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ORIGINAL ARTICLE

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

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

Since the end of the Little Ice Age around 1850 many alpine glaciers have retreated (Maisch, 2000), exposing new terrain for soil formation. Consequently, glacier forefields represent chronosequences of different soil development stages offering an ideal system to study the development of functional microbial communities in soil. Initial stages of the glacier chronosequences are characterized by low plant diversity and abundance (Chapin *et al.*, 1994; Tscherko *et al.*, 2005; Hammerli *et al.*, 2007), which increase over time and reach their maxima at sites being ice-free for more than

200 years. Consequently, the amount of available carbon (C) and nitrogen (N) is low during initial ecosystem development. Sigler and Zeyer (2002) reported highest bacterial richness at initial soils (ice-free for 2–10 years). This observation is in agreement with studies reporting highest diversity of functional groups, such as nitrate reducers and N-fixing microbes. (Deiglmayr *et al.*, 2006; Duc *et al.*, 2009; Schmalenberger and Noll, 2009). Moreover, initial sites are often dominated by microbes belonging to the *r*-strategists being able to respond quickly to changing environmental conditions, whereas with continuing succession a shift to *k*-strategists occurs that rather pursue the strategy of maintenance (Sigler and Zeyer, 2002). In contrast to these studies, some reports found no significant correlation between soil age and diversity (Edwards *et al.*, 2006) or increasing diversity (Tscherko *et al.*, 2003; Nemergut *et al.*, 2007).

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

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

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

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

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

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

Materials and methods

Sampling site
Sampling took place along the forefield of the Damma glacier (46°38′20″N and 8°28′00″E) in
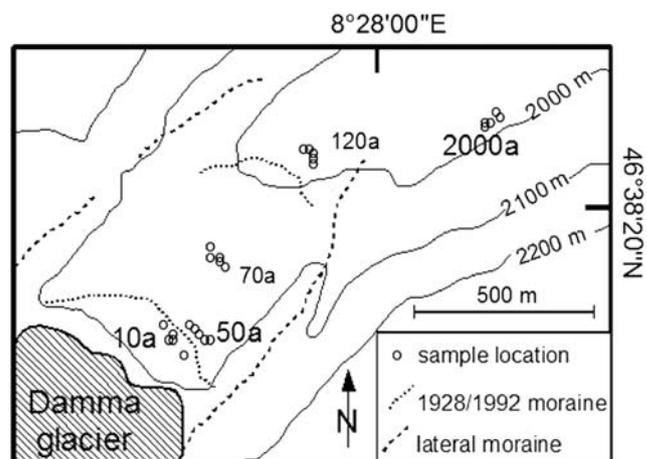
Figure 1 Site map illustrating the Damma glacier forefield. Circles mark the sampling locations. Five circles representing the soil samples of the same developmental stage are labeled with the corresponding sample code: 10, 50, 70, 120 and 2000a.

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

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

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

Table 1 Nitrogen deposition at alpine ecosystems in central Switzerland

Type of deposition	Area, source of data	Deposition (kg N ha^{-1} a^{-1}) or (kg C ha^{-1} a^{-1})			
		Dry N	Wet NH$_4^+$-N	Wet NO$_3^-$-N	Total N
Dry and wet deposition within a year (models)	Central Switzerland[a]	—	—	—	10–15
	Central Switzerland[b]	2.2	4.5	3.5	—
	Central Switzerland[c]	—	5–7.5	2.5–3.5	—
Particulate deposition on snow (experimental)		Particulate N[d]		Particulate C[d]	
	Forefield Damma glacier, this study	0.63 (0.18)		7.5 (1.5)	
	Forefield Stein glacier, this study	0.77 (0.08)		11.8 (0.4)	
	Forefield Tiefen glacier, this study	0.24 (0.05)		4.0 (0.7)	

