Diss. ETH No° 18327

DIAZOTROPHIC MICROORGANISMS IN THE ROOT ZONE OF ALPINE PIONEER PLANTS: POPULATIONS, FUNCTIONS AND INTERACTIONS

A dissertation submitted to the

SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH

LAURENCE DUC

ZURICH, 2009

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Diazotrophic microorganisms in the root zone of alpine pioneer plants: populations, functions and interactions

A dissertation submitted to the
SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH

for the degree of
Doctor of Sciences

presented by

LAURENCE DUC

Dipl. Biologist, University of Lausanne

born on April 20, 1978

citizen of Forel (FR)

accepted on the recommendation of

Prof. Dr. Josef Zeyer, examiner

Prof. Dr. Hans-Martin Fischer, co-examiner

Dr. Ralph Schlapbach, co-examiner

Dr. Stefan Neuenschwander

2009

“Savoir s’étonner à propos est le premier pas fait sur la route de la découverte”

Louis Pasteur

SUMMARY

Forefields created by receding glaciers represent unique and sensitive environments as well as natural chronosequences in which biological succession and soil development can be studied. Similarly to plants and animals, microbial communities are subject to successional development. The microbial succession has important effects on soil development and plant establishment as microbes play key roles in nutrient cycling. In young glacier forefield soils, nitrogen is a limiting nutrient, especially in the rhizosphere of pioneer plants where additional carbon is made available through root exudation. Under nitrogen-limited conditions the ability to fix nitrogen might represent a selective advantage for nitrogen-fixing microorganisms (diazotrophs). Symbiotic nitrogen fixation participates significantly to nitrogen accumulation in alpine soils. However, symbiotic plant species are rarely found in early stages of the chronosequence. Therefore, free-living diazotrophs can be expected to be competitive in young glacier forefields and to stimulate the growth of pioneer plants interacting with them. The aim of this thesis was to obtain new insights on the role and ecology of microbial and particularly of free-living diazotrophic communities in the dynamic and highly heterogeneous glacier forefield environment. Special emphasis was given to the impact of pioneer plant presence on these communities.

In the first part of this thesis, the spatial extent of plant-microorganism interactions in the young Damma glacier forefield was assessed by determining microbial cell counts, enzyme activities, as well as the eubacterial community structure at different distances from the plant. Results indicated that microbial cell number and enzymatic activity per cell were increasing in presence of the plant, despite an unaffected microbial community structure. This effect extended beyond the root zone, at least 20 cm around pioneer plants.

The second part of the study focuses on free-living diazotrophic communities in Damma glacier forefield soils. Asymbiotic nitrogen fixation was assessed using the acetylene reduction assay. This activity was positively influenced by the presence of the plant. Free-living diazotrophic diversity and population structure were determined by the assemblage of NifH sequence libraries. The phylogenetical analysis revealed 45 unique NifH phylotypes broadly distributed among the *nifH* phylogeny. The NifH diversity was higher than those formerly observed in other environments, confirming the hypothesis of free-living diazotrophs being relevant and potentially contributing to nitrogen input in this nitrogen-limited environment. In contrast to observations on eubacterial communities, free-living diazotrophic diversity decreased significantly in presence of the plant. NifH sequences related to methanotrophic microorganisms and *Cyanobacteria* were particularly frequent.

Based on the phylogenetical analysis mentioned above, a *nifH* oligonucleotide microarray was developed for the monitoring of free-living diazotrophs in glacier forefield soils. A total of 946 probes (20 nts) designed *in silico* were validated experimentally using the Geniom® One in situ synthesis platform. This probe set covered 35 NifH phylotypes out of the 45 found in glacier forefield soils and delivered consistent and accurate data. This experimental approach proved to be highly suitable and recommended for probe selection.

This new tool was then applied to investigate the effect of variable environmental parameters on of free-living diazotrophic populations. Plant presence revealed to have a stronger impact at the beginning of the vegetation period compared to that observed in September and to buffer the impact of the season. Both nitrogen deposition and temperature increase had higher impacts in rhizosphere soils compared to bulk soils, probably due to their positive influence on plant growth. This observation indicates strong carbon limitation in glacier forefield soils. Finally, *Methylocystis* related bacteria were mostly responding to environmental variations. These methanotrophic microorganisms able to use atmospheric methane as sole carbon source seem to be particularly adapted to extreme nutritional and climatic conditions found in glacier forefields. Therefore, they might play a key role in maintaining nitrogen fixation in these harsh environments.

RÉSUMÉ

Les moraines frontales crées par le retrait des glaciers constituent des environnements uniques et fragiles, ainsi que des chronoséquences naturelles permettant l'étude des successions biologiques et du développement du sol. Similairement à la flore et la faune, les communautés microbiennes sont sujettes au développement sucessionel. Les successions microbiennes sont supposées avoir un effet important sur le développement du sol et l'établissement de la végétation, dû principalement au rôle clé joué par les micro-organismes dans les cycles nutritques du sol. La quantité d'azote présent dans les jeunes moraines est limitée, spécialement dans la rhizosphère des plantes pionnières, où les exsudats racinaires représentent une source de carbone additionnelle. Dans de telles conditions de carence azotée, l'aptitude à fixer le diazote atmosphérique peut représenter un avantage sélectif pour les bactéries fixatrices d'azote. La fixation symbiotique de l'azote participe significativement à l'accumulation de l'azote dans les sols alpins. Pourtant, les espèces de plantes symbiotiques sont rares dans les jeunes stades de la chronoséquence. Dès lors, on peut s'attendre à ce que les bactéries libres fixatrices d'azote soient particulièrement compétitives dans cet environnement et qu'elles stimulent la croissance des plantes pionnières interagissant avec elles. L'objectif de cette thèse était d'obtenir un nouvel aperçu sur le rôle et l'écologie des communautés microbiennes, en particulier des communautés fixatrices d'azote dans l'environnement dynamique et hétérogène formé par les jeunes moraines frontales. Une attention spéciale a été portée sur l'influence des plantes pionnières sur ces communautés.

Dans un premier temps, l'étendue spatiale des interactions plante-bactéries dans la moraine frontale du glacier Damma a été déterminée par l'évaluation du nombre de cellules microbiennes, des activités enzymatiques et de la structure de la communauté eubactérienne à différente distance de la plante. Les résultats ont indiqué un nombre de cellules microbiennes et une activité enzymatique par cellule croissantes en présence de la plante, malgré une communauté microbienne stable. Cet effet a été identifié au delà de la zone racinaire, à un minimum de 20 cm de distance des plantes pionnières.

La seconde partie de cette étude se focalise sur les communautés de bactéries libres fixatrices d'azote de cette même moraine frontale. La fixation biologique de l'azote a été mesurée grâce à la méthode de réduction de l'acétylène. Cette activité s'est révélée être positivement influencée par la présence de la plante. La diversité des bactéries libres fixatrices d'azote et la structure de leur population a été évaluée par l'assemblage de bibliothèques de clones *nifH*. L'analyse phylogénétique a dévoilé 45 phylotypes uniques, largement distribués au sein de la phylogénie *nifH*. La diversité NifH s'est révélée être plus grande que celles trouvées dans d'autres environnements, confirmant ainsi l'hypothèse de l'importance de bactéries libres fixatrices d'azote et de leur potentielle contribution à l'enrichissement en azote des moraines frontales. En contraste aux observations sur les communautés eubactériennes, une diversité des bactéries libres fixatrices d'azote plus petite a été décelée en présence de plantes. Finalement, un grand nombre de séquences NifH ont pu être associé aux bactéries méthanotrophes et aux *Cyanobacteria*.

En se basant sur l'analyse phylogénétique mentionnée ci-dessus, un microarray *nifH* a été conçu pour l'étude des bactéries libres fixatrices d'azote dans les moraines frontales. Un total de 946 sondes (20 nucléotides), créées *in silico*, on été validées expérimentalement en utilisant la plateforme de synthèse in situ Geniom® One. Cet assortiment de sondes couvrant 35 phylotypes NifH sur les 45 trouvés dans la moraine frontale du glacier Damma a délivré des résultats cohérents et précis. Cette approche expérimentale a prouvée être appropriée et de ce fait, est recommandée pour la sélection de sondes. Ce nouvel outil a été appliqué afin d'évaluer l'effet de paramètres environnementaux variables sur les populations libres fixatrices d'azote. La présence de plantes a révélé avoir un impacte plus grand au début de la période de végétation comparé au mois de septembre. Nos résultats ont également montré que la présence de plantes atténue l'impacte de la saison. La déposition d'azote et l'augmentation de la température ont induit des effets plus grands dans le sol rhizospherique que dans le sol simple, probablement dû à leur influence positive sur la croissance de la plante. Cette observation indique une forte carence en carbone dans les jeunes sols périglaciaires. Finalement, les bactéries apparentées au genre *Methylocystis* ont montré une forte réponse aux variations environnementales. Ces bactéries méthanotrophiques capable d'utiliser le méthane atmosphérique comme seule source de carbone semblent être particulièrement adaptées aux conditions nutritiques et climatiques extrêmes observées dans les moraines frontales. On peut de ce fait supposer qu'elles jouent un rôle important dans le maintient de la fixation biologique de l'azote dans ces environnements particulièrement rudes.

1 INTRODUCTION

1.1 Glacier forefield environment

1.1.1 Establishment of the chronosequence

A chronosequence is defined as a sequence of interrelated variables that differ in certain properties as a result of time. Chronosequences situated within the bounds of alpine glacier forefields (Fig. 1-1 A) represent successions of soils differing by their age, texture, chemical and biological compositions. They arose after the retreat of ice, starting in 1850 (Fagan, 2000; Zemp et al., 2006), as a result of the local climate warming. Since then, ice has been retreating apart from an expansion between 1972 and 1992, affecting a fraction of alpine glaciers, as illustrated for the Damma glacier in Fig. 1-1 B. These chronosequences allow the study of biological communities establishment and soil formation. Moreover, due to their high sensitivity, they might represent important indicators of the impact of climate change on local soil development mechanisms.

