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High Diversity of Diazotrophs in the Forefield of a Receding Alpine Glacier

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Abstract Forefields of receding glaciers are unique and sensitive environments representing natural chronosequences. In such habitats, microbial nitrogen fixation is of particular interest since the low concentration of bioavailable nitrogen is one of the key limitations for growth of plants and soil microorganisms. Asymbiotic nitrogen fixation in the Damma glacier (Swiss Central Alps) forefield soils was assessed using the acetylene reduction assay. Free-living diazotrophic diversity and population structure were resolved by assembling four NifH sequence libraries for bulk and rhizosphere soils at two soil age classes (8- and 70-year ice-free forefield). A total of 318 NifH sequences were analyzed and grouped into 45 unique phylotypes. Phylogenetic analyses revealed a higher diversity as well as a broader distribution of NifH sequences among phylogenetic clusters than formerly observed in other environments. This illustrates the importance of free-living diazotrophs and their potential contribution to the global nitrogen input in this nutrient-poor environment. NifH diversity in bulk soils was higher than in rhizosphere soils. Moreover, the four libraries displayed low similarity values. This indicated that both soil age and the presence of pioneer plants influence diversification and population structure of free-living diazotrophs.

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Introduction

Most alpine glaciers have been retreating since the end of the Little Ice Age around 1850 [30, 69]. This retreat exposed large areas to colonization by microorganisms, plants, and animals [19, 47, 70, 76, 77]. These glacier forefields provide unique opportunities to study biological succession, biogeochemical weathering processes, and the conversion of glacial till into fertile soils along the chronosequence (from the glacier to the valley).

The pioneer plants colonizing bare glacier forefields are crucial for soil formation, as their root exudates and decaying biomass are the main sources of organic matter in the developing soils [34, 61]. Moreover, root systems of pioneer plants contribute to slope stabilization [52]. In recently deglaciated environments, nutrient shortage is the main limitation for plant growth [39]. Organic acid anions (e.g., malate, citrate, oxalate) released in the rhizosphere can act as ligands, thus increasing the uptake of mineral nutrients [61]. Soil bacteria are also responsible for the release of organic acids [45] and chelators (e.g., siderophores) [66], facilitating the mineral nutrient acquisition

by plants. Moreover, some of them can suppress plant pathogens through their competitive and biocontrol abilities [36]. Overall, plants receive considerable benefits from soil microbial communities [33] and in turn stimulate microbial growth and activity through the exudation of highly nutritive substances. This phenomenon, known as the rhizosphere effect, has previously been studied in the forefields of the Lyman glacier (Washington, USA) [70] and the Rotmoosferner and Ödenwinkelkees glaciers (Austria) [26, 81, 82]. In the Damma glacier forefield (Switzerland), two different scales have been explored. Edwards et al. [29] observed a decrease of plant influence on soil bacterial communities along the chronosequence, while Miniaci et al. [63] showed that this influence extended at least to 20-cm distance around pioneer plant patches.

The nitrogen cycle plays a central role in soil ecosystems and in particular in developing soils. Nitrogen deposition is low in the Swiss Central Alps, where this study was conducted. The study location receives a relatively low atmospheric nitrogen input of less than 10 to 15 kg nitrogen per hectare and year, in contrast to many sites in the Swiss plateau receiving 25 kg to more than 40 kg per hectare and year, as given by the Swiss Federal Office for the Environment (www.bafu.admin.ch). The study by Korner et al. [53] (2,470 m, Swiss Central Alps), as well as a preliminary study on the Damma glacier [10] suggested that the growth of microbial communities in alpine grassland was limited by mineral nitrogen availability and carbon supply. Under nitrogen-limited conditions, microbial nitrogen fixation represents a selective advantage for nitrogen-fixing bacteria (diazotrophs) and, indirectly, for the interacting pioneer plants. Symbiotic nitrogen fixation has been extensively studied particularly in agricultural soil [44]. However, only few studies have focused on nitrogen fixation in glacier forefields. For example, Kohls et al. [50] studied the impact of symbiotic nitrogen fixation on plant succession and soil formation and Jacot et al. [42] showed that symbiotic nitrogen fixation provides 70% to 95% of the nitrogen requirements of legumes in the Swiss Alps. Even less is known about the role and the phylogeny of free-living diazotrophs in such environments. This group may be particularly important in the early stages of the chronosequence, as only few pioneer plant species form symbioses with nitrogen-fixing bacteria [43]. These plants may take advantage of indirect interactions with free-living diazotrophs.

Nitrogen fixation is a complex and energy-intensive process, which requires the interaction of several gene products including the nitrogenase structural proteins NifD, NifK, and NifH. The *nifH* gene, which encodes the iron protein of the nitrogenase complex (nitrogenase reductase), represents a valuable marker gene for the study of diazotrophs. The amino acid sequence of the NifH protein is highly conserved [16], and an extensive database of sequences retrieved from multiple environments is available [88]. Comparison of 16S rRNA and *nifH* phylogenies gives no strong evidence for lateral *nifH* gene transfer [88] although some anomalies were observed [59, 62]. For example, Dedysh et al. [25] mentioned that diverse microorganisms may have acquired their nitrogen fixation capability from lateral transfer of an α-proteobacterial *nifH* gene.

The *nifH* phylogeny was classified into four clusters (clusters I–IV) [20]. This phylogenetic classification, based on amino acid sequences, has proven to be reasonable [12, 48, 55] and was updated and rearranged by Zehr et al. [88]. The latter study on *nifH* phylogeny constitutes a good basis for further phylogenetic studies of diverse environments.