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

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

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

Soil carbon and nitrogen content
Fresh soil and fumigated soil (see below) was extracted in 0.01 M calcium chloride solution by 45 min horizontal shaking. Soil-to-liquid ratio was 1:2 for the 10–120a samples and 1:3 for the 2000a samples. The following parameters were measured for each soil filtrate (Whatman 595 ½, Dassel, Germany): dissolved organic carbon and nitrogen on DIMA-TOC 100 (Dima Tec, Langenhagen, Germany), nitrate (NO$_3^-$) using Spectroquant Nitrate-Test kit (Merck, Darmstadt, Germany) and ammonium (NH$_4^+$) using Nanocolor kit (Macherey-Nagel, Düren, Germany). Microbial carbon (C$_{mic}$) and microbial nitrogen (N$_{mic}$) were determined by the fumigation-extraction method (Vance et al., 1987; Joergensen, 1996). Total organic carbon of air-dried ground soil was determined on a CN-analyzer (Leco CNS2000, St Josephs, MI, USA). Total organic carbon was measured as total C, as the soil contains no carbonates. Total nitrogen was determined by the persulfate oxidation method as described for soil extracts (Cabrera and Beare, 1993). Approximately 200 mg (2000a) to 500 mg (10a) of ground soil was autoclaved (60 min at 121 °C) in 10 ml of oxidizing reagent. Total nitrogen was then measured as nitrate by ion chromatography (Dionex DX-320, IonPac AS11-HC column, Sunnyvale, CA, USA). All analyses for soil C and N content were performed in technical triplicates.

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

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

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

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

Potential denitrification activity was determined as reported by Luo et al. (1996). Three replicates of 10 g fresh soil were saturated with 0.1 mM glucose and nitrate solution. The headspace of the serum bottle was flushed with helium and 10% acetylene

was added. N_2O was analyzed by gas chromatography on Shimadzu GC-14B (Düsseldorf, Germany). The soil was incubated at 20 °C and headspace samples were taken after 3 and 6 h. Preliminary experiments showed that in all soils considered, the N_2O production was a linear function of time from 0 to 6 h (data not shown). It was also found that in these assays the denitrification rates were identical in the presence and absence of chloramphenicol (data not shown).

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

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

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

Results

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

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

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

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

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

Potential enzyme activities

Potential enzyme activity was related to soil dry weight as well as the amount of extracted DNA to obtain a biomass-independent parameter (Figure 2). Generally, potential enzyme activities related to dry soil increased along the chronosequence. N-fixation activity was below the detection limit in samples 10–70a (detection limit 0.2 pmol N h^{-1} g^{-1}) but ranged between 1 and 3 pmol N h^{-1} g^{-1} in samples 120 and 2000a. Relative N-fixation activity was similar in samples 120 and 2000a. As proxy for potential mineralization activity, protease and chitinase assays were performed. Significant increases of protease activity from 5 to 25 nmol MUF h^{-1} g^{-1} as well as of chitinase activity from 14 to 72 nmol MUF h^{-1} g^{-1} were observed along the chronosequence. At the same time, relative mineralization activity tended to decrease, however, the trend was not significant. Potential nitrification activity increased significantly from 0.1 (10a) to 2.3 nmol NO$_2^-$-N h^{-1} g^{-1} (2000a). The same pattern was observed for potential denitrification activity, where activities increased from 1.8 to 40 nmol N$_2$O-N h^{-1} g^{-1} The relative nitrification and denitrification activities resulted in similar pattern but did not reach significant levels in most cases.

Abundance of functional genes

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

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

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

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

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

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

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

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

dance at site 2000a. Relative gene abundances showed comparable patterns for denitrification genes except *nirS*, which decreased along the chronosequence.

