This pattern was observed at the chronosequence of the Damma glacier (Chapter 2) as well as at the BSCs at Lieberose dune (Chapter 4). Therefore, this sequence of the N cycle development appears to be a general rule; and similar mechanisms might trigger the sequence of development in both systems. One of the most important factors, the emergence of primary producers in the system, is discussed in the following section.

6.2 Primary producers kick-start microbial succession

Primary producers such as BSCs, lichens, bryophytes, or plants produce biomass from inorganic compounds using the energy of light. This process converts the light energy into chemical energy, i.e. reduced organic matter, which will then serve as an energy source for heterotrophic organisms.

To use the general term *primary producer* is necessary in the context of initial ecosystems, because different systems are characterised by different primary producers that stabilise the initial soil surface and perform photosynthesis. In the Andes at 5000 m altitude, BSC dominated by cyanobacteria are the pioneer primary producers for an extended period of time (years) before mosses and lichens supersede the BSC (Schmidt et al., 2008). In other systems, algae (Kastovska et al., 2005) or lichens (Belnap et al., 2001) were described as dominant primary producers before plants colonised the ecosystem. In contrast, at the Damma glacier plants establish in the system within five years (Miniaci et al., 2007).

The establishment of primary producers marks a distinct change in microbial succession—the shift from the heterotrophic stage to the transition stage (Figure 6.2). In the heterotrophic stage, primary production is very low, since only a few cyanobacteria or algae might be present (Kastovska et al., 2005; Nemergut et al., 2007; Duc et al., 2009b). Therefore, in the heterotrophic stage most microorganisms will live of allochonous and recalcitrant carbon. This has been demonstrated in other systems (Hodkinson et al., 2002; Bardgett et al., 2007; Sattin et al., 2009) and at the Damma glacier using the radiocarbon method to date the carbon dioxide emitted from soil respiration (Smittenberg et al., 2012). During this stage, heterotrophic respiration is the dominant process over primary production. However, in the transition stage, primary producers colonise the system and biomass is produced *in situ*. This changes a number of characteristics of the habitat and the living conditions of the microbial communities, such as: *i)* the quality and quantity of the available carbon, *ii)* the emergence of new niches, *iii)* the potential for symbiosis, and *iv)* the physical stabilisation of the soil.

Primary producers increase the carbon availability in soil and so affect conditions for microbial growth in two ways. First, above ground, debris and litter produced by bryophytes and higher plants is falling to the ground (Zaady, 1996; Widmer et al., 1999). Second, below ground, root exudates (Grayston et al., 1997) and fine root turnover (Aber et al., 1985) contribute to C input to the soil. These different inputs give indication of the different niches to exploit the C source and suggest different quality of the C. While root exudates are characterised as low molecular weight C (Grayston et al., 1997), litter is mainly composed of cellulose and hemicellulose (Vivanco and Baluška, 2012). In the soil chronosequence at the Damma glacier (Chapter 2) as well as in the BSC at the Lieberose dune (Chapter 4) an accumulation of organic C in the soil indicated an increasing C availability due to the presence of primary producers. Moreover, the controlled experiment on the impact of plant presence on the N cycling microbial communities (Chapter 3) supports the hypothesis that