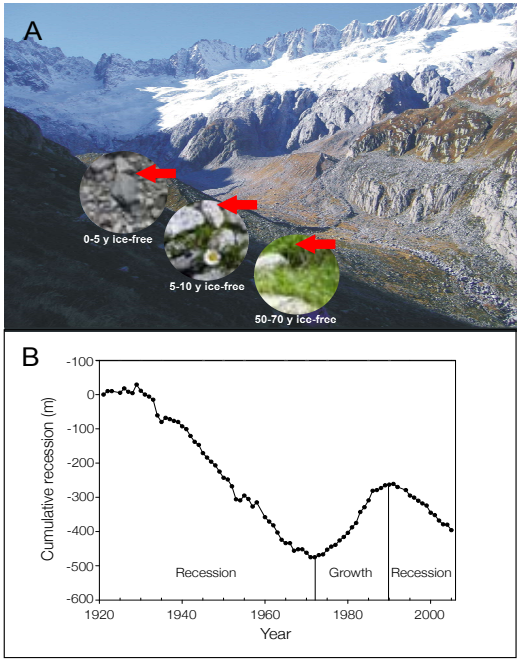


Figure 1-1 A. Picture of the Damma glacier forfield (Swiss Central Alps). B. Cumulative recession of the Damma glacier from 1922 to 2005.

1.1.2 Climatic and nutritional status

Glacier forefields are characterized by harsh climatic and nutritional conditions. The climate encountered in these high altitudinal environments is extreme and highly variable. The rise of solar radiation (Barry, 1992) and light intensity (Landolt, 1992) with increasing altitude leads to day surface-temperatures of up to 40°C at 2100 m (Miniaci, 2007). Additionally, the low air humidity and density (Barry, 1992) allow high thermal radiation during the night and therefore restrain the preservation of heat energy (Landolt, 1992). Precipitations are also irregularly distributed during the year. Northern alpine regions receive the majority of the precipitation during winter time (Brunetti et al., 2006). Nevertheless, climatic studies predict changes in alpine precipitation regime, with less snow expected in winter and heavy rains during spring time (Horton et al., 2006). The actual regime leads to alternation between frequent inundations due to snow melt and heavy rains followed by periods of drought accentuated by solar radiation and winds (Landolt, 1992).

Young glacier forefields are nutrient poor environments constituted mainly of rocks, gravels and sediment. Minerals are released either mechanically by rock fragmentation, frost and erosion, or biologically, through the discharge of organic acid anions (Marschner, 1995) and chelators (e.g., siderophores) (Neilands and Leong, 1986) by plants and soil bacteria, respectively.

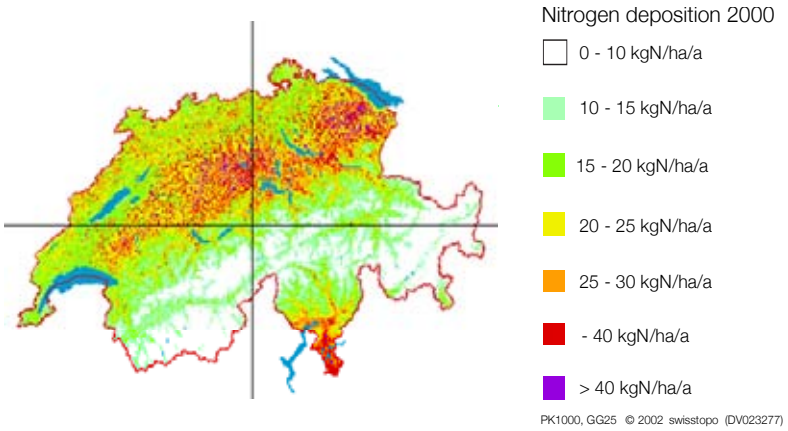


Figure 1-2 Map of nitrogen deposition in Switzerland (2000). Source: Swiss Federal Office of the Environment (www.bafu.admin.ch).

In the early chronosequence, low amounts of nitrogen and carbon compounds are carried and delivered by wind and precipitation (Hodkinson et al., 2002), or are released in spring during the melt of ice and snow (Williams and Melack, 1991; Bowman, 1992). Fig. 1-2 depicts nitrogen deposition status in Switzerland, with more than four times higher concentrations in agricultural areas compared to high alpine regions. Along the chronosequence, total soil nitrogen and carbon content increase due to the development of soil floral, fauna and microbial communities (Sigler et al., 2002).

1.1.3 Colonization of the chronosequence

Glacier forefields are rapidly colonized by microorganisms (Fig. 1-3). Dispersal occurs through wind, animals and glacial water in the case of bacteria present in and on the glacier ice. In contrast to the common statement alleging that primary successions always begin with autotrophy, heterotrophic bacteria were shown to precede autotrophs in glacier forefields (Tscherko et al., 2003a). These bacteria feed on carbon pools present beneath or at the surface of glaciers (Bardgett et al., 2007), as well as on organic material deposited by wind (Hodkinson et al., 2002). Autotrophic communities establish directly after this heterotrophic phase and actively build up organic matter through carbon and nitrogen fixation. This nutrient enrichment constitutes the basis for the colonization by organisms at higher trophic levels.

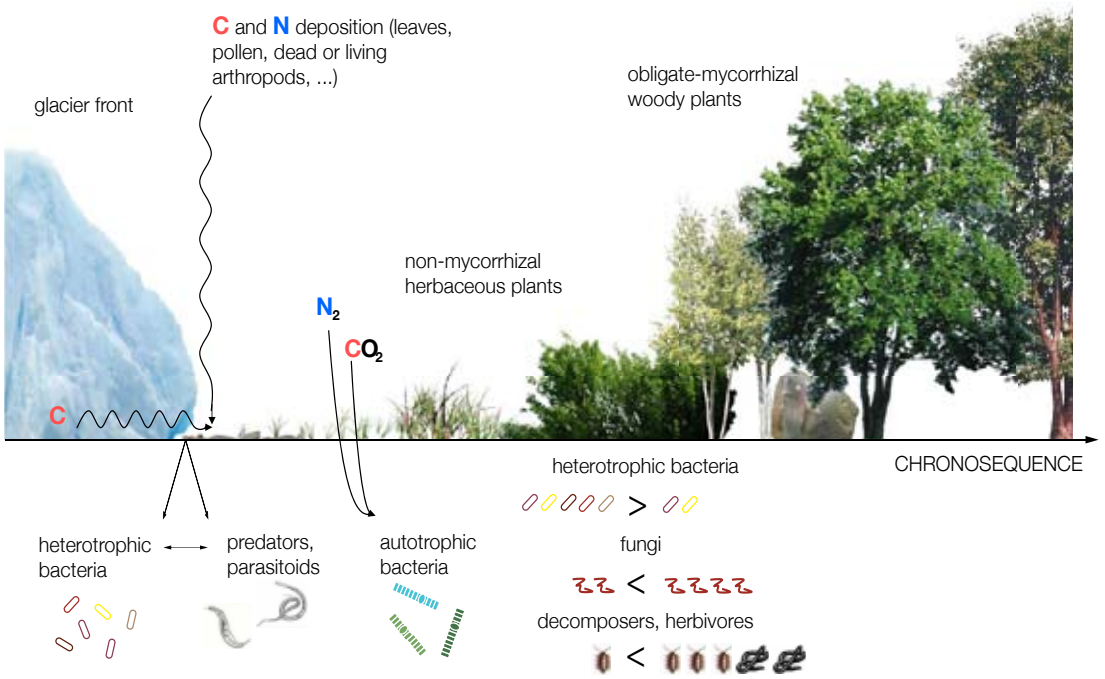


Figure 1-3 Succession of biological colonizers along the chronosequence

Vegetation also changes with age. In the young chronosequence, herbaceous pioneer plant populations are dominated by non-mycorrhizal species, while in the mid-chronosequence facultative-mycorrhizal species become more frequent. Finally, shrubs and trees having obligate requirement for arbuscular mycorrhizal fungi dominate the vegetation in the late chronosequence (Bardgett et al., 2005). This shift directly influences the soil microbial communities. In the early chronosequence, fast growing plants produce high quality litter, hence favoring bacteria and leading to a “fast and leaky” nutrient

cycle. In the late chronosequence slow growers generate a lignin-rich litter promoting a “slow” and highly conservative food web dominated by fungi (Bardgett and Walker, 2004). Regarding faunal communities, predators, parasitoids, and detritivores first appear. They feed on organic material deposition and microbial pools. Herbivores and decomposers arrive afterwards with the development of autotrophic communities.

1.1.4 Seasonal variation

Soil microbial biomass is highly dynamic over the year with a maximum in winter time (Fig. 1-4) (Schadt et al., 2003). In autumn, plant decaying biomass provides high quality carbon and nitrogen sources, and therefore promotes microbial growth. Easily degradable carbon compounds (e. g., carbohydrates) are rapidly used mainly by soil bacteria, explaining their higher proportion over fungi. In winter, microbial biomass still increases (Bardgett et al., 2005). It is dominated by fungi due to the higher proportion of less easily degraded carbon compounds (e. g., low molecular weight phenolic compounds). After snowmelt microbial biomass declines dramatically, releasing nitrogen in the form of protein. This decline might be explained by the depletion of carbon sources, as well as the exposition to highly fluctuating soil temperatures. In early summer, plant growth is sustained by microbial nitrogen. In return, plants release carbon in their root exudates promoting the recovery of microbial growth.