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

Discussion

The initial state
Initial ecosystems are characterized by low nutrient content and scarce vegetation (Sigler and Zeyer, 2002; Tscherko *et al.*, 2004; Yoshitake *et al.*, 2007). For the Damma glacier it was shown that the initial sites (10a) have less than 10% vegetation cover, whereas the developed sites (120–2000a) have a closed plant cover (Hammerli *et al.*, 2007; Hans Goransson, personal communication). Thus, the input of organic material by root exudates or plant litter might be low at the initial sites of the glacier forefield. Hence, N fixation and autotrophic CO_2 incorporation may have a crucial role for ecosystem development (Kohls *et al.*, 1994). Interestingly, our
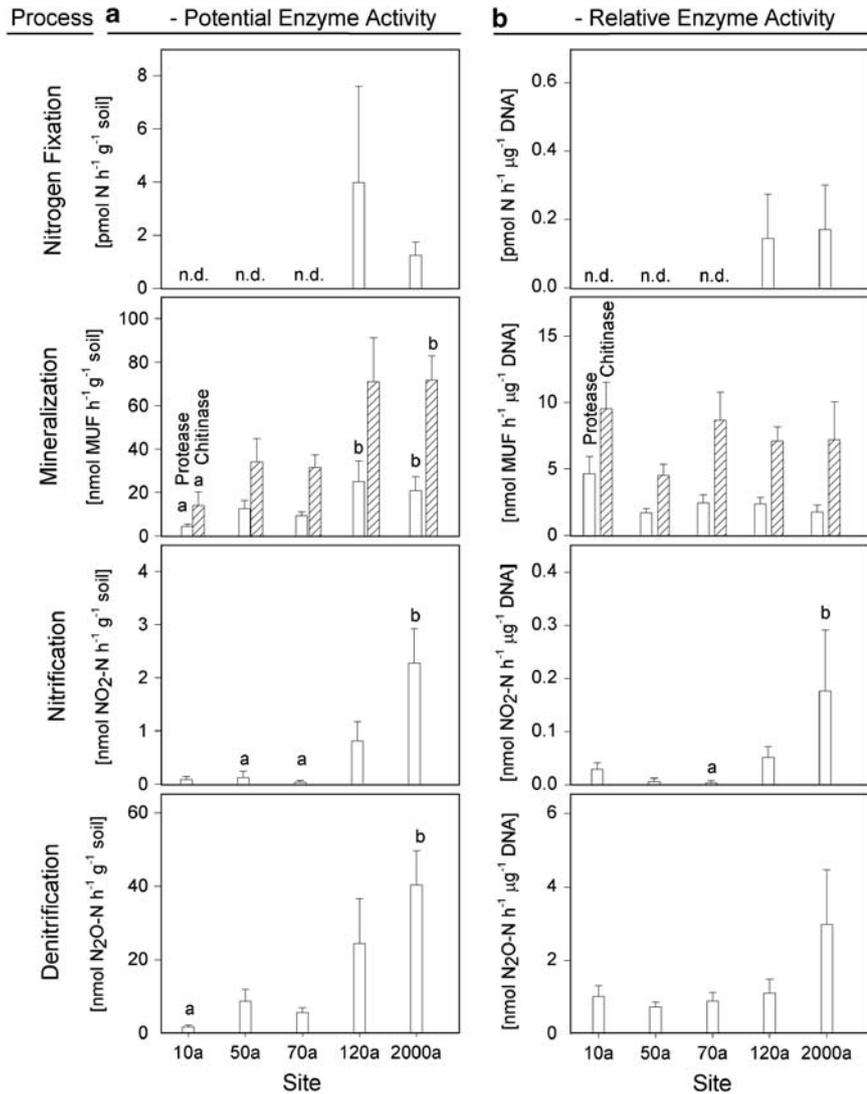
Figure 2 Potential (**a**) and relative enzyme activities (**b**) for nitrogen fixation, mineralization, nitrification and denitrification are displayed ($n = 5$, error bars represent standard error of means). Only significant differences as revealed by one way ANOVA on ranks ($P < 0.05$) are indicated by different letters. n.d. indicates that all five replicates of potential N fixation were below the detection limit of $0.2\,pmol\,N\,h^{-1}\,g^{-1}$ soil.