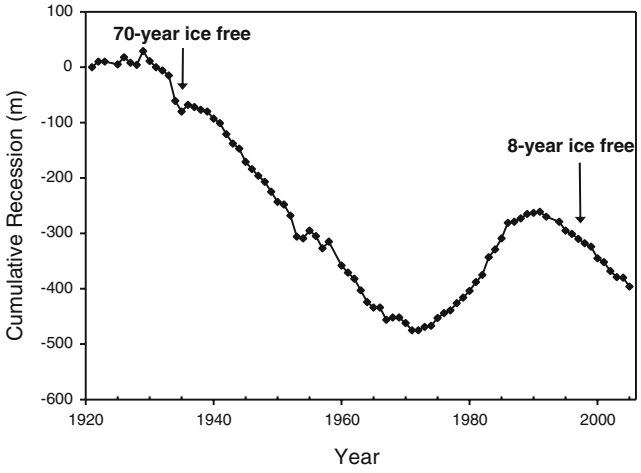
Numerous previous studies have used the *nifH* gene to assess diazotroph diversity in soil (e.g., [41, 85, 87]), including arctic soil [27], but there are no previous studies in alpine glacier forefields.

The aim of this study was to verify that asymbiotic nitrogen fixation was effectively taking place in the Damma glacier forefield and to determine the NifH diversity and distribution, providing for the first time a survey on free-living diazotrophic diversity in high alpine soils. We further hypothesized that due to their unique ability to grow in nitrogen-limited environments in presence of a suitable energy source, they would react strongly to the key factors influencing soil parameters in the forefield environment: soil age and plant influence. To test this hypothesis, we sampled bulk soil and rhizosphere soil of three prominent pioneer plants from two successional stages. We retrieved *nifH* sequence information from glacier forefield soils using a cloning and sequencing approach and estimated NifH diversity by applying rarefaction analysis. The glacier-specific NifH sequences were aligned to publicly available sequences and subjected to phylogenetic analysis to determine their association with the *nifH* clusters as given by Zehr et al. [88].

Materials and Methods

Damma Glacier Forefield

The study site is the forefield of the Damma glacier (8° 27′ N, 46° 38′ E) near Göscheneralp (Central Switzerland) at an altitude of approximately 2,000 m above mean sea level. The glacier has been receding since 1850, apart from an intermittent advancement between 1972 and 1992 (corresponding to the intermediate moraine; Fig. 1). Since the beginning of systematic measurement in 1921, the glacier has been receding at a mean rate of 10 m year^{-1}



Year	Number of 70-year ice free forefield samples	Type of soil	Number of 8-year ice free forefield samples
2004	5	Bulk soil	3
	8	Rhizospheres oil	15
2005	3	Bulk soil	3
	6	Rhizospheres oil	6

Figure 1 Cumulative recession of the Damma glacier from 1922 to 2005. *Arrows* indicate the two types of soil age sampled in this study. Adapted and expanded from Edwards et al. [29]

(http://glaciology.ethz.ch/swiss-glaciers/, [3–6]). The exposition of the forefield is SW to NE with a slope of 25%. The site is characterized by a mean annual air temperature of between 0°C and 5°C and a mean annual precipitation of 2,400 mm [79]. The parent rock of the forefield is granite.

Sampling

Sampling took place during two consecutive summers. Samples from 2004 were collected for acetylene reduction assay and phylogenetic analysis and samples from 2005 were used for soil characterization and phylogenetic analysis. Two sampling sites were selected before and after the intermediate moraine according to the two soil age classes defined by Hämmerli et al. [38]. The two sites corresponded to 8- and 70-year ice-free forefield (year of deglaciation 1997 and 1935) and were situated at 60 and 350 m from the glacier tongue, respectively. Soil collection sites were localized using a Geographic Information System (ArcMap™) and Global Positioning System (GPS). In total, 14 bulk soil samples and 35 rhizosphere soil samples from three pioneer plant species (*Leucanthemopsis alpina*, *Poa alpina*, and *Agrostis* sp.) were collected (Fig. 1). Plants were selected to be solitary or at least to be the dominant plant species within the sampled vegetation patches.

Bulk soil samples were collected with a spatula to a depth of approximately 5 cm and transported in plastic bags on ice to the laboratory within 8 h. Pioneer plants, including root systems, were collected with a spade and then vigorously shaken to remove nonadherent soil. All three plants had extensive root systems. The grasses develop a very dense mass of long and thin roots, while the less compact root system of *L. alpina* is composed of thicker roots. In the laboratory, rhizosphere soil adhering to the roots was then collected and used for further analysis.

Chemical and Physical Characterization of Soils

Soils ranged from coarse sand (8-year ice-free forefield) to well-defined layered soils (70-year ice-free forefield). Nitrate was analyzed using a Dionex DX-320 ion chromatograph (Dionex, Sunnyvale, CA, USA) in 0.01-M $CaCl_2$ soil extracts [63]. Soil pH was measured in the same extracts. Total carbon (C_{tot}) and nitrogen (N_{tot}) contents were determined by combustion of 1 g ground soil at 1,200°C, using a Leco 2000 CNS device elementary analyzer (Leco, Krefeld, Germany) following manufacturer's instructions. Available ammonium was determined photometrically after 2-M KCl soil extraction [65]. Soil samples were dried and weighed to determine soil water content. Effects of soil age and plant presence on soil properties as well as the interaction soil age × plant presence were examined through two-way analysis of variance (ANOVA) using the JMP software [75].