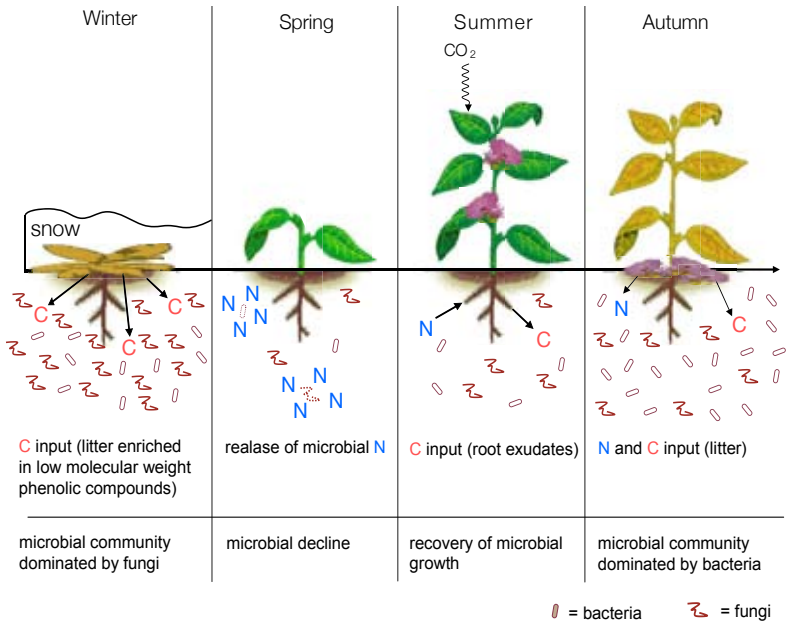


Figure 1-4 Seasonal dynamic of soil microorganisms related to the aboveground vegetation. Adapted from Bardgett et al. (2005).

1.1.5 Soil development and stabilization

Plants and microorganisms are substantially responsible for the weathering of minerals and the mobilization of elements by taking up ions and excreting organic acids and siderophores (Marschner, 1995). Concurrently, they represent the main source of organic nitrogen and carbon in the young glacier forefield, and are therefore key players in soil formation. Carbon accumulation rate has been shown to decrease along the chronosequence reaching a steady state after thousands of years (Bardgett et al., 2005). In the forefield environment, glacial till is rapidly conversed in less than hundred years to loamy sand with a top humic layer. The evolution along the older chronosequence is much slower (Sigler and Zeyer, 2002a). Additionally, plants improve dramatically soil stability through their extended root systems.

The quality of microbial mediated weathering and soil-formation processes is likely to be disturbed by strong climatic variation. Additionally, the climate warming presently observed in the Alps intensifies the glacier-retreat rate and the loss of permafrost area, which results to the exposure of large undeveloped and unstable soil area (Bauder et al., 2008). Both biotic and abiotic changes might increase the number of severe landslides and rockfalls, similar to those observed in the last few years in the Swiss Alps.

1.2 Microbial diversity

1.2.1 Species concept

Biologists always had the wish to find solid rules to decide which organisms are genomically and phenotypically similar enough to be considered part of the same species (Doolittle and Papke, 2006). Indeed, species taxonomic definitions simplified tremendously the communication between people and more specifically between researchers. The most common definition of a species is a group of interbreeding organisms able to produce fertile offspring. However, this definition might be problematic. For example, considering three populations X, Y, and Z, with population Z interbreeding with X and Y, but populations X and Y unable to interbreed, one can discuss if these populations might be considered as one or two species. Therefore, the definition of species is arbitrary and depends largely on the focus of the research.

Nevertheless, this definition can only be used for sexually reproducing organisms and does not fit to microbial populations. Microbiologists generally use the “genetic species” concept, stating that two organisms belong to the same species if they share a high level of genetic similarity, this level being arbitrary (Doolittle and Papke, 2006). Unfortunately, this concept does not simultaneously support genetic and ecological models of bacterial diversification and adaptation (Kassen and Rainey, 2004). Indeed, in microbiology, evolutions of genotype and phenotype do not necessarily correspond. In fact, mutation and selection are not the only driving factors leading to microbial genetic diversity. Lateral gene transfer (LGT), through the acquisition of plasmids, chromosomal genes or mobile genetic islands, rapidly creates subpopulations displaying high genetic similarity but differing in their life style (e. g., acquisition of pathogeneicity) (Doolittle and Papke,

2006). Therefore, strains of the same species may have divergent genome size (gene content, number of gene copies), while sharing high gene sequence similarity. This phenomenon leads to incoherencies in phylogenetic affiliation when considering different marker genes (Fig. 1-5) and indistinct species-cluster boundaries.

It seems that processes influencing microbial genomic coherence are numerous and might act both in a synergistic and an antagonistic way, making difficult to predict their final impact (Kassen and Rainey, 2004). The most convenient way to proceed is to comprehend microbial diversity as phylogenetic relationships within and between species, as often described by phylogenetical trees. Nevertheless, such trees give only a partial insight of microbial diversity and strongly differ depending on the part of the genomes considered to do the phylogenetic analysis.

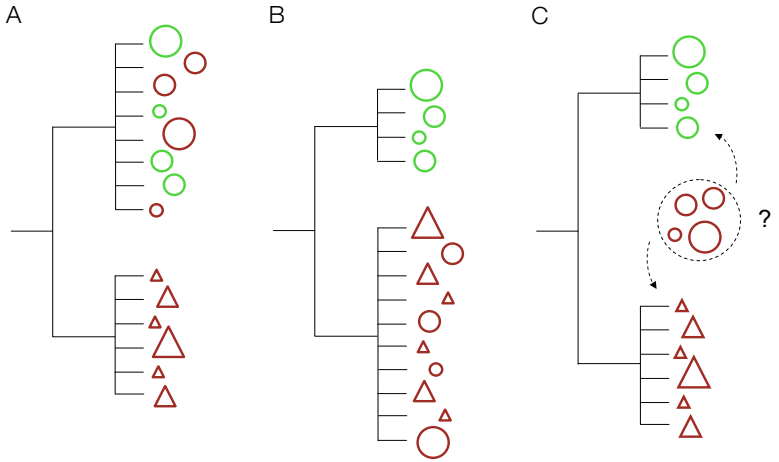


Figure 1-5 Phylogenetic analysis of microbial population considering either affiliation of the gene coding for the form (A), for the color (B) or the entire genome (C). Triangle and circle sizes illustrate the variability of the genome size.

1.2.2 Emergence and role of microbial diversity

Microbial communities are highly diverse. The niche exclusion principle explains such a large diversity (Hardin, 1960). A niche is defined as a unique habitat regarding physico-chemical properties. A niche offers unique conditions of growth and therefore supports only one type of bacteria. Environmental abiotic heterogeneity increases the number of niches. At a microbial scale, the number of different microhabitats is vast and results in a strong diversification of bacteria. Microbial species can also create new niches for other types of bacteria, by modifying the environment through their metabolic activities. Spatial heterogeneity favored the emergence of specialists, while temporal variation was shown to select for generalists (Rainey et al., 2000). The initial invasion of niches, comprehending bacterial diversification or phenotypic in-

novation, is mainly explained by gene duplication and changes in gene regulation rather than in gene structure (Kassen and Rainey, 2004).

Biodiversity significantly influences natural ecosystems. For example, plant diversity has been shown to be positively correlated to the stability and the productivity of ecosystems (Tilman, 2000). In fact, more diverse plant communities maximally exploit spatiotemporal resources and niches, and respond more flexibly to environmental variation. Actually, biodiversity might be considered as one of the major factors influencing the ecosystem functioning, in the same way as species composition, disturbance regime, soil type and climate (Tilman, 2000). Similarly, one can hypothesise that in heterogeneous and variable environments, diverse soil microbial communities would perform better and more evenly than communities showing low diversity. Soil microorganisms play an essential role in organic matter transformation and nutrient fluxes, and thereby strongly influence the soil functioning. The composition of microbial communities has been shown to be sensitive to disturbance and to recover slowly (Allison and Martiny, 2008) (Fig. 1-6). Compositional shifts leaving behind unexploited gaps might strongly affect the soil functioning. Functional redundancy of soil microbial communities might mitigate this phenomenon. Indeed, highly diverse communities showing high rates of redundancy seem to rapidly colonize empty niches and close functional gaps (Ekschmitt and Griffiths, 1998; Nannipieri et al., 2003). Nevertheless, in this field, observations are often contradictory and knowledge about microbial taxa, their function and their differential response to disturbance is poor (Allison and Martiny, 2008). Therefore, the real impact of diversity on the resilience (the capacity to recover after disturbance) and the resistance (the capacity to endure disturbance) of microbial communities, and thereby on soil functioning, is still unclear.

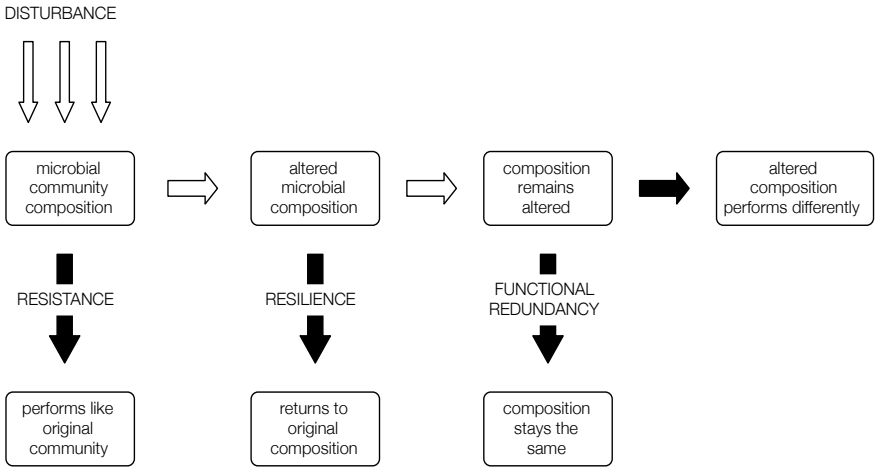


Figure 1-6 Chart illustrating roles of resistance, resilience and functional redundancy in the maintenance of microbial composition and function. Adapted from Allison and Martiny (2008).