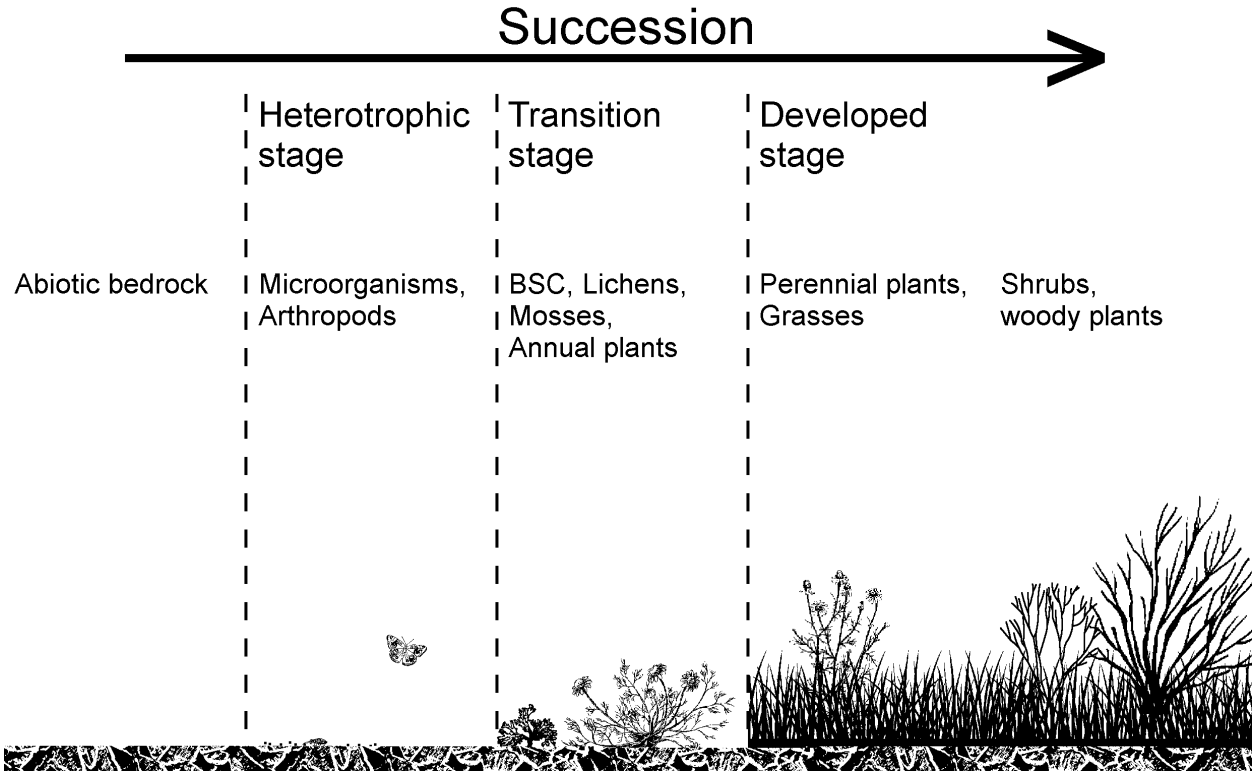

Figure 6.2: Refined schematics of succession: the heterotrophic stage is void of primary producers, in the transition stage primary producers colonise the system, and in the developed stage a closed plant cover is established.

colonisation by plants affects the microorganisms. Similarly, field studies by Miniaci et al. (2007) at the forefield of the Damma glacier and lab incubations by Duc et al. (2011) suggested a strong dependence of the microorganisms on C inputs from plants.

As primary producers establish in the system, new niches are formed that are available to be colonised by microorganisms. On the one hand, the primary producers interact with their soil environment by the chemical exchange of solutes (ions, nutrients, sugars) (Jones, 1998). For example, the C input leads to a C gradient from high to low C environment that is followed by the formation of other gradients, such as oxygen, pH, and redox potential (Edwards et al., 2006; Miniaci et al., 2007). Other gradients might form due to specific ion uptake by the primary producers, e.g., nutrient depletion in the rhizosphere (Grayston et al., 1997). On the other hand, the physical penetration of the soil by the roots forms new niches, e.g., preferential flow paths or high water potential within the rooting zone (Ishikawa and Bledsoe, 2000).

In addition, primary producers and microorganisms will engage in symbiosis. For example, mycorrhizal fungi depend on a phototrophic symbiont to establish. At the chronosequence of the Damma glacier the development of fungal communities was affected by the colonisation of primary producers, and mycorrhizal fungi tended to increase with plant cover (Zumsteg et al., 2011). Similarly, bacteria are involved in symbiosis with primary producers. The effect of plant presence on the N cycling microbial community was studied in the rhizosphere experiment (Chapter 3). The abundance of most functional genes was higher in the rhizosphere soil compared to the bulk soil. Moreover, the processes of nitrification and denitrification appeared to be closely linked to plant presence (Chapters 2, 3, and 4), indicating a close association between the plant and the microorganisms involved. A detailed study on the distribution of the microorganisms in the rhizosphere would help contribute to understanding these complex interactions.