Acetylene Reduction Assay

Potential asymbiotic nitrogenase activity was estimated using the acetylene reduction assay (ARA) [14]. Briefly, field-moist bulk soil and rhizosphere soil (roots, stem, and leaves included) samples were weighed and placed in glass bottles sealed with gas-tight neoprene septum lids (Supelco, Bellefonte, PA, USA) to facilitate the sampling of gas. The headspace was flushed for 15 min with N_2, before the addition of 10% (*v*/*v*) acetylene (C_2H_2). The bottles were incubated in a climate chamber at 15°C with 14 h of artificial light per day. Ethylene (C_2H_4) production was measured after 5 min, 24, 48, and 54 h following acetylene addition, by injecting 0.5-ml headspace into a 8000 GC gas chromatograph (Carlo Erba Instruments, Milan, Italy) equipped with a flame ionization detector and a Hayesep N capillary column (BGB Analytik, Auwil, Switzerland) at 75°C. The rate of ethylene production was derived from the slope of the linear regression of ethylene concentration versus time. Effects of soil age and plant presence on the acetylene reduction activity were tested as described above.

Nucleic Acid Extraction and PCR Amplification

DNA was extracted from 0.7 g of (fresh weight) soil using a bead-beating method as described previously [68]. The extracted nucleic acids were dissolved in 50 μl TE buffer

(10 mM Tris–HCl (pH 8), 1 mM ethylene diamine tetraacetic acid). DNA concentration in extracts was determined using a Quant-iT™ PicoGreen dsDNA Kit (Invitrogen) and a Synergy plate reader (BIO TEK, Vinooski, USA).

For amplification of *nifH* genes, a nested polymerase chain reaction (PCR) approach was performed according to Bürgmann et al. [15, 85]. The initial PCR was performed with the forward primer *nifH*-forA (5′-GCIWTITAYGG NAARGGNGG-3′) and the reverse primer *nifH*-rev (5′-GCRTAIABNGCCATCATYTC-3′), followed by a semi-nested PCR reaction using the forward primer *nifH*-forB (5′-GGITGYGAYCCNAAVGCNGA-3′) and the same reverse primer.

Cloning and Sequencing

Clones libraries of amplified *nifH* fragments were created for four samples each of both 8-year soils and the 70-year rhizosphere soil, as well as seven samples of 70-year bulk soil. The amplicons of the nested PCR reactions were loaded on a 1% agarose gel. DNA bands of 370 bp were excised and purified with Qiagen gel extraction kit (Qiagen AG, Hilden, Germany). The products were cloned using a TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. Clones were selected randomly and sent to GATC Biotech AG (Konstanz, Germany) for sequencing.

Sequence Data and Phylogenetic Analysis

Sequence data processing was carried out using the Sequencher™ (Gene Codes Corporation, Ann Arbor, USA) software package and closest relatives were determined with a Basic Local Alignment Search Tool search [1] at National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Translated NifH amino acid sequences were organized in separate libraries for each type of soil. As this study focused on the impact of plant presence and not on plant species, sequences originating from rhizosphere soil from the three different pioneer plant species were combined into a single 8-year and 70-year rhizosphere soil library, respectively. Three hundred eighteen Damma glacier NifH sequences were merged with 3,324 sequences retrieved from the Pfam Fer4_NifH database (2005; http://www.sanger.ac.uk) [2], and 4,298 sequences were kindly provided by B. D. Jenkins (Department of Cell and Molecular Biology, University of Rhode Island, Kingston). The two latter groups of sequences might contain overlaps. Sequence alignments, similarity matrix calculation, and tree construction were performed using the ARB software package [58]. A phylogenetic inference tree was calculated at the protein level using the neighbor-joining algorithm [74] and a Kimura correction [49], considering 107 amino acid positions. Tree topology confirmation was achieved using the PHYLIP Protein-Parsimony package (version 3.6a3). Highly variable regions within the sequences were excluded using a 30% amino acid frequency filter. The *nifH* phylogeny defined by Zehr et al. [88] was used as backbone for tree construction.

Diversity Measurements and Statistical Analyses

Amino acid sequences with at least 97% sequence similarity were grouped in unique NifH phylotypes, according to sequence similarity data retrieved from ARB. Rarefaction curves were constructed using the EstimateS software package (version 8.0) [21] and total NifH phylotype richness of the four libraries was estimated using Chao1 [17] as a nonparametric indicator. EstimateS generated 50 runs (individual randomization) for each rarefaction curve. These replicates were used to test the effect of soil age and plant presence on NifH phylotype number using a two-way ANOVA, as well as Student and Tukey HSD tests using the JMP software [75]. Diversity and evenness are given by the Shannon–Wiener diversity index, $H' = -\sum (p_i)(\ln p_i)$, where p_i is the proportion of ith phylotype, and the Shannon evenness, $E_H = H'/\ln S$, where S is the total number of phylotypes. Similarity between libraries was evaluated using the Sorensen similarity index $C_s = 2j/a + b$, where j is the number of common species in two libraries and a and b are the numbers of phylotypes of both libraries.

Nucleotide Sequence Accession Numbers

The *nifH* gene sequences retrieved from the Damma glacier forefield were deposited in the GenBank database under Accession No. EF988336–988349, EF988351–988362, EF988364–988377, EF988379–988420, EF988422–988428, EF988430–988549, EF988552–988615, EF988617, EF988619–988622, and EU305256–EU305295.