1.3 Diazotrophic microorganisms

1.3.1 Mechanism, genetics and regulation of biological nitrogen fixation

Biological nitrogen fixation (BNF) corresponds to the reduction of atmospheric nitrogen (N_2) to biologically available ammonium, as shown by the following stoichiometry:

$$N_2 + 8\,H^+ + 8\,e^- \longrightarrow 2\,NH_3 + H_2$$
$$(16\text{-}24\ ATP \rightsquigarrow 16\text{-}24\ ADP + 16\text{-}24\ Pi)$$

In diazotrophs, prokaryotes that have the ability to fix nitrogen, BNF is catalysed by the nitrogenase complex composed of the Fe protein (dinitrogenase reductase) and the MoFe protein (dinitrogenase). The Fe protein is a 64 kDa dimer encoded by the *nifH* gene. The MoFe protein has a molecular mass of 230 kDa and comprises two heterodimers encoded by *nifD* and *nifK* genes. The Fe protein couples ATP hydrolysis and the transfer of electrons, originating from catabolic processes, to the MoFe protein (Fig. 1-7). The latter contains the substrate-binding site (cofactor FeMoco). This cycle is repeated eight times, in order to transfer enough electrons to fully reduce the FeMo-cobound substrate N_2 (Postgate and Eady, 1988). Two Mo-independent dinitrogenases have been described, in which vanadium (*vnfH* + *vnfDK*) or iron (*anfH* + *anfDK*) replace molybdenum (Smith and Eady, 1992). Enzymes involved in these three alternative systems have similar biochemical properties and requirements: low potential reductants, ATP and anoxic conditions.

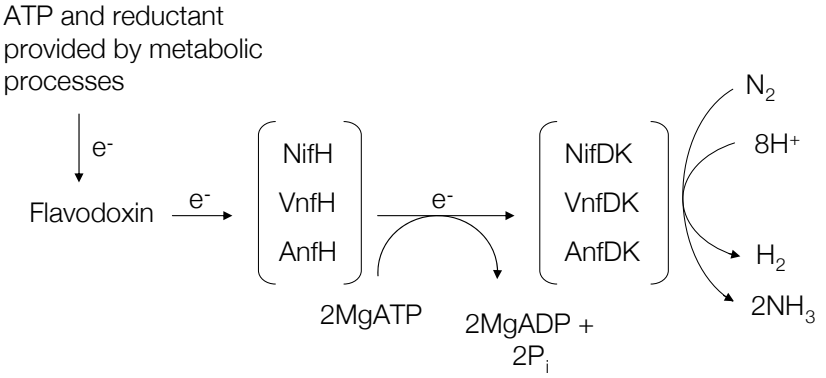


Figure 1-7 Electron transfer during biological nitrogen fixation as found in Klebsiella pneumoniae. A total of eight electrons and at least 16 molecules of ATP are required per N_2 molecule reduced. NifH, VnfH and AnfH correspond to the Fe protein in the molybdenum-, vanadium- and iron-nitrogenase systems. NifDK stand for the MoFe protein in the molybdenum-nitrogenase system, while VnfDK and AnfDK correspond to analogous proteins in the vanadium- and the iron-nitrogenase systems.

With 16 ATP used to reduce one molecule of N_2, BNF is an energy-demanding process. The multiple regulation strategies attest of the necessity to control such a costly process (Halbleib and Ludden, 2000).Expression of the *nif* genes is activated by the regulator protein NifA. Despite the conserved mode of action of this protein, its regulation is diverse among diazotrophs and might occur at the transcriptional or post-translational levels. In *Klebsiella pneumoniae*, the synthesis of NifA is activated by the Ntr system (system controlling the global nitrogen regulation in bacteria) in response to a low cellular nitrogen status (Fig. 1-8) (Arcondeguy et al., 2001). The product of the co-transcripted *nifL* gene modulates the activity of NifA. Under high oxygen or high ammonium status, the oxidized form of the NifL flavoprotein binds to NifA and inhibits its activity. The reduction of NifL and the relief of the inhibition depend on the respiratory activity and the availability of reducing equivalents (Halbleib and Ludden, 2000). In *Rhizobium meliloti*, *nifA* expression is controlled by an oxygen sensitive two-component regulatory system involving *fixL* and *fixJ* (Halbleib and Ludden, 2000). Finally, several species (e. g., *Rhodospirillum rubrum*, *Rhodobacter capsulatus*, *Azospirillum brasiliense*) present a post-translational regulation system able to rapidly and reversibly inactivate the nitrogenase complex under energy-limiting or nitrogen-sufficient conditions, through ADB-ribosylation of the Fe protein (Halbleib and Ludden, 2000).

Strategies against oxygen threat are multiple. For example, *Azotobacter vinelandii* is able to lower the ambient oxygen concentration through an intensive oxidative metabolism, while *Azospirillum* builds up a barrier of extracellular polysaccharides to prevent the entrance of oxygen. Further, *Gluconobacter diazatrophicus* produces a protective protein inferring oxygen tolerance to the nitrogenase (Marchal and Vanderleyden, 2000).

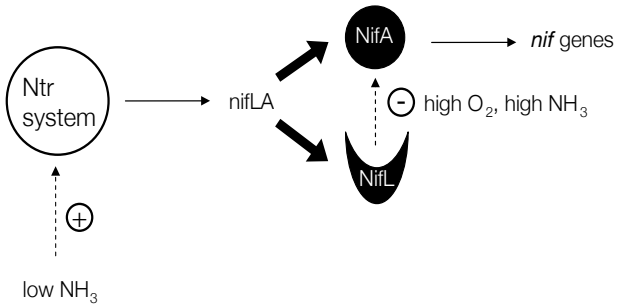


Figure 1-8 Transcriptional regulation of nif genes in Klebsiella pneumoniae.

In total, BNF involves around 20 genes and 20 kb of DNA (Fig. 1-9). The *nifHDK* operon constitutes a good marker for the study of diazotrophs as it is expressed and regulated in response to environmental parameters controlling BNF. Despite that the nitrogenase system can be found in three different forms, previous diversity studies were mostly focused on the molybdenum-dinitrogenase reductase and its encoding gene (*nifH*).

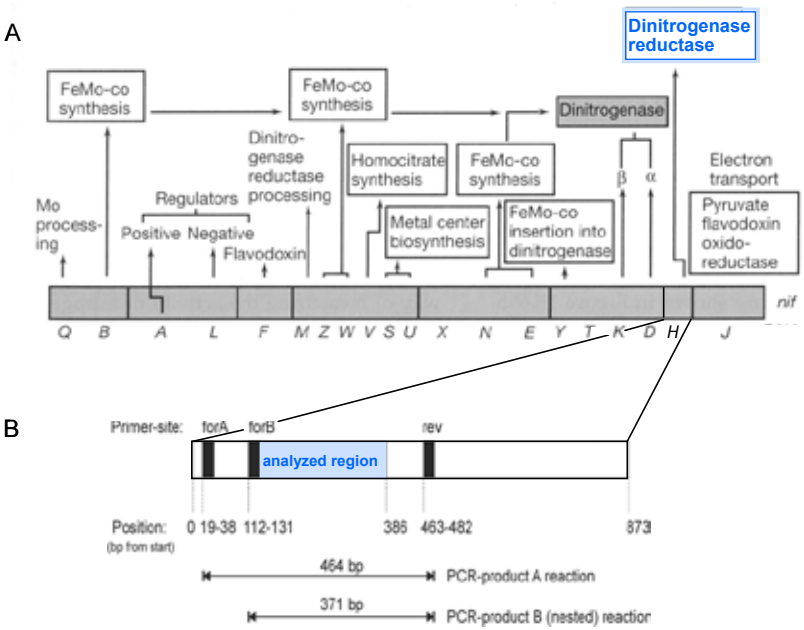


Figure 1-9 Physical map of the nif gene cluster in the genome of Klebsiella pneumoniae and functions of the respective products (A), and amplification strategy of the nifH gene (B). Adapted from Madigan and Martinko (2006).

1.3.2 Phylogeny and ecology

The ability to fix nitrogen is broadly but sporadically distributed amongst the prokaryotic microbial world and does not correspond to any phylogenetic affiliation. Nitrogenase genes are found in both *Archaea* and *Bacteria* clades, suggesting an early origin or a lateral gene transfer. Nevertheless, the consistency between phylogenies based on 16S rRNA genes and the *nifH* gene gives no strong support for lateral gene transfer (Zehr et al., 2003a). Gene duplication events most probably led to the two Mo-independent nitrogenases. Other similar events might explain the few divergences found in the *nifH* phylogeny. To better resolve the phylogeny of diazotrophs, *nifD* and *nifK* phylogenies should be combined with the analysis of the *nifH* gene.

Diazotrophs are characterized by a high metabolic versatility. They might be obligate anaerobes (e. g., *Clostridium*, *Desulfovibrio*), facultative anaerobes (e. g., *Bacillus*, *Vibrio*) or aerobes (e. g., *Azotobacter*, *Herbaspirillum*), but necessarily need anaerobic or microaerobic conditions to fix nitrogen (Postgate and Eady, 1988). Some species have the ability to form a symbiosis with plants and are located in root or stem nodules (e. g., *Rhizobium*, *Frankia*). These diazotrophs play an important role in agriculture through their symbioses with legumes. Leguminous plants produce 20% of the protein

consumed worldwide. Their symbiosis with nitrogen-fixing rhizobia can partially replace the need of fertilizers (Burris and Roberts, 1993b).

Free-living diazotrophs are known to perform oxygenic and anoxygenic photosynthesis, and might also display chemo-heterotrophic and –lithotrophic metabolisms. They have been observed within a wide range of temperatures in diverse habitats like invertebrates (e. g., *Desulfovibrio gigas*), acquatic environments (e. g., *Nodularia spumigena*), marshes (e. g., *Microcoleus chtonoplastes*), stromatolites (e. g., *Lyngbya lagerhemmii*), plants (e. g., *Rhizobium leguminasorum*), and soils (e. g., *Pseudomonas fluorescens*) (Postgate and Eady, 1988).