6.3 Development of biogeochemical cycles at the Damma glacier chronosequence

The chronosequence approach of *space-for-time substitution* allows us to study processes of ecosystem development that naturally occur in time frames of decades to centuries (Stevens and Walker, 1970), and has been frequently used in soil science, ecology, and botany. The limitations of space-for-time substitution have always been a matter of controversy, however in combination with alternative approaches and reasonable interpretation of the chronosequence data, significant scientific contributions have been made (Fukami and Wardle, 2005).

At the Damma glacier forefield a chronosequence covering 120 years was established (Duc et al., 2009b) and allowed us to study the development of biogeochemical cycles. The focus of this dissertation is the development of the microbial N cycle. However, close interactions of the N cycle with other geochemical cycles of carbon (C), phosphorus (P) and sulfur (S) exist and will be considered in this section.

The N cycle develops from an open N cycle towards a closed N cycle (Chapter 2; Odum, 1969). In the youngest soil, N originates mainly from wet and dry deposition. The N deposition in the forefield is in the range of 15 kg ha^{-1} a^{-1} (Chapter 2) and the isotopic signature of the N in the youngest sites indicates an atmospheric origin of the N (Smittenberg et al., 2012). Most of the deposited N is not transformed in the soil but quickly leached from the system. Therefore, the N cycle is termed *open*. At the developed site, the N cycle is closed. At

this stage the N is maintained within the compartments soil organic matter, soil fauna, and vegetation. Major loss paths during the developed stage might be leaching and denitrification (Figure 6.1).

The colonisation of the Damma forefield by primary producers is a turning point for the N cycling microorganisms (see section 6.2) as well as the entire ecosystem. This is because primary producers fixate C in biomass and the ecosystem C fluxes start to grow exponentially (Dümig et al., 2011; Smittenberg et al., 2012). C availability increases and stimulates microbial activity. Microbial activity in turn accelerates the nutrient turnover. In the case of N, C availability triggers the fixation of N. Duc et al. (2011) demonstrated in an experiment that plant presence alleviated microbial C limitation and N fixation was stimulated. Moreover, availability of C increases soil respiration rates and nutrients that are bound to the organic matter are released (Gülland, 2012).

The P and the S cycle are of particular interest. In contrast to N, P and S are present in the bedrock. Therefore, two sources are available: *i)* P and S bound to the minerals in the bedrock, and *ii)* P and S present in the soil organic matter.

The dissolution and availability of P was the focus of studies within the BigLink project (Bernasconi et al., 2011) at the Damma glacier, for two reasons. First, in terrestrial ecosystems the primary P source is the minerals in the bedrock. Therefore, mineral weathering, i.e. the interaction of the microorganisms with the mineral surface, might represent the first biological activity after glacier retreat. Second, in natural systems P next to N is frequently found to limit plant growth. Work by Göransson et al. (2011) showed that neither plants nor microorganisms were P limited in the forefield. Isotopic analysis on the P source supported the view that the minerals are the major P source within the forefield (Tamburini et al., 2010). In mineral dissolution experiments bacteria isolated from the forefield were screened for P dissolution potential (Lapanje et al., 2011).

Since S is a macronutrient, significant amounts are required to maintain the high ecosystem productivity that was measured at the Damma forefield. Borin et al. (2010) pointed out the importance of sulfuroxidising bacteria as first colonisers in microbial communities at the Midtre Lovénbreen glacier, Svalbard. This would indicate that sulfate is released from the bedrock at a very early successional phase and might therefore be plant available early on. A study on desulfonating bacteria, i.e. bacteria that release sulfate from organic matter (Cook et al., 1998), found very high diversity at the Damma forefield (Schmalenberger and Noll, 2010). Therefore, it was hypothesised S might be a limiting nutrient. Measurements of S were found to be comparatively low in

the youngest soils (0.3 μg S g^{-1} soil) and S content only increased in soils after more than 50 years of soil development (≈1.2 μg S g^{-1} soil, Noll and Wellinger, 2008). The deposition of S is estimated to be 200 to 350 mg SO_x m^{-2} a^{-1} (Nyiri et al., 2009), however detailed analysis on S sources in the Damma forefield are lacking.