Results

Soil Biogeochemistry

The analysis of selected soil biogeochemical characteristics revealed significant differences for nitrate, ammonium, water, and DNA content between the two soil age classes, as well as between bulk soil and rhizosphere soil (Table 1). Nitrate concentration decreased significantly with soil age and to a lesser extent in the presence of plants. Water content was slightly higher in the 70-year ice-free forefield and increased significantly in presence of plants. DNA

Table 1 Biogeochemical properties and nitrogenase activity for the four types of soil

Parameters [units]	8-year ice free		70-year ice free		ANOVA		
	Bulk soil	Rhizosphere soil	Bulk soil	Rhizosphere soil	*p* value		
					Soil age	Plant presence	Factors interaction
N_{tot} [μg N/g dry soil]	4.7±3.4	38.37±9.3	10.45±8.84	529.4±524.22	0.2459	0.1997	0.2563
Nitrate [μg N/g dry soil]	0.04±0.03	0.022±0.012	0.02±0.007	0.015±0.003	0.0594[a]	0.0961[a]	0.3309
Ammonium [μg N/g dry soil]	0.58±0.05	0.55±0.01	0.55±0.03	0.75±0.29	0.0724[a]	0.0905[a]	0.0246[b]
C_{tot} [μg C/g dry soil]	155.1±203.6	668.2±50.1	233.7±179.0	10,016.83±10,800.8	0.1248	0.0958[a]	0.1307
DNA [ng DNA/g dry soil]	2.6±0.4	156.4±46.4	5.9±3.2	4,338.2±826.0	<0.0001[c]	<0.0001[c]	<0.0001[c]
Water content [%]	5.3±3.8	16.2±2.6	7±4.6	20.3±5.2	0.0693[a]	<0.0001[c]	0.4351
pH	4.7±0.2	4.9±0.3	4.7±0.1	4.8±0.5	0.5980	0.1249	0.5699
Nitrogenase activity [nmol C_2H_4/day·g dry material]	0.054±0.043	0.265±0.525	0.098±0.095	1.948±2.529	0.1275	0.0715[a]	0.1472

[a] Significant at 0.1 level
[b] Significant at 0.05 level
[c] Significant at 0.01 level

content increased significantly with soil age and was lower in bulk soil than in rhizosphere soil: 60-fold and 700-fold lower in the 8-year and 70-year ice-free soils, respectively. Nevertheless, both effects cannot be differentiated as their interaction was highly significant. Similarly, ammonium was significantly affected by both factors but their respective contribution cannot be distinguished. The pH was not significantly affected by the presence of plant or by soil age. The impact of plant presence on total carbon and total nitrogen contents was significant. However, C_{tot} and N_{tot} values were out of the validity range of the CNS elementary analyzer and therefore have to be interpreted with caution.

Nitrogenase Activity

Acetylene reduction rates were low but above the detection limit in most samples, with a few outliers showing higher rates (Table 1). ARA values increased significantly in presence of plants ($p<0.1$) despite high standard deviations for both rhizosphere soils. This variability seemed not to be related to the three different plant species but to plant individuals (data not shown).

Rarefaction Analysis

The 318 aligned NifH amino acid sequences were grouped into 45 unique NifH phylotypes at 97% sequence similarity level. The lowest number of sequences ($n=74$) in the 8-year bulk soil library was used as a threshold for comparison of phylotype richness according to rarefaction analysis of the four libraries. Richness at $n=74$ was higher in bulk soil than in rhizosphere soil regardless of soil age (Fig. 2). The ANOVA on the 50 EstimateS replicates ($n=74$) showed that both plant presence ($p<0.0001$) and soil age ($p=0.077$) had significant and marginally significant effects, respectively, on NifH phylotype number. The interaction of these two factors was significant ($p=0.0007$). According to Student and Tukey HSD tests, 8-year and 70-year rhizosphere soils

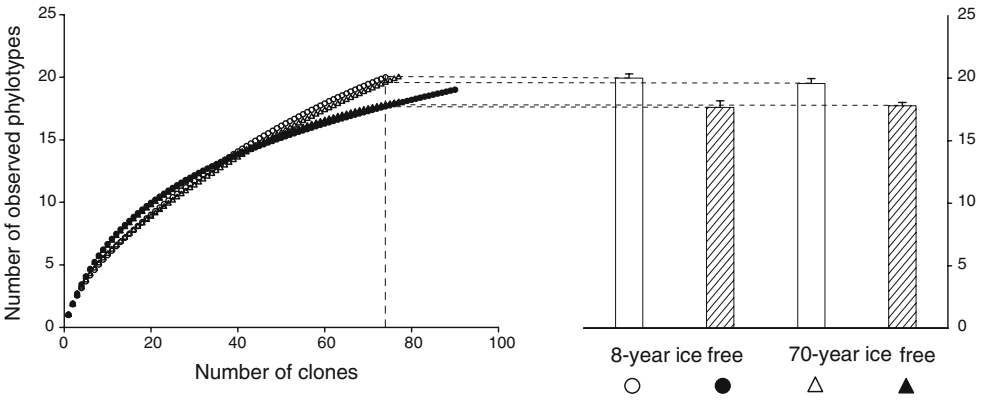


Figure 2 Species richness of the four NifH sequence libraries. Symbols: 8-year bulk soil (*empty circles*), 70-year bulk soil (*empty triangles*), 8-year rhizosphere soil (*filled circles*), and 70-year rhizosphere soil (*filled triangles*). *Left*: Rarefaction curves were constructed using EstimateS software package at 97% amino acid similarity level. *Right*: Species richness at $n=74$. *Error bars* correspond to Sobs standard error (Mao Tau) [22]

were similar, while 8-year and 70-year bulk soils were significantly different.