data revealed no potential N-fixation activity and the lowest *nifH* gene copy numbers per gram soil in the 10a soil. This is supported by the studies of Duc *et al.* (2009) and Nemergut *et al.* (2007) in which the lowest N fixation rate was measured in the bulk soil of the initial stage of the Damma glacier (Switzerland) and Puca glacier (Peru) forefield, respectively. In contrast, mineralization of organic material seemed to be the driver for N cycling at the initial stage of ecosystem development, indicated by the highest potential activities for proteases combined with highest relative *aprA* gene copy numbers (based on the amount of extracted DNA). Although other genes like *nirK* and *amoA* AOB showed similar or even higher gene copy numbers at the initial sites, the potential denitrification and nitrification rates were extremely low. This implies the presence of inactive nitrifying and denitrifying

populations, attributed to the shortage of ammonium and nitrate. Obviously, at the initial stage of the glacier forefield, sufficient N and C are provided for mineralization: (i) allochthonous organic material (plant debris, insects and so on) is deposited in the forefield, as revealed by our and other measurements (Hodkinson *et al.*, 2002; National Air Pollution Monitoring Network (NABEL), 2008; Nyiri *et al.*, 2009), (Table 1), (ii) the cryoconite holes in the glacier ablation zone possibly inhabit small food-webs of *cyanobacteria* and heterotrophs, which were relocated to the glacier forefield by the glacial stream after snowmelt (Schmalenberger and Noll, 2009; Xiang *et al.*, 2009) and (iii) microbes might feed on ancient recalcitrant C (Hodkinson *et al.*, 2002; Bardgett *et al.*, 2007). In addition, significant inputs of nutrients may be due to atmospheric dry and wet deposition of N and C species (Table 1).
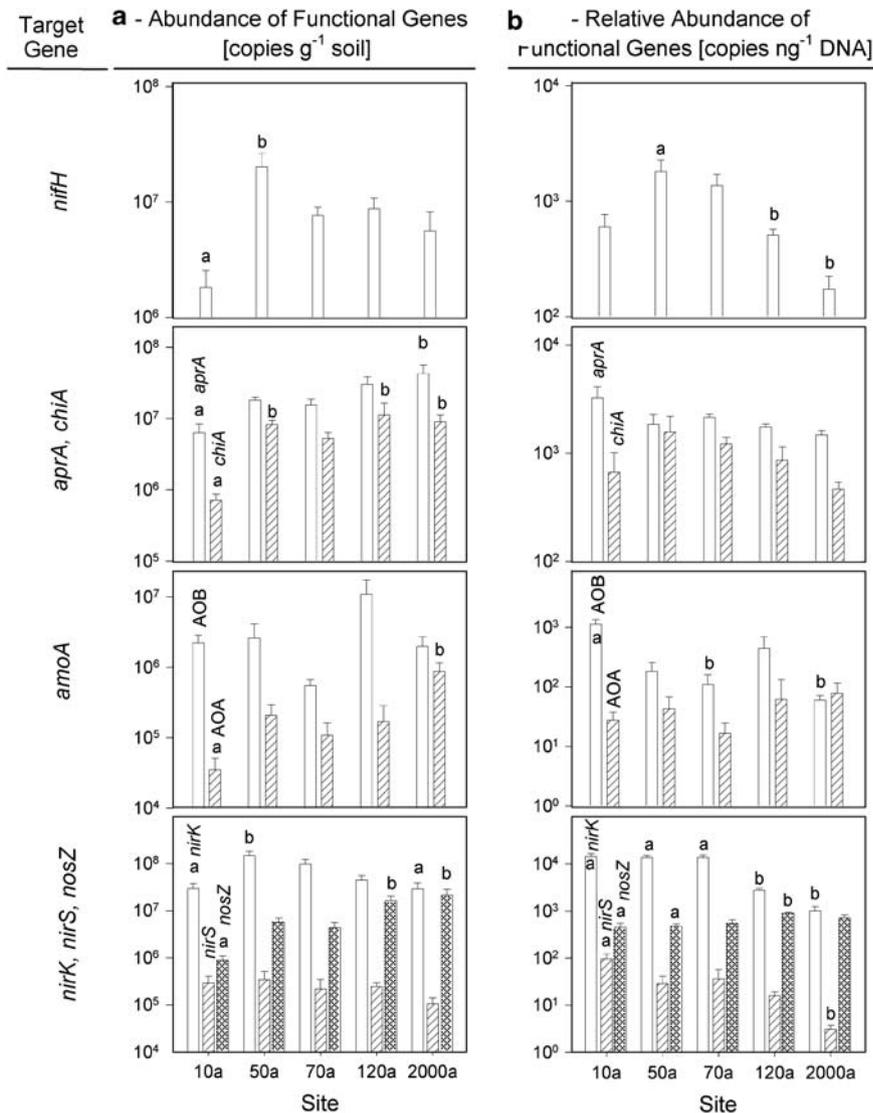
Figure 3 Gene copy numbers of functional genes involved in major steps of the nitrogen cycle (*nifH*, *aprA*, *chiA*, *amoA* AOB, *amoA* AOA, *nirK*, *nirS* and *nosZ*) are displayed related to gram soil (**a**) and nanogram DNA (**b**) ($n = 5$, error bars represent standard error of means). Only significant differences as revealed by one way ANOVA on ranks ($P < 0.05$) are indicated by different letters.