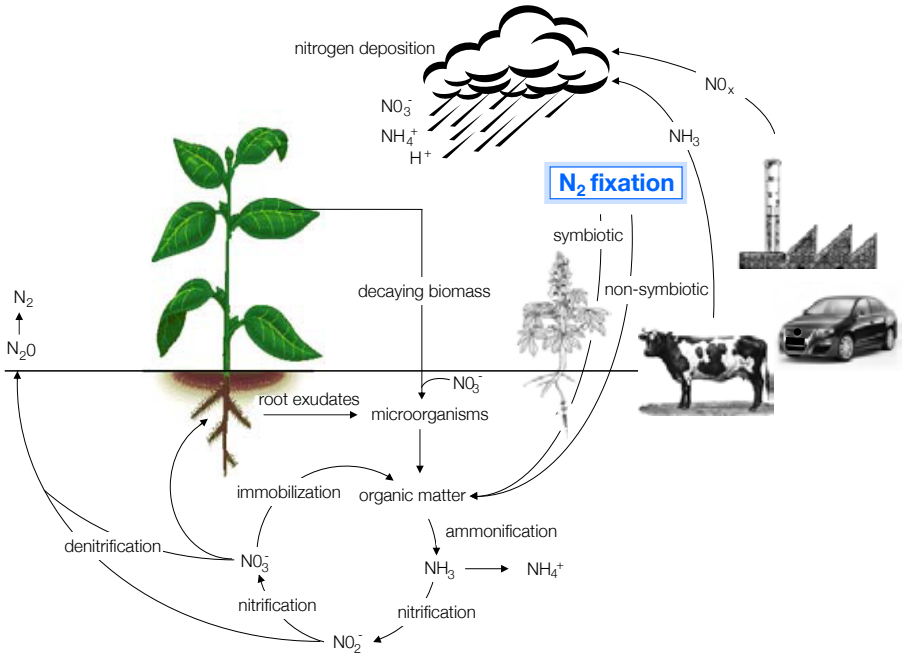


Figure 1-10 Chart of the soil nitrogen cycle processes.

Except for photoautotrophic *Cyanobacteria*, free-living diazotrophs only fix nitrogen in presence of a suitable carbon source. In the soil, the plant rhizosphere constitutes a favorable habitat for diazotrophs. Via the uptake of nitrates, plants lower the soil nitrogen pool and they simultaneously provide carbon sources through their root exudates and their decaying biomass (Fig. 1-10). Diazotrophic rhizobacteria feed on these carbon sources and in turn stimulate plant growth through the enhancement of available soil nitrogen. They may induce additional plant growth by synthesizing phytohormones (e. g. auxin, cytokinine- and gibberelin-like coumpounds), increasing nutrient uptake by the plant, enhancing plant stress resistance and in some cases by acting as biocontrol agents (Dobbelaere et al., 2003).

1.4 Methods for the study of microbial diversity

A first category comprises the so called fingerprinting methods. These technologies do not allow to determine precisely species present in a sample but generally give valuable global information on changes in microbial population structure, dynamics and diversity. These techniques are well suited for comparison-based studies. They are often favored because of their rapidity to deliver results and their high throughput. The most frequently used fingerprinting methods are listed bellow:

–Denaturing gradient gel electrophoresis (DGGE): Differentiation of similarly sized DNA fragments according to their differential melting properties under increasingly denaturing conditions (usually increasing formamide/urea concentrations). Each species gives rise to one band (Muyzer et al., 1993; Muyzer and Smalla, 1998).

–Restriction fragment length polymorphisms (RFLP): Separation of DNA fragments based on their size after their cleavage with restriction enzymes. Organisms or samples are differentiated on the basis of their banding patterns (Poly et al., 2001a).

–Terminal restriction fragment length polymorphism (T-RFLP): Separation of 5'-end-labeled DNA fragments through a capillary electrophoresis based on their size after their cleavage with restriction enzymes. In general, each organism of a community contributes to one terminal fragment of a distinct length (Osborn et al., 2000).

–Phospholipid fatty acid analysis (PLFA): Analysis of extracted, fractionated and methylated PLFAs through capillary gas-chromatography. Some PLFA might be assigned to specific phylogenetic groups. Only viable cells are detected (Frostegard and Baath, 1996; Zelles, 1999).

The cloning-sequencing methodology constitutes the second category. It comprehends: i) the cloning of PCR products in competent *Escherichia coli* cells to isolate and amplify different DNA sequences, and ii) the sequencing of these unique DNA segments. This method is a very sensitive approach with a high resolution to investigate in detail environmental samples. Naturally, its sensitivity depends directly on the number of clones analyzed. It delivers strong results at the species level, but is highly time consuming. With the actual development of new sequencing facilities allowing metagenomic analysis, with less time and cost, the cloning-sequencing approach seems to be one of the most promising tools to study microbial diversity.

The last category includes hybridization methods:

–Fluorescence *In Situ* Hybridisation (FISH): Hybridization of fluorescent probes to fixed microbial cells in order to detect specifically phylogenetic groups. Labeled cells are viewed under the epifluorescence microscope. This method allows to

study spatial arrangement and to quantify microbial population (Amann et al., 2001).

–Oligonucleotide microarray: Hybridization of fluorescently labeled DNA to nucleic acid probes, chemically attached to a substrate (Bodrossy and Sessitsch, 2004).

Both methods enable to characterize the composition and the function of microbial communities at the species level. They are relatively complex and require extensive initial phylogenetic work during the development phase. Additionally, the microarray technology presents a throughput comparable to that of fingerprinting methods.

1.5 Oligonucleotide microarrays

1.5.1 General concept

The microarray technology allows the parallel detection and quantification of multiple nucleic acid molecules with a high throughput. It was first developed in medical research to perform whole genome gene expression analysis. In clinical microbiology, it has been used to detect one or a few microorganisms out of many, at the level of strains, species, genera or even clades. This technology also presents a great potential in environmental studies, allowing to grasp the high spatiotemporal versatility of microbial communities, and therefore to study in detail their phylogeny and functions. In combination with physico-chemical data, microarray monitoring of biogeochemical processes enables a better understanding of soil ecosystems.

Environmental microbial diagnostic microarrays are divided into three types. Phylogenetic oligonucleotide microarrays are typically based on the 16S rRNA phylogenetic marker gene. The high conservation degree of the 16s rRNA gene limits the resolution above the species level. Such microarrays have been used to characterize phyla (e.g., *Alphaproteobacteria* (Sanguin et al., 2006)), processes (e. g., sulphate reduction (Loy et al., 2002)) or ecosystems (e. g., compost (Franke-Whittle et al., 2005)). Other highly conserved housekeeping marker genes (e. g., *rpoβ*, *recA*, *gyrB*) might alternatively be used. These genes offer a higher resolution but their respective sequence databases are poor compared to that of the 16s rRNA gene.

Functional gene arrays target genes with a specific function (e. g., *pmoA*, methane oxidation (Bodrossy et al., 2003b)) and generally display a better resolution than phylogenetic oligonucleotide microarrays. PCR-amplified gene fragments are usually used as targets, thus decreasing the complexity of hybridized DNA samples and allowing the detection of minor species. However, this initial PCR amplification has been shown to alter the original proportion of DNA sequences (Polz and Cavanaugh, 1998).

Both types of microarrays can be built with short (15-30 nts) or long (40-70 nts) oligonucleotide probes. Short probes are more specific allowing the discrimination of species, whereas long probes are less specific but more sensitive. This increased sensitivity may allow to avoid target amplification prior to hybridization.

Finally, community genome arrays use entire fragmented genomes as probes. They work without prior PCR amplification but are only useful for cultivated bacteria. These arrays are commonly used to study gene expression and regulation, and the profiling of new genes.

The general work flow of environmental microbial diagnostic microarrays is illustrated in Fig. 1-11. Shortly, microbial gene sequences, retrieved from the environment of interest, are merged in genetic databases containing all known sequences of the particular gene. The sequences are aligned and compared allowing the *in silico* design of probes specifically targeting defined groups of sequences. Probes are then spotted or synthesized on the microarray substrate, and their quality (specificity and sensitivity) is experimentally tested by the hybridization of labeled specific DNA targets. Once the probes have been successfully validated the microarray can be used to detect, in environmental DNA extracts, sequences similar to the genetic groups defined *in silico*.

1.5.2 Technical aspect

Microarray substrates are generally made of glass, silicon or plastic. Two main type of immobilisation can be differentiated. The so-called spotted microarrays are constituted of pre-synthesized oligonucleotides, which bound covalently on the coating layer of planar surfaces. Oligonucleotides can be deposited by contact printing involving pins or microstamps (Barbulovic-Nad et al., 2006), or sprayed at the surface using piezoelectric inkjet dispenser systems. The uniformity of oligonucleotide spots is one of the main limitations of this type of immobilization. Nevertheless, it is essential for the reproducibility and the robustness of the technology, as spots composed of too dense negatively charged oligonucleotides have been shown to repulse labeled targets (Gao et al., 2004).

The second type is the *in situ* synthesis of oligonucleotides on microarray substrates (Fig. 1-12). One of the mainly used techniques is the photolithography. Here the substrate is covalently modified to provide an initial synthesis site constituted of hydroaxylalkyl groups. Linkers presenting photolabile protecting groups (PLPG) are first deposited. Specific regions are excited with UV light using masks or micromirrors to remove PLPGs. Reactive 5'-ends can then bind the next 5'-end protected nucleophosphoramidite monomers (A, T, C or G). Successive rounds of protection and deprotection lead to the extension of oligonucleotide probes (Gao et al., 2004).

Alternatives to this technique are, for example, the electrochemical deprotection of nucleophosphoramidite, or the *in situ* synthesis using printing technologies involving piezoelectric inkjet and masks (Gao et al., 2004).