The Chao1 estimates of total NifH phylotype richness values were in the same range as the observed number of phylotypes and confirmed the trend found for richness at n=74 (Table 2). Rhizosphere soil showed higher Shannon–Wiener evenness than bulk soil, while diversity values ranged between 2.16 and 2.46, with lower values in bulk soil due to the lower evenness (Table 2).

Phylogenetic Characterization

All NifH amino acid sequences derived from the glacier forefield were assigned to the NifH phylogenetic clusters defined by Zehr et al. [88]. Diazotrophs present in the Damma glacier forefield were highly diverse with respect to phylogeny (Figs. 3 and 4). All previously described NifH clusters [20] were represented except the *Archaea* (cluster IV) which are not targeted by the primers used in this study.

The four libraries varied greatly in their phylotype composition as illustrated by Fig. 3 and the low Sorensen similarity indices (Table 3). Briefly, the similarities between libraries from different age classes were intermediate, while similarity between bulk soil and rhizosphere soil in 8-year ice-free forefield was higher, and similarity between bulk soil and rhizosphere soil in the 70-year ice-free forefield was lower (Table 3). Shannon evenness indices were smaller for the two bulk soil libraries than for rhizosphere soil (Table 2). In both case, more than half of the NifH sequences belonged to few phylotypes that were almost exclusive to these two libraries.

The majority of the NifH sequences (80%) were affiliated with cluster I. Within this cluster, most of them (35%) were related to subcluster IK (α-, β-, γ-*Proteobacteria*; 57%, 15%, and 28%, respectively). This subcluster was most abundant in the rhizosphere soils (59% and 25% of sequences in 8- and 70-year soil, respectively). A large number of these NifH sequences were related to *Methylococcus capsulatus* (22 sequences, 97% amino acid sequence similarity level) and *Methylocystis sp*. (30 sequences, 99% amino acid sequence similarity level). Almost all (96%) of these 52 NifH sequences were found in rhizosphere soils and two thirds of them were retrieved from the 8-year soil samples. Thirteen NifH sequences were related to potentially symbiotic nitrogen-fixing bacteria *Ideonella* sp. (>97% similarity). Subcluster IA (δ-*Proteobacteria*) was the second most important subcluster within cluster I (27%). These sequences occurred predominantly in bulk soil and were related to *Geobacter uraniumreducens*, *Geobacter bemidjensis*, or *Geobacter lovleyi* at more than 96%. Subcluster IB, corresponding to *Cyanobacteria,* recovered 19% of the NifH sequences in cluster I. These sequences occurred with high abundance in both rhizosphere libraries, and this was the most abundant group in 70-year rhizosphere soil. In this subcluster, nine sequences were closely related to *Nostoc* sp. (100% similarity), one to *Nostoc muscorum* (96% similarity), four to *Aphanizomenon* sp. (99% similarity), six to *Scytonema* sp. (98% similarity), five to *Pseudanabaena* sp. (98% similarity), and two to *Anabaena cylindrica* (100% similarity). Additionally, 22 sequences were weakly affiliated with *Oscillatoria* sp. (93% similarity). Further sequences were grouped within subcluster IE (*Firmicutes*) which occurred with high abundance in the 8-year bulk soil library (31%, related to *Paenibacillus durus* with 94% similarity), IP (β-*Proteobacteria*), and uncultured subclusters (i.e., IC and IG).

Cluster III was the second prominent cluster with 23.6% of all NifH sequences. Within this cluster, two thirds of the sequences did not group into any known subcluster and were classified in two new subclusters Damma1 and Damma2 (Fig. 4.). The remaining sequences clustered within the subclusters IIID (*Firmicutes*), IIIC (*Archaea*), and IIIQ (uncultured). Cluster II contained only a few sequences (1.6%) belonging to subcluster IIC (α-, γ-*Proteobacteria*, *Spirochaetes*) and IIE (*Firmicutes*).

Table 2 NifH phylotype richness and diversity estimators in the four NifH libraries

	8-year ice free		70-year ice free	
	Bulk soil (n=74)	Rhizosphere soil (n=90)	Bulk soil (n=77)	Rhizosphere soil (n=77)
Number of phylotype represented by NifH sequences[a]	20	19	21	18
Number of NifH phylotypes extrapolated[a, b]	26	24	31	19
95% confidence interval[a, b]	22–47	20–47	23–64	18–28
Shannon–Wiener diversity (H')	2.22	2.46	2.16	2.32
Shannon evenness (E_H)	0.74	0.84	0.71	0.80