Plants as competitors for nutrients

With the first appearance of plant patches it could be expected that C will be provided in moderate quantities by rhizodeposition and exudation. However, it has rather been postulated for developing ecosystems that plants and microbes compete for nutrients, like N and phosphorus, as on the one hand nutrient availability is low and on the other hand plant performance and exudation is reduced (Chapin et al., 1994; Hammerli et al., 2007). Although ammonium and nitrate concentrations increased two- to sixfold from site 10a to the transient soils (50 and 70a), these values are still more than 10 times lower than values from pasture sites (Chronakova et al., 2009), confirming the assumption that strong competition for N still exists. Thus, it is likely that with the first occurrence of plant patches at site 50a, the *nifH* gene

abundance reached its maximum even if the potential N-fixing activity was still negligible. However, it has to be taken into account that the used assay only covered the performance of free-living heterotrophic N fixers and did not consider symbiotic N fixation by *Rhizobia*, plant-associated N fixation or phototrophic N fixation. The observation that the activity of free-living N fixers is low at this stage of ecosystem development is not surprising, as the amounts of dissolved organic carbon in the bulk soil of the 50 and 70a sites were low and consequently, C sources were missing for the energy-consuming process of N fixation (Zehr et al., 2003). In this regard Duc et al. (2009) showed that once C-rich nutrients were delivered like exudates in the rhizosphere of plants, N-fixation activity significantly increased. Hence, it can be speculated that mainly symbiotic and plant-associated N fixing
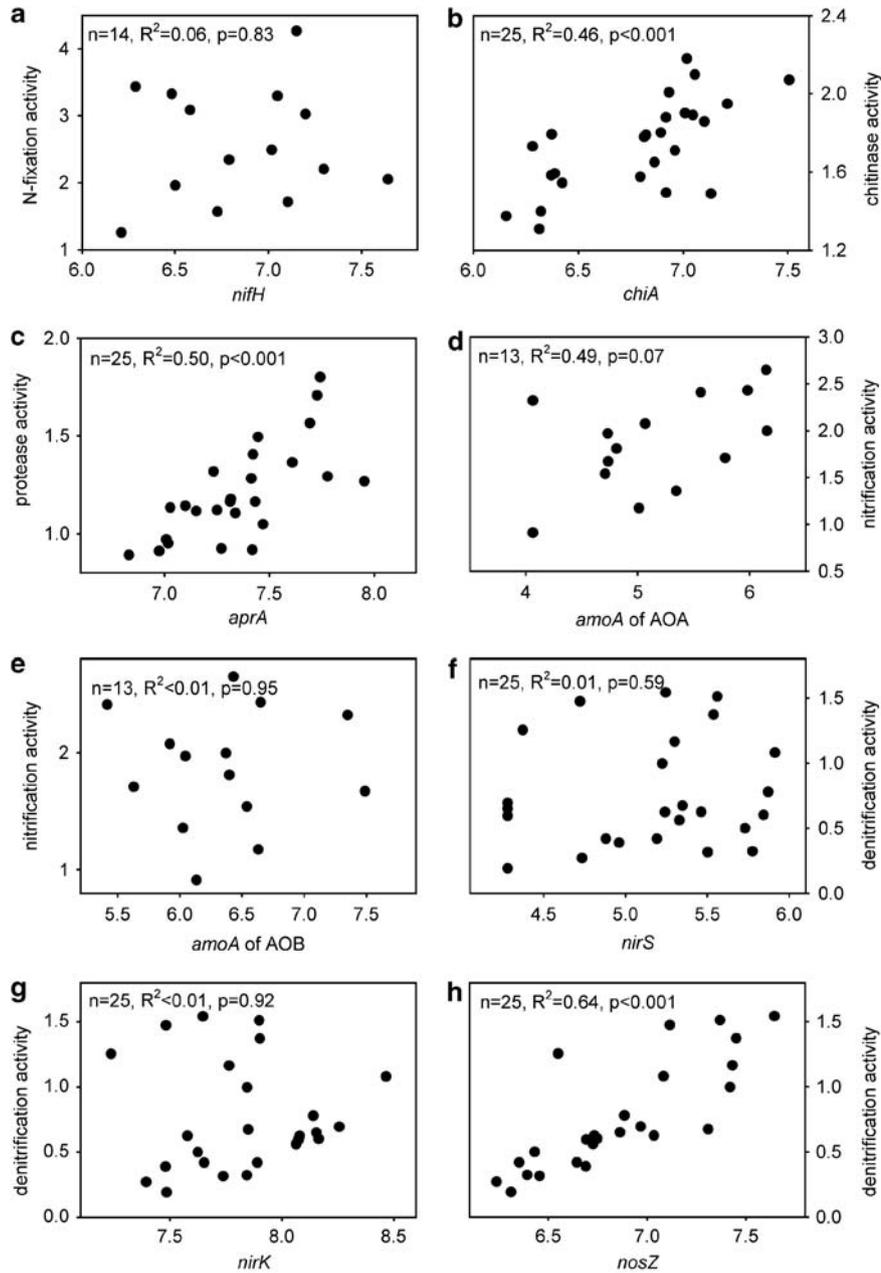