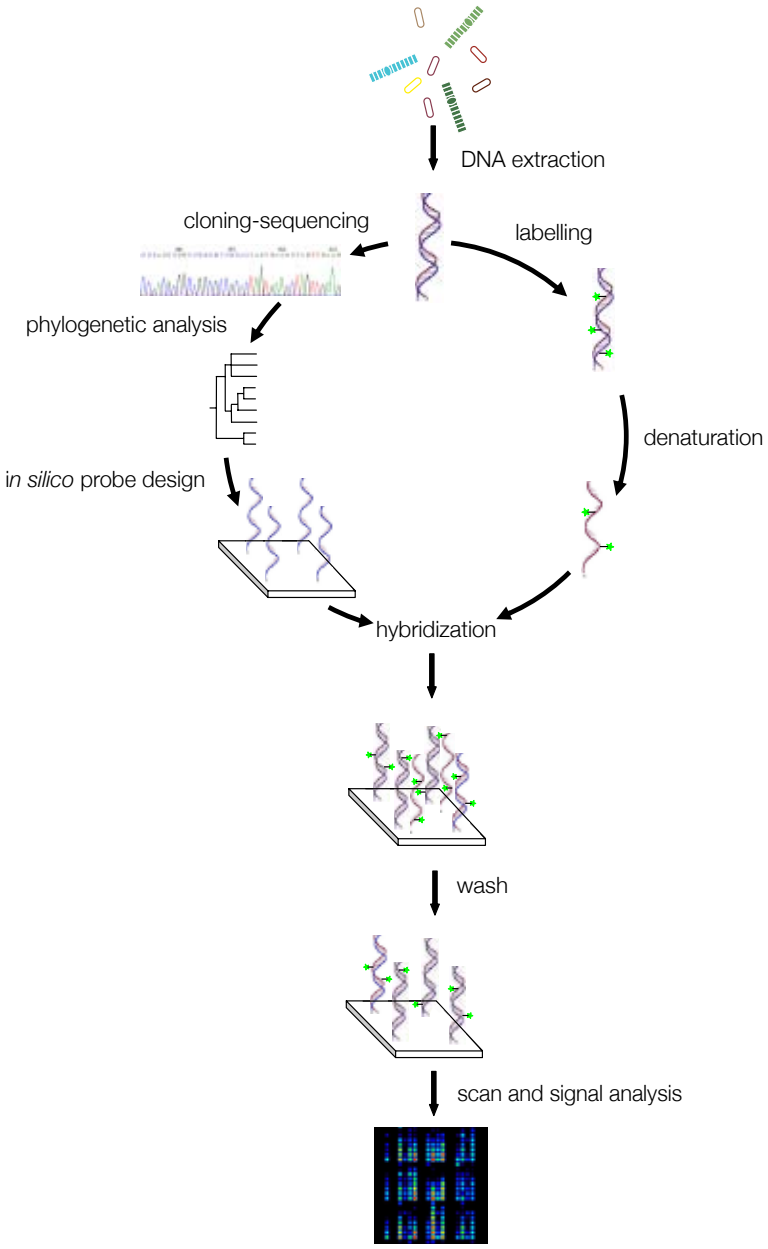


Figure 1-11 Experimental approach of environmental microbial diagnostic microarrays.

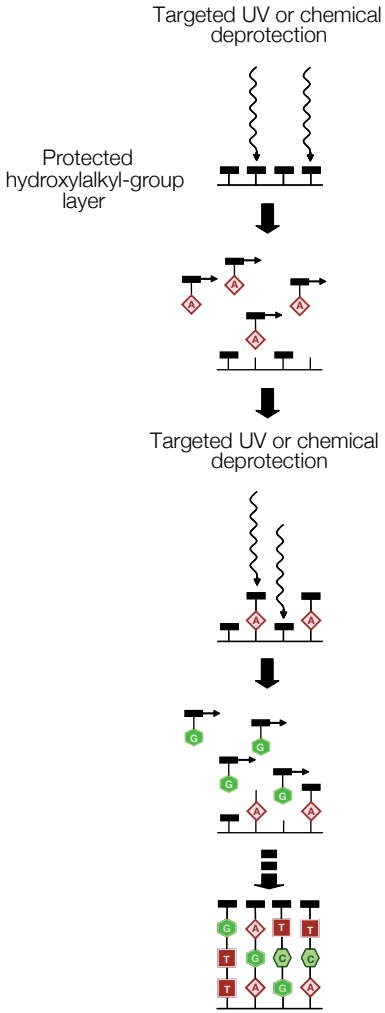


Figure 1-12 Oligonucleotide microarray fabrication with in situ synthesis.

The *in situ* synthesis technology has the advantage to be a standardized and automated process and therefore guarantees a better regularity and quality of oligonucleotide spots. Common substrates are two (e.g., planar glass slide) or three dimensional (e. g., microchannels, beads) (Verpoorte, 2003). The latter enhance the surface and decrease interactions between probes and substrate (steric hindrance).

The hybridization is generally fulfilled by static or dynamic incubation of labeled DNA targets at the microarray surface. A better controlled hybridization can be performed using microelectronic arrays (Heller et al., 1999). This method produces reconfigurable electric fields on the microarray surface allowing the transport of labeled DNA molecules to any test sites. The hybridization efficiency is enhanced by increasing the concentration of DNA target at the test site. Similarly, the introduction of spacers between the microarray surface and the beginning of the probe's sequences lowers the steric hindrance and consequently improves the hybridization (Halperin et al., 2006).

Detection approaches are also multiple. Fluorescent dyes (e. g., Cy3 and Cy5) are usually used to label DNA targets. The latter are then detected by conventional scanning fluorescence microscopy, total internal reflection technique (excitation of fluorescent dyes by an evanescent field) or fiber-optic sensor (immobilization of probes at the end of optical fibers) (Bally et al., 2006). Label-free detection through surface plasmon resonance imaging represents also an interesting technique. It permits the reuse of the microarray and avoids disadvantages of labeling, e. g. high cost, time consuming and biases due to photobleaching.

1.5.3 Computational aspect

In parallel to technical considerations, probe and experimental designs, as well as signal analysis constitute challenging procedures. The design of specific and sensitive probes displaying similar thermodynamic behavior is conflictual. It might be optimized by modifying the length of probes or by using a hybridization buffer containing tertiary amine salts. Nevertheless, in most cases optimal hybridization and washing conditions remains specific to probe sequences. Predictors of the thermodynamic behavior of probes are commonly used in microarray studies, even if none of them is able to completely predict the specificity of hybridization (Pozhitkov et al., 2006). Much effort is still needed to better understand immobilization, steric hindrance and hybridization processes in order to improve the reliability of *in silico* design and predictions. Other current strategies which ameliorate the reliability of microarray analyses are the use of multiple probes targeting unique phylogenetical groups (Astrand et al., 2008), hierarchical design (Loy et al., 2002), multiple marker genes or mismatch controls, where unspecific probe signals are used to normalize specific signals (Ferrantini and Carlon, 2008).

In microarray studies, great care has to be given to the conception of the experimental design (Churchill, 2002). Biological replicates are essential to reduce biological and technical variability resulting from target preparation. Due to the high costs of microarray technology, replicates are frequently pooled just before the hybridization. Moreover, signal intensities have to be normalized to control extraneous factors. Normalization with positive controls (probes binding all sequences susceptible to be detected) eliminates bias resulting from target characteristics (e.g., secondary structure) and minimize variability between microarrays, whereas normalization with negative controls (probes not binding any target susceptible to be detected) or background intensities allows to minimize variability within a single microarray (Schadt et al., 2005).

The large amount of data involved in microarray experiments requires solid bioinformatics tools. Software like Cluster 3.0 (Human genome Center, University of Tokyo, Japan) and Array Designer (TeleChem International Inc., Sunnyvale, USA) are commonly used to design specific probes for gene expression medical studies. These software work with alignment containing a maximum of 200 sequences. They are therefore not appropriate to develop probes targeting specifically microorganisms in environmental studies. In the latter, the ARB software package (Ludwig et al., 2004) is popular as it allows the design of specific probes using alignments containing several thousands of sequences. A second type of software is needed for image analysis (e. g., ArrayPro 4.5, Media Cybernetics Bethesda, USA; GenPix Pro, Axon, Foster City, USA). Finally, a multitude of different software might be used to achieve data analysis in gene expression studies (e. g., GEPAS (Tarraga et al., 2008), EXPANDER (Shamir et al., 2005), ImaGene, Redwood, USA). In contrast, only four algorithms for significance-based microorganisms detection in environmental diagnostic microarray experiments can be found in the literature (Urisman et al., 2005; Watson et al., 2007; Wong et al., 2007; Rehrauer et al., 2008).

1.6 Outline of the thesis

Through their key role in nutrient cycling, microorganisms present in glacier forefield environments may have important effects on soil development and plant establishment. Nitrogen is a limiting nutrient at least in certain microenvironments of the young glacier forefield, e.g. in the rhizosphere of pioneer plants where additional carbon is made available through root exudation. Under such conditions, free-living diazotrophs might be highly competitive and strongly influence nitrogen accumulation in young glacier forefields. Factors that control nitrogen fixation activity as well as the composition and the diversity of free-living diazotrophic populations in glacier forefield soils are currently unknown. The primary goal of this research was to enlarge our knowledge of such populations and particularly to investigate their response to plant presence.

In chapter 2, the impact of pioneer plant presence on biomass, functions and diversity of microbial populations in young Damma glacier forefield soils was investigated.

Chapter 3 focuses on the study of free-living diazotrophic populations at two time points along the chronosequence, in presence or absence of pioneer plants. This study required the construction of *nifH* gene clone libraries for the four types of soils. Diversity and distribution of translated NifH sequences were then assessed through phylogenetical analysis using the ARB software (Ludwig et al., 2004).

This analysis constitutes the basis for the development of the *nifH* microarray described in chapter 4. This tool was specially designed to study free-living diazotrophs in glacier forefield environments. Specific probes, targeting NifH phylotypes defined in chapter 3, were designed using ARB and tested on the Geniom® One microarray platform. The consistency of the *nifH* microarray was tested by comparing hybridization results with those obtained on a second microarray platform. Its accuracy was assessed through a comparison with cloning-sequencing experiments.

Chapter 5 describes the application of the *nifH* microarray to determine impacts of environmental variation on free-living diazotrophic population structure.

2 EFFECTS OF PIONEERING PLANTS ON MICROBIAL STRUCTURES AND FUNCTIONS IN A GLACIER FOREFIELD

Miniaci C., Bunge M., Duc L., Edwards I., Bürgmann H., Zeyer J.

Biology and Fertility of Soils (2007) 44(2): 289-297.