n Number of NifH sequences analyzed in respective NifH library

[a] Number of different (<97% sequence similarity) NifH sequences

[b] Number of phylotypes extrapolated using the richness estimator Chao1

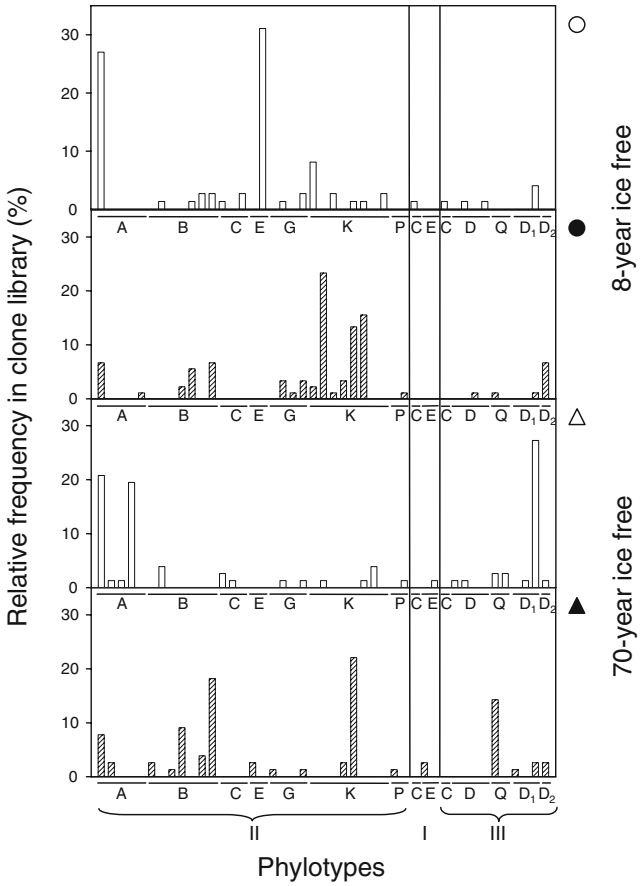


Figure 3 Relative frequency of phylotypes in individual NifH libraries, classified in phylogenetic clusters as defined by Zehr et al. [82], based on the phylogenetic analysis shown in Fig. 4. Symbols: 8-year bulk soil (*empty circles*), 70-year bulk soil (*empty triangles*), 8-year rhizosphere soil (*filled circles*), and 70-year rhizosphere soil (*filled triangles*)

Discussion

Nitrogenase Activity and Soil Biogeochemistry

Acetylene reduction rate measurements indicated that asymbiotic nitrogen fixation occurs in the Damma glacier forefield soils. The higher activity in rhizosphere soils is in agreement with previous observations on other enzyme activities [63, 81] and may be related to the elevation of substrate and nutrient concentration through the exudation of carbon-rich substances by the roots, as well as to low abiotic stress (e.g., reduced temperature and moisture fluctuation) due to vegetation cover [63, 81].

The analysis of selected biogeochemical parameters confirmed that the Damma glacier forefield is a heterogenous environment, significantly affected by soil age and the presence of pioneer plants, as discussed previously [29, 77]. Interestingly, nitrate decreased significantly along the chronosequence, while ammonium concentrations seemed to be stable in bulk soil and to increase in rhizosphere soil. The nitrate pool is probably depleted due to both the immobilization by plants and microorganisms and the absence of nitrification. In fact, Verhagen et al. [83] and Bengtson and Bengtsson [8] reported that nitrifying bacteria are outcompeted by heterotrophic bacteria in ammonium-poor environments. In contrast, microbial nitrogen fixation and ammonification may explain the sustainability of the ammonium pool.

NifH Diversity

Comparative studies on soil NifH diversity have to be performed with caution, as different studies have used different primer pairs for the *nifH* gene amplification. Nevertheless, NifH diversity (H') values in Damma glacier forefield soils were surprisingly high compared to diversity found in other studies [27, 72, 73, 85]. The methods used in these studies, restriction fragment length polymorphism (RFLP) and terminal restriction fragment length polymorphism (T-RFLP), may bias diversity measurements. RFLP has a low genotyping resolution, while T-RFLP may lead to an overestimation of the diversity due to incomplete restriction digestion [67]. The cloning–sequencing analysis used here has a high phylogenetic resolution but is of course also subject to potential PCR bias. NifH diversity values presented in this study were comparable to those found by Izquierdo and Nusslein [41] who used a similar analytical approach. The Shannon–Wiener diversity index found in this study were intermediate to those found for arctic tundra soils ($H'=1.97$) and tropical forest ($H'=2.41$)[41]. Thus, despite extreme nutritional and climatic conditions, diazotrophs in the glacier forefield establish with similar diversity as those found in more favorable environments.

Previous work at the Damma glacier forefield suggested a higher bacterial diversity in bulk soil compared to rhizosphere soil [29]. Our results were in agreement with this observation, showing a significantly higher NifH phylotype richness in bulk soil. It may result from a higher diversity of the microhabitat [35, 72], as well as more fluctuating growth conditions [82]. Nevertheless, the competition for carbon and nitrogen resources in the nutrient-poor glacier forefield bulk soil may lead to strong selection pressure on the bacterial community. The lower Shannon evenness value for both bulk soil libraries supports this assumption.

Additionally, the Chao 1 estimator predicted the highest diversity in 70-year bulk soil. One possible explanation may be the intermediate disturbance hypothesis [23], stating that environments with intermediate rate of disturbance display highest diversity. In the present study, 70-year bulk soil may correspond to this intermediary state as it undergoes less abiotic disturbance than 8-year bulk