Figure 4 Correlations of the gene copy numbers and the corresponding potential enzyme activities are displayed for nitrogen fixation (**a**), mineralization (**b**, **c**), nitrification (**d**, **e**) and denitrification (**f**, **g** and **h**). The graphs plot log-transformed copy numbers vs log-transformed enzyme activities. In the top left corner of each plot the parameters of the regression analysis, including *P*-values of *t*-tests, are provided.

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

Along with the increase in plant abundance and diversity (Hammerli *et al.*, 2007) also other organisms establish in the developing soils. Especially the abundance of fungi increased along glacial chronosequences (Ohtonen *et al.*, 1999; Bardgett and Walker, 2004). Thus, the observed increase in *chiA* gene abundance and potential chitinase activity were not surprising. These findings are further in-line with data presented by Tscherko *et al.* (2004) who revealed highest *N*-acetyl-β-glucosaminidase

activity in the 75a transient soils of the Rotmoosferner glacier. On the contrary, *aprA* gene abundances did not change, underlining the high relevance of chitin as alternative C and organic N source (Olander and Vitousek, 2000), which might result from the deposition of dead insects (Hodkinson *et al.*, 2002).

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

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

Plants as driver for nitrogen turnover

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

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

Linking potential enzymatic activities to gene abundance

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

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

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

The chronosequence approach

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

Conclusion

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

Acknowledgements

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