2.1 Abstract

This study investigates the small-scale spatial impact of the pioneering plant *Leucanthemopsis alpina* (L.) Heywood (*L. alpina*) on biological and chemical–physical parameters in an early successional stage of a glacier forefield. Considering the frequent occurrence of isolated patches of this pioneer plant in the forefield of the Damma glacier (Switzerland), we hypothesized that the impact of the plant would establish gradients in nutrients, and microbial community structure and activity that may be of importance for the successional processes occurring in the forefield. Our results indicated that, in young successional soils, the rhizosphere effect of *L. alpina* plant patches can influence bacterial cell numbers and activities not only within the root zone, but even at 20 cm distance from the plant. Microbial cell counts, active cells, and saccharase, glucosidase, and acid phosphatase activities revealed significant distance effects, decreasing from soil directly underneath the plant to soils at 20 and 40 cm distance. Soil chemical and physical parameters did not exhibit significant trends. Fingerprinting analysis of amplified 16S rDNA fragments was used to characterize the microbial community. A selective effect of the plant on the microbial community could not be shown because the bacterial communities were similar regardless of distance to the plant.

2.2 Introduction

The majority of Swiss glaciers are currently receding as a consequence of global warming (Haeberli and Beniston, 1998). Since 1850, around the end of the little Ice Age, European glaciers lost about 50% of their original glaciated area (Zemp et al., 2006). The forefield of receding glaciers is initially vegetation-free, with low nutrient content (Sigler and Zeyer, 2002b; Darmody et al., 2005). In a continuous glacier retreat, greater distance from the termini corresponds to longer periods of ice-free exposure, resulting in a successional chronosequence (Matthews, 1992).

Whereas the succession of flora and fauna has been extensively studied in this system (Matthews, 1992; Chapin et al., 1994; Kaufmann, 2001; Caccianiga and Andreis, 2004; Hodkinson et al., 2004), few studies addressed bacterial processes in glacier forefields (Ohtonen et al., 1999; Sigler and Zeyer, 2002b; Bardgett and Walker, 2004; Tscherko et al., 2004). Sigler and Zeyer (2002b), using molecular methods, reported that community structures and metabolic functions change along the chronosequence of a glacier forefield. Tscherko et al. (2003b) demonstrated that microbial functional diversity increased along a glacier forefield chronosequence.

As plant-cover is one of the most obvious gradients in a glacier forefield, microbial activity is likely to vary with vegetation. Plants translocate between 5 and 60% of their photosynthetically fixed carbon into the rhizosphere, which may stimulate microbial communities and their associated enzymatic activities (Marschner, 1995; Walker et al., 2003). However, along a glacier forefield chronosequence, the interaction between microorganisms and vegetation might differ at the various successional stages. Tscherko et al. (2004) investigated microbial community structure and enzymatic activities in the rhizosphere and bulk soil of *Poa alpina* plants at various successional stages of a glacier forefield. In an additional study, Tscherko et al. (2005) investigated the interrelationship of different successional plant communities with microbial diversity and enzymatic activity patterns, providing evidence that microbial community structure and enzymatic activity patterns are strongly conditioned by the successional stage, as well as the carbon and nitrogen content of the forefield soils. The relationship between chronosequence and microbial community structure in bulk and rhizospheric soils of the pioneering plant *L. alpina* was investigated by Edwards et al. (2006). A major focus of this study was the changing patterns of rhizodeposits (in the form of organic acids and sugars) in the *L. alpina* rhizosphere and how they can condition the structure of microbial communities, as well as their ecological strategies.

Patchy vegetation is a common observation in early successional stages. A question not addressed by the previous literature is the spatial extent of plant microbial interaction in these environments. We hypothesized that pioneer plants provide hot spots of microbial activity that extend into the surrounding bulk soil, leading to gradients in soil chemistry, microbial activity, and microbial community structure. To address this question we chose *L. alpina* because it is one of the earliest and most abundant vascular plant pioneers in the young Damma glacier forefield soils and because there are previous studies on the plant–microbial interactions for this plant (Edwards et al., 2006). The current work therefore focuses on resolving the spatial effect of *L. alpina* on microbial cell counts, community structure, and enzyme activities

in an early successional soil. We applied chemical–physical measurements (e.g., pH, dissolved carbon, and nitrogen parameters) to describe the conditions for the microbial community. Microbiological (microscopic counts of total and active cells) and molecular [fingerprinting of bacterial 16S rRNA genes using restriction fragment length polymorphisms (RFLP) and denaturing gradient gel electrophoresis (DGGE)] methods describe the size and structure of the microbial community, respectively, and enzymatic activity assays (saccharase, glucosidase, phosphatase, and urease) were selected to reflect major microbial processes related to nutrient cycling and energy metabolism. In combination, these data provide detailed information about chemical and biological gradients around isolated patches of *L. alpina* plants.

2.3 Material and methods

2.3.1 Field site and sample collection

The research site at the terminus of the Damma glacier is located in the Central Alps, in the canton of Uri, Switzerland (N 46°38.177' E 008°27.677'), about 2100 m above sea level. The climate in this area is characterized by a short vegetation period, and about 2400 mm precipitation per year (Sigler and Zeyer, 2002b). Between July and October 2003, we noted very large day–night temperature fluctuations, the observed maximum and minimum soil surface temperatures ranged between 38 and 0°C. At the research plot, soil conditions varied between fine fluvial sands and coarse sandy areas.

Sampling took place shortly after snow melt in May 2003. Plants were collected from three isolated patches of *L. alpina* that were situated about 60 m distant from the glacier terminus, corresponding to a deglaciation time of 5 to 10 years. *L. alpina* occurred at a frequency of about 1000 individuals per hectare at this location. The whole plants including roots were collected for determination of root biomass. The soil attached to the roots together with soil from within 10 cm of the plants was collected and arbitrarily considered as plant center soil. In addition, samples were taken along two transects from two further distance classes, at 20 cm and at 40 cm distance from the *L. alpina* plants. For each distance class, up to four samples were collected from the 0–5 cm soil layer; larger rocks prevented sampling in some cases. The samples were sieved (2 mm) and homogenized, and roots were manually separated from soil samples. Subsamples were dried at 105°C for 24 h and subsequently weighed for dry mass determination of root material and soil.

2.3.2 Analysis

Soil chemical properties were analyzed by extracting field moist soil samples with 0.01 M $CaCl_2$ (ratio 1:5 of soil/extractant). Samples were shaken for 1 h in an overhead shaker. Nitrate, phosphate, total sugars, and organic acids were determined in $CaCl_2$ extracts. Ion chromatography with suppressed conductivity detection (DX-100, Dionex, Sunnyvale, CA, USA) was used to measure nitrate, phosphate, lactate, acetate, propionate, formate, butyrate, pyruvate, oxalate, and citrate in their anionic forms (Edwards et al., 2006). Total soluble sugars were determined by acid hydrolysis as glucose

equivalents (Chabrerie et al., 2003). Available NH_4^+ was measured colorimetrically by extracting soil samples with 2 M KCl (ratio 1:5 of soil/extractant) shaken for 1 h at room temperature (Mulvaney, 1996). Soil pH was measured (MP 225, Mettler-Toledo, Greifensee, Switzerland) by diluting 5 g of air-dried soil in 15 ml 0.01 M $CaCl_2$ solution. Total organic C (TOC) and total N were determined by combustion of finely ground air-dried soil samples using a LECO 932 CHNS device (Leco, Krefeld, Germany).

Bacterial cells were fixed in field moist soil samples (1 g) with 4% paraformaldehyde in phosphate-buffered saline solution (pH 7). Total bacterial cell numbers were determined after 4′6-diamidino-2-phenylindole (DAPI) staining using an epifluorescence microscope (Zarda et al., 1997). Active bacterial cells were counted after fluorescein diacetate (FDA) hydrolysis by bacterial cells based on the method cited by Alef et al. (1995) with minor changes. Briefly, field moist soil samples (5 g) were diluted in 5 ml of 60 mM phosphate buffer (pH 7.6) and shaken with 1.2 g of sterile glass beads (0.5 mm) for 2 h. This suspension was further diluted (1:10) with sterile distilled water, and FDA was added to a final concentration of 10 µg ml^{-1}. The diluted and FDA-stained samples were shaken (2 min) and centrifuged (2 min; 10000×g). The supernatant was removed and excess FDA was washed off by flushing with 60 mM phosphate buffer. Cells were resuspended in phosphate buffer (1 ml), and 10 µl of the supernatant was dried on microscopic slides for 10 min at 37°C. Active fluorescent bacterial cells were determined microscopically by counting 10 fields per slide and sample.

DNA was extracted from triplicate field moist soil samples (0.7 g each), using the bead-beating method previously described by Sigler et al. (Sigler et al., 2002) and pooling triplicate extracts for subsequent PCR amplification. For analysis of RFLP, 16S rRNA gene fragments were amplified using oligonucleotides targeting bacterial sequences using the EUB 338 (5′-ACT CCT ACG GGA GGC AGC-3′)/uni-b-rev (5′-GAC GGG CGG TGT GTR CAA-3′) primerset (Amann et al., 1995). PCR products were purified with equal volumes of chloroform and reprecipitated with isopropanol. Subsequently, the PCR products (2 µl) were digested with HaeIII (2U; Promega, Madison, WI, USA) during overnight incubation at 37°C. RFLP gels (12% Bis-/acrylamide) were loaded with 3 µl of the digested PCR products that were separated for 3.5 h at 200 V. For RFLP analysis, the DCode Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA) was used, the running buffer was 1×TAE buffer (0.04 M Tris base, 0.02 M of sodium acetate, and 1.0 mM EDTA, pH 7.4) at 35°C. The gel was stained for 30 min with GelStar® (Cambrex Bioscience, Baltimore, MA, USA), 1:10000 in 1×TAE buffer. The restriction patterns were photographed under UV light using the GelDoc 2000 system and Quantity One® software (Bio-Rad). After using a rolling disk algorithm for background correction, band intensities and relative band position were determined. Digital image data were exported to the statistical software SPSS 11.0. Hierarchical cluster analysis was performed using the Ward's method in SPSS.