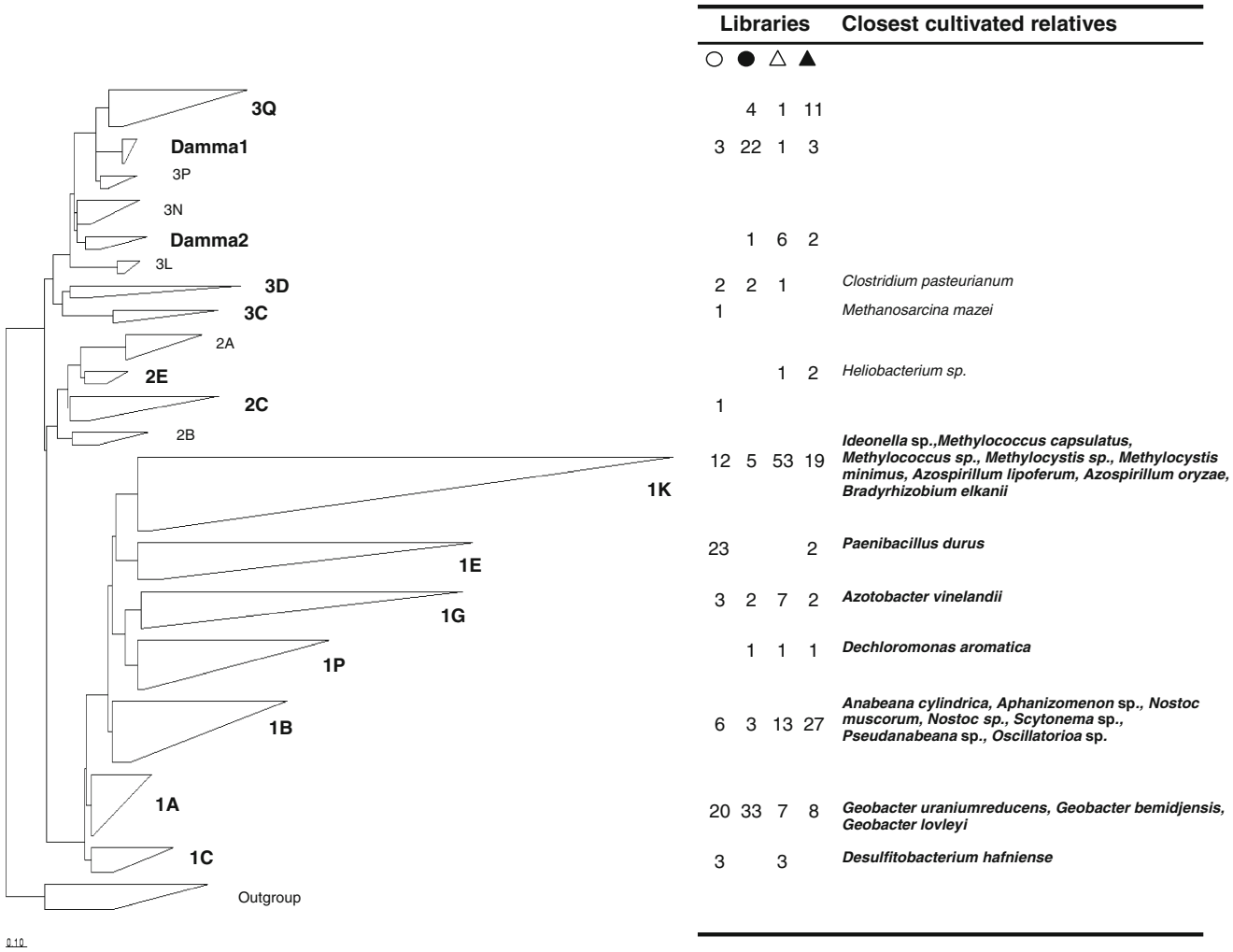


Figure 4 Phylogenetic characterization of NifH sequences detected in the Damma glacier forefield soils. Presence, in respective subclusters, is given by the absolute number of NifH sequences related to the four libraries. The dendrogram is based on 1,713 aligned amino acid sequences deduced from DNA sequences and was calculated using the neighbor-joining algorithm. Thirty-seven sequences affiliated with Cluster IV (*Archea*) were used as outgroup. Species in *bold* type showed more than 95% similarity (amino acid level) with clones retrieved from the glacier forefield

soil (higher carbon and nitrogen availability, physical protection due to the dense surrounding vegetation) but more than rhizosphere soil where conditions are more favorable and stable (high nutrient availability, less moisture and temperature fluctuation) [82]. Similar findings are reported for a nutrient-rich agricultural soil by Marilley and Aragno [60] who showed that low rates of disturbance associated with rhizosphere soils (e.g., higher substrate availability) lead to communities which are dominated by a few species.

Only few studies addressed the direct comparison between nitrogenase activity and diazotrophic diversity. Deslippes et al. [28] observed a poor relationship between *nifH* diversity and nitrogenase activity in natural environment. In a laboratory pot experiment, Bürgmann et al. [13] described more diverse active diazotrophic populations related to higher nitrogen fixation rates. Nevertheless, active NifH diversity and nitrogenase activity in natural environment have never been related. Methods used in the present study do not allow the direct comparison of activity and active diversity.

Table 3 Sorensen similarity (C_s) matrix for the four types of soil by using NifH phylotypes identified in the NifH libraries

Type of soil		8-year ice free		70-year ice free	
		Bulk soil	Rhizosphere soil	Bulk soil	Rhizosphere soil
8-year ice free	Bulk soil	1.00			
	Rhizosphere soil	0.51	1.00		
70-year ice free	Bulk soil	0.39	0.40	1.00	
	Rhizosphere soil	0.32	0.43	0.26	1.00

Relative frequencies of phylotypes per library are not taken into account

NifH Phylogenetic Distribution

Clone distribution among phylogenetic clusters and Sorensen's index revealed a low degree of overlap between NifH libraries of the different soils. This indicated a high heterogeneity in the Damma glacier forefield environment. Furthermore, it implied that soil age and presence of pioneer plants may have promoted strong shifts in the community structure.