6.4 Accounting for heterogeneity: matching sampling setup and research question

Within the discipline of environmental microbiology, researchers are working on scales that cover several orders of magnitude. On the scale of microbial cells, gradients in the μm-range, e.g., nutrient- and redox-gradients play an important role. Other processes affecting microbial activity might cover scales on the meter- to km-range. For example site exposition and geomorphology will affect soil temperature, precipitation, and soil texture. In combination, these different gradients will form a heterogeneous environment with diverse niches.

Considering the environmental heterogeneity is the basis of successful research. Without prior knowledge of the properties of the studied system, it is not possible to design an unbiased sampling scheme that will reflect the environment. Therefore, a solid research question is needed to derive the requirements of sampling setup, experimental setup and statistical sampling design. The research question needs to consider the environmental heterogeneity in all its aspects and the setup in turn needs to precisely capture the requirements of the research question.

In Chapters 2 and 4 the research questions focussed on the development of the N cycle. Accordingly, the sampling strategies needed to reflect different phases of soil development. In both systems, heterogeneity is high. At the Damma forefield soil texture and soil moisture regime vary significantly on a scale of 0.1-1 m. A major part of the heterogeneity is caused by the creek, which exhibits high seasonal and diurnal variation in flow regime. During snow melt vast areas of the forefield are flooded and soil substrate is redistributed causing sorting of the soil grains. On a daily basis, however, water flow is lowest during early morning and highest after mid-day, causing constantly changing water potential in the hydrolytically connected soils. At the Lieberose dune, heterogeneity is visible by the patchiness of BSC growth that is caused by random disturbances of the BSC by wind or animals as well as the microtopography of the sand.

At the Damma forefield four soil ages within the chronosequence (10a, 50a, 70a, and 120a) and one reference (>2000a) close-by the forefield were sampled

(Chapter 2). To account for the heterogeneity of the glacier forefield each of the sampling ages was replicated five times by sampling approximately 10 m apart (5 × 5-setup), for a total of 25 samples. Overall, this sampling approach was successful and confirmed hypothesis three; we were able to detect significant differences in nutrient content, microbial abundance, and microbial activity along the forefield, reflecting the gradient of soil development. Similarly, at the Lieberose site the 5 × 5-setup was successful in detecting different phases of soil development. Despite high variation between the replicates, significant differences in nutrient and chlorophyll content were detected (Chapter 4).

In contrast to the 5 × 5-setup, the BigLink project used a continuous sampling approach, comprised of 24 randomly placed sampling sites (no replication) within and close-by the glacier forefield (Bernasconi et al., 2011). By using this sampling setup, the carbon budget could be modelled in detail and it was found that the glacier advance in the 1980s did affect the soil formation in a different way than was previously thought (Smittenberg et al., 2012). These specific findings would have not been possible using the 5 × 5-setup used in Chapter 2. Nevertheless, the drawback of sampling continuously is that there are no replicates of each soil developmental phase available and conclusions such as those drawn in Chapter 2 or 4 would not be possible.

A plant-centred setup was used in Chapter 3 in order to address the question of how plant-microbe interactions impact the N cycle at different phases of soil development. The setup included pots filled with initial (10 a) and developed soil (120 a), which received *Leucanthemopsis alpina* plants (treated pots) or no plants (control pots). Previously, Miniaci et al. (2007) used a plant-centred setup to study the effect of *L. alpina* in the initial soil of the glacier forefield. Soil was sampled with increasing distance (10 cm, 20 cm, 40 cm) from the plant. Comparing both approaches, the latter would not be suitable to investigate the plant effect in developed soil because, in the field, the heterogeneity and plant abundance at the developed site is very high. Therefore, an experimental setup was the ideal choice to answer this research question (Chapter 3).

6.5 Functional gene analysis: promising tool

For decades, the analysis of microbial communities had to rely on cultivation dependent methods that were limited, because of the low culturability rate of microorganisms (Osborn, 2005). Therefore, with the advent of cultivation independent methods an array of new experimental possibilities have emerged and have been exploited. Today, a number of DNA- and RNA-based techniques

are widely applied in the field of environmental microbiology (Osborn, 2005; Bothe, 2007).