The total of 14 NifH subclusters observed in the four libraries represents a much broader distribution of NifH sequences as formerly described in forest soil [73, 85] and cultivated soil [72], where the entire NifH diversity was related to *Proteobacteria*. The diazotrophic community found in the Damma glacier, especially in 8-year bulk soil, was much more comparable to that found in arctic soil by Deslippe and Egger [27], who likewise found a high number of *Firmicute*-related microorganisms (subcluster IE) in an arctic glacial lowland soil. Interestingly, eight phylogenetic subclusters, not depicted as soil subclusters by Zehr et al. [88], and two new subclusters of uncultivated diazotrophs were found in Damma glacier forefield soils. The latter two subclusters represented 12% of total NifH sequences and were represented in four and three of the four libraries, respectively (Fig. 3). All these observations highlight the fact that asymbiotic NifH diversity has been mainly studied in the marine environment and knowledge for soils, especially in extreme environments, is limited.

Sequences affiliated with two methanotrophic bacteria, *M. capsulatus* and *Methylocystis* sp., represented 16.4% of the total diversity, and two thirds of them were found in the young forefield. Various upland soil methanotrophs have a high affinity for CH_4 and are able to oxidize atmospheric CH_4 even at low concentrations [7, 51]. That may explain a high competitiveness in the studied carbon-poor environment. Alternatively, plant exudates may be fermented to methane in anaerobic microniches, which in turn may stimulate the growth of methanotrophs in the rhizosphere. This hypothesis is supported by the phylogenetic data showing the almost exclusive presence of methanotrophs in the more humid and carbon-rich rhizosphere soils. However, as mentioned by Dedysh et al. [25] the affiliation of the NifH sequence from the γ-*Proteobacteria M. capsulatus* with α-*proteobacterial* sequences may result from *nifH* gene lateral transfer. Therefore, it cannot be entirely ruled out that these sequences actually originated from other closely related α-*Proteobacteria.*

Twenty-one percent of all NifH sequences were grouped in subcluster IA and were related to *G. uraniumreducens*, *G. bemidjensis*, and *G. lovleyi*. Similar sequences have already been retrieved from bulk and rhizosphere soil samples [37, 88]. *Geobacter* species are able to gain energy from Fe(III) reduction using organic acids or acetate as electron donors [57] Representatives of the Geobacteraceae are also able to use oxygen as alternative electron acceptor, giving them competitive advantages in oxic–anoxic boundaries [56]. Glacier forefield soils typically undergo periods of inundation after snow melt and heavy rainfall, followed by periods of drought due to high light intensity and strong winds. These frequent fluctuations between aerobic and anaerobic conditions may favor the spreading of *Geobacter* species.

Cyanobacterial NifH sequences were also well represented in the glacier forefield soils. *Cyanobacteria* have already been described as an important source of nitrogen input in arctic [18, 54] and alpine [31] environments. Furthermore, they are known as primary colonizers of recently deglaciated soil [24, 46]. Nevertheless, while *Cyanobacteria* are well studied in aquatic and symbiotic systems [9, 71], very little is known about cyanobacteria under the soil surface [84]. The cyanobacterial community found in the glacier forefield was very similar to that found in mature biological soil crusts (BSCs) from the Colorado plateau and Chihuahuan desert [86], but crust formation was not evident at the sampling sites. *Cyanobacteria* are known to form symbioses with mosses (e.g., Azolla, Hepatica) [78], which are frequently associated with vegetation patches in the glacier forefield. Cross contamination with traces of moss biomass or BSCs cannot be ruled out but seems unlikely especially for rhizosphere samples. Another explanation may be the high metabolic versatility of *Cyanobacteria*. Half of cultivated *Cyanobacteria* are facultative heterotrophs, with light remaining the energy source [80], or chemoorganotrophs [11]. Nine of our cyanobacterial sequences were related to *Nostoc* sp., a species able to perform aerobic respiration [80]. Gantar et al. [32] showed that *Nostoc* sp. and *Anabaena* sp. were able to form both loose and tight association with the roots of wheat. Half of the NifH sequences clustering with *Cyanobacteria* were closely related (99%) to NifH sequences retrieved from the rhizosphere of a transgenic tomato plant [40] that were distantly affiliated with facultative fermentative *Oscillatoria* sp. [64]. Fermentative conditions may occur locally in the rhizosphere of pioneer plants, where 89% of these sequences were found. Therefore, one can hypothesize that *Cyanobacteria* may also be found living heterotrophically in close but asymbiotic association with pioneer plants and that their abundance is directly or indirectly linked to the concentration and the composition of root exudates.

Conclusion

This study for the first time demonstrated activity and diversity of nitrogen-fixing bacteria in an alpine glacier

forefield. The considerable diversity as well as the broad distribution of NifH sequences retrieved from the glacier forefield chronosequence were unexpected and indicated that in its entirety this environment supports one of the most diverse diazotrophic communities studied so far. The association of methanotrophic and cyanobacterial diazotrophs with rhizosphere soils and *Geobacter* relatives with bulk soil indicated that interesting and diverse ecological strategies were associated with different habitats, warranting further study. This study demonstrated that, in accordance with our hypothesis, the presence of pioneer plants reduced the diversity of free-living diazotrophs and directly or indirectly influenced their community structure. Soil age was a less important factor, affecting significantly free-living diazotrophs only in bulk soil.

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