Microbial communities are normally studied by their phylogenetic composition based on the 16S/18S rRNA gene (Osborn, 2005). However, the phylogeny carries only limited information on the niche preferences or the functional capabilities of a microorganism. For example, from the presence of cyanobacteria, based on 16S rRNA gene information, it is not possible to conclude the presence of an N fixing microorganism, because there are cyanobacteria that are not able to perform N fixation (Zehr et al., 2001). In contrast, from the presence of the marker gene for N fixation—*nifH*—it is possible to infer the function as well as the phylogeny of the microorganism. Recently, Burke et al. (2011) demonstrated the significance of analysis based on functional genes. They studied microbial succession on the phylosphere of a macroalgae and found that succession followed a pattern at the level of the functional genes; in contrast, no pattern in 16S rRNA gene could be observed.

In Chapters 2, 3, and 4 functional gene analysis has successfully been employed in studying the abundance of N cycling microorganisms. Differences in gene abundance significantly discriminated phases of soil development. For some of the studied processes, gene abundance was significantly correlated to potential enzyme activities (Chapter 2). However for other processes this relationship did not hold. Further work is necessary to establish molecular techniques to identify functional key players. One possibility might be to analyse gene expression, rather than gene abundance (Bürgmann et al., 2003; Turk et al., 2011), e.g., by reverse transcription qPCR (Bustin, 2004a).

In Chapter 5 the One-Point-Calibration (OPC) method to analyse qPCR data was developed. The method takes into account that amplification efficiency of the standard and the sample might be different, and corrects the quantification estimate for efficiency differences. The experiments verified hypothesis four that accounting for efficiency differences between different templates increases the accuracy. When analysing artificial mixtures of two different templates, the samples were accurately quantified using the OPC method, while the standard curve method was less accurate. In the future, the OPC method might help to detect significant differences between samples more readily, as inter-sample variability is reduced.

The aim of environmental microbiology is to understand microbial interactions with their environment and to identify key players in microbial communities. To achieve this, the analysis of functional genes is one promising tool. A number of marker genes representing different functions have successfully been used for community analysis (Kandeler et al., 2006; Henry et al., 2006; Schauss et al.,

2009). Analysing those marker gene in gene expression analysis will significantly improve our understanding of the ecological role of key players.

6.6 Outlook

Intensive research on the microbial ecology of pioneer sites opened the view into further areas of research that were beyond the focus of this dissertation, however, might be of interest in future studies.

The data presented in this dissertation are based on quantitative analysis of microbial populations. Abundance of functional genes and potential enzyme activity have been measured to draw conclusions about the development of the N cycle. One aspect that has been neglected, for the purpose of feasibility, is the qualitative community analysis. In order to identify the key-microorganism of each process, it would be necessary to sequence the functional genes. In particular, in face of the diversity of microorganisms that catalyse the same process, future studies should consider to combine quantitative and qualitative analysis.

BSC occur globally widespread, however, at the Damma glacier and other Swiss glaciers they are rather rare and occur in small patches only (personal observation). Two phenomena might explain this. First, the occurrence of BSC might be limited to a certain soil texture because light penetration, surface area, and water potential within the soil depend on soil texture. Second, the disturbances might be to frequent to allow the establishment of BSC. Experimental testing of this hypotheses is enabled by the fact that soil texture in forefields of granite bedrock covers a broad range, and patches of well sorted particle sizes exist.

One main finding of this dissertation is the occurrence of three stages of functional succession: *i)* heterotrophic stage, *ii)* transition stage, and *iii)* developed stage. Since the heterotrophic stage has previously been described in other systems too (Bardgett, 2000; Hodkinson et al., 2002), it can be hypothesised that the heterotrophic stage has a beneficial effect on the establishment of primary producers. Future experiments might involve microcosms experiments (0.1-1 mL) of defined mixtures of heterotrophic and autotrophic (cyanobacteria/algae) microorganisms to elucidate nutrient turnover and exchange of axenic and mixed populations. Working hypothesis would be that a general mutualism between heterotrophs and autotrophs exists.

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