We obtained PCR products for DGGE using the primers F 968-GC (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3′) and R-1401 (5′-CGG TGT GTA CAA GAC CC-3′) as described by Nübel et al. (Nübel et al., 1996). Each PCR reaction contained 1×PCR buffer, 2 mM MgCl, 0.2 mM of each deoxyribo-

nucleotide triphosphate, 0.5 μM of each primer, 0.2 mg ml^{-1} BSA, 1 U of Taq polymerase (Invitrogen, Carlsbad, CA, USA), and 1 ng of template DNA. PCR products were screened on a 1% agarose gel. Equal amounts of PCR products were electrophoresed on a 8% polyacrylamide denaturing gel (35–60% denaturant gradient consisting of urea and formamide as defined by Muyzer et al. (1993). Gels were run in 1 × TAE at 60°C for 5 h at 200 V using the DCode System, stained, and analyzed as described for RFLP.

Shannon diversity index was calculated from DGGE band intensity data according to

$$H = -\sum_{j=1}^{x} pi \ln pi$$

with *pi* being the proportional intensity of band *i* to total band intensity. Richness is the number of identified phylotypes (identifiable DGGE bands per lane).

We measured five different extracellular enzymatic activities involved in carbon, nitrogen, and phosphorus cycling. All measurements were made in triplicates. Saccharase activity was determined according to the method by Schinner and von Mersi (1990), which was adapted for glacier forefield soils. Briefly, field moist soils (5 g) were mixed with 5 ml of sucrose solution (1.2%) and 5 ml of acetate buffer (2 M, pH 5.5). After incubation for 3 h at 50°C, the released reduced sugars were determined photometrically at 690 nm. β-Glucosidase activity was quantified according to the method described by Tabatabai (1994). Samples were measured by colorimetric determination of p-nitrophenol released by β-glucosidase after incubation in a p-nitrophenyl-β-D-glucoside solution. We quantified urease activity according to Kandeler and Gerber (1988) by colorimetrical determination of the ammonia released after incubation of field moist soil samples (5 g) with 2.5 ml urea solution (Kandeler and Gerber, 1988). Estimations of alkaline and acid phosphatase activity were performed according to the method described by Tabatabai (1994). For both measurements, field moist soil (1 g) was mixed with 4 ml of alkaline (pH 11) or acid (pH 6.5) modified universal buffer accordingly, together with toluene (0.25 ml) and 1 ml of p-nitrophenyl phosphate solution (15 mM). All samples were incubated for 1 h at 37°C in the dark. The reaction was stopped by adding $CaCl_2$ at a final concentration of 0.05 M, and NaOH at 0.2 M. The phosphatase activity, assayed by p-nitrophenol release, was measured photometrically.

2.3.3 Statistical analysis

Statistical analysis was carried out using the SPSS 11.0 software package. Mean values are given ±1 standard error of the mean. The significance threshold was set to 0.05 for the *p* value. To test for the distance effects on chemical and physical parameters and on microbial abundance, we applied one-way ANOVA on each parameter set (n=25), with distance classes as fixed factors [0 cm (n=3), 20 cm (n=11), 40 cm (n=11)]. To test for pair wise differences between distance classes, we applied unpaired sample *t* tests to analyze the differences of means. We applied hierarchical clustering

to investigate microbial community fingerprints using the Ward's linkage method. Distances were recorded as squared Euclidian distances.

2.4 Results

The measured chemical and physical soil properties showed no significant effect with distance from the plant (Table 2-1), although all measures of soil carbon were highest in plant center soil. The measurements revealed the acidic character of the soil, with a pH of 5.03 of the plant center soil of L. alpina. TOC values were generally low, ranging from 285.89 µg C (g dry soil)$^{-1}$ at 0 cm distance to 127.77 µg C (g dry soil)$^{-1}$ at 40 cm distance from the plants. Extractable organic carbon was dominated by organic acids [240 to 270 µg (g dry soil)$^{-1}$], with lactate and formate being the most abundant acids in all samples. Total sugar values were more than a magnitude lower [10 to 13 µg glucose (g dry soil)$^{-1}$]. NH_4^+-N values fluctuated between 5.06 and 2.87 µg (g dry soil)$^{-1}$. NO_3^-–N values ranged between 18.06 and 18.69 µg (g dry soil)$^{-1}$. Soluble phosphate was around 100 µg (g dry soil)$^{-1}$ in plant center soil and below detection at further distance from the plant.

Table 2-1 Chemical and physical properties across the investigated distance classes of central L. alpina plants in the Damma glacier forefield

	Distance from plant			ANOVA	
	0 cm (n = 3)	20 cm (n = 11)	40 cm (n = 10)	$F_{1,24}$ ratio	p-value[a]
Soil water content (%)	8.01 ± 2.03	8.32 ± 1.37	9.58 ± 1.75	0.22	0.81
pH	5.03 ± 0.1	4.91 ± 0.03	4.92 ± 0.02	1.67	0.21
Carbon parameters					
TOC [µg C (g dry soil)$^{-1}$]	285.89 ± 165.06	221.11 ± 66.67	127.77 ± 38.53	0.46	0.64
Total sugar [µg (g dry soil)$^{-1}$][b]	13.08 ± 3.58	10.94 ± 1.37	10.42 ± 1.32	0.32	0.73
Total organic acids [µg (g dry soil)$^{-1}$][b]	344.62 ± 23.11	283.91 ± 17.81	313.56 ± 29.35	0.86	0.44
Lactate [µg (g dry soil) $^{-1}$][b]	268.03 ± 9.62	240.43 ± 10.81	248.00 ± 14.82	0.53	0.60
Formate [µg (g dry soil)$^{-1}$][b]	76.59 ± 13.62	43.48 ± 8.10	65.56 ± 16.16	1.18	0.33
Nitrogen parameters					
Total nitrogen [µg N (g dry soil)$^{-1}$]	<100[c]	<100[c]	<100[c]	–	–
Nitrate [µg N (g dry soil)$^{-1}$][b]	18.12 ± 0.79	18.69 ± 0.85	18.06 ± 0.86	0.16	0.86
Ammonium [µg N (g dry soil)$^{-1}$]	4.70 ± 2.66	2.87 ± 0.53	5.06 ± 1.84	0.69	0.52
Phosphate [µg (g dry soil)$^{-1}$][b]	108.32 ± 62.54	<24[d]	<24[d]	–	–

Values are given ± 1 standard error of the mean. The distance effect was tested with ANOVA
[a] Significance threshold 0.05
[b] Measurements were done in 0.01 M $CaCl_2$ extracts
[c] Below detection limit [100 µg N (g dry soil)–1]
[d] Below smallest standard concentration [24 µg (g dry soil)–1]

In contrast to chemical data, cell count data showed significant trends with distance from the plant (Table 2-2). Root biomass significantly decreased with distance from the plant (one-way ANOVA, $p < 0.01$), but 20 and 40 cm were not significantly different (*t* test) (Table 2-2). DAPI counts were highest in the plant center soils [7.88 × 108 cells (g dry soil)$^{-1}$] and significantly decreased to about 50% at 40 cm. Active bacteria counts (as measured by FDA) also decreased significantly with distance (one-way ANOVA, $p = 0.01$). On average, about 30% of the total bacterial cells were detected as active, with no significant effect of distance from the plant. Only DAPI (*t* test, $p < 0.01$) and active bacteria (*t* test, $p < 0.05$) showed significant differences between 20 and 40 cm samples.

Table 2-2 Biological properties across three investigated distance classes of central L. alpina plants

	Distance from plant			ANOVA	
	0 cm ($n = 3$)	20 cm ($n = 11$)	40 cm ($n = 10$)	$F_{1,24}$ ratio	*p* value[a]
Biomass					
Root biomass [mg (g dry soil)$^{-1}$]	1.35 ± 0.95	0.05 ± 0.03	0.03 ± 0.01	8.937	0.001
DAPI [10^8 cells (g dry soil)$^{-1}$]	7.88 ± 0.58	5.54 ± 0.45	3.87 ± 0.34	12.383	<0.001
Bacterial activity					
FDA [10^8 cells (g dry soil)$^{-1}$]	2.02 ± 0.15	1.56 ± 0.12	1.23 ± 0.11	5.753	0.010
FDA/DAPI [%]	25.70 ± 1.01	29.18 ± 2.66	33.36 ± 3.72	0.850	0.441
Enzyme activities					
Saccharase activity [nmol glucose (g dry soil h)$^{-1}$]	119.46 ± 47.32	40.68 ± 8.66	29.25 ± 7.91	7.534	0.003
Glucosidase activity [nmol phenol (g dry soil h)$^{-1}$]	677.53 ± 231.62	152.60 ± 27.54	101.60 ± 17.63	20.375	<0.001
Urease activity [nmol N (g dry soil h)$^{-1}$]	101.59 ± 21.3	127.37 ± 24.39	99.09 ± 21.57	0.438	0.651
Alkaline phosphatase activity [nmol phenol (g dry soil h)$^{-1}$]	57.80 ± 6.94	31.64 ± 6.01	34.63 ± 5.86	2.265	0.127
Acid phosphatase activity [nmol phenol (g dry soil h)$^{-1}$]	482.02 ± 124.75	262.16 ± 28.82	264.93 ± 22.20	5.801	0.009
Enzyme activities per FDA stained cells					
Saccharase activity [fmol glucose (cell h)$^{-1}$]	0.57 ± 0.19	0.29 ± 0.08	0.24 ± 0.07	2.029	0.155
Glucosidase activity [fmol phenol (cell h)$^{-1}$]	3.25 ± 1.04	1.00 ± 0.17	0.80 ± 0.11	14.873	<0.001
Urease activity [fmol N (cell h)$^{-1}$]	0.50 ± 0.08	0.89 ± 0.19	0.79 ± 0.14	0.636	0.539
Alkaline phosphatse activity [fmol phenol (cell h)$^{-1}$]	0.28 ± 0.02	0.20 ± 0.03	0.30 ± 0.05	1.626	0.219
Acid phosphatse activity [fmol phenol (cell h)$^{-1}$]	2.32 ± 0.49	1.79 ± 0.24	2.32 ± 0.25	1.332	0.285

Values are given ± 1 standard error of the mean. The distance effect was tested with ANOVA
[a] Significance threshold 0.05

RFLP analysis revealed complex restriction patterns (Fig. 2-1). Cluster analysis revealed no clear distance effects; overall, all samples showed a high degree of similarity. Closely related patterns tended to originate from the same plant, but the plant or site effect was likewise marginal (Fig. 2-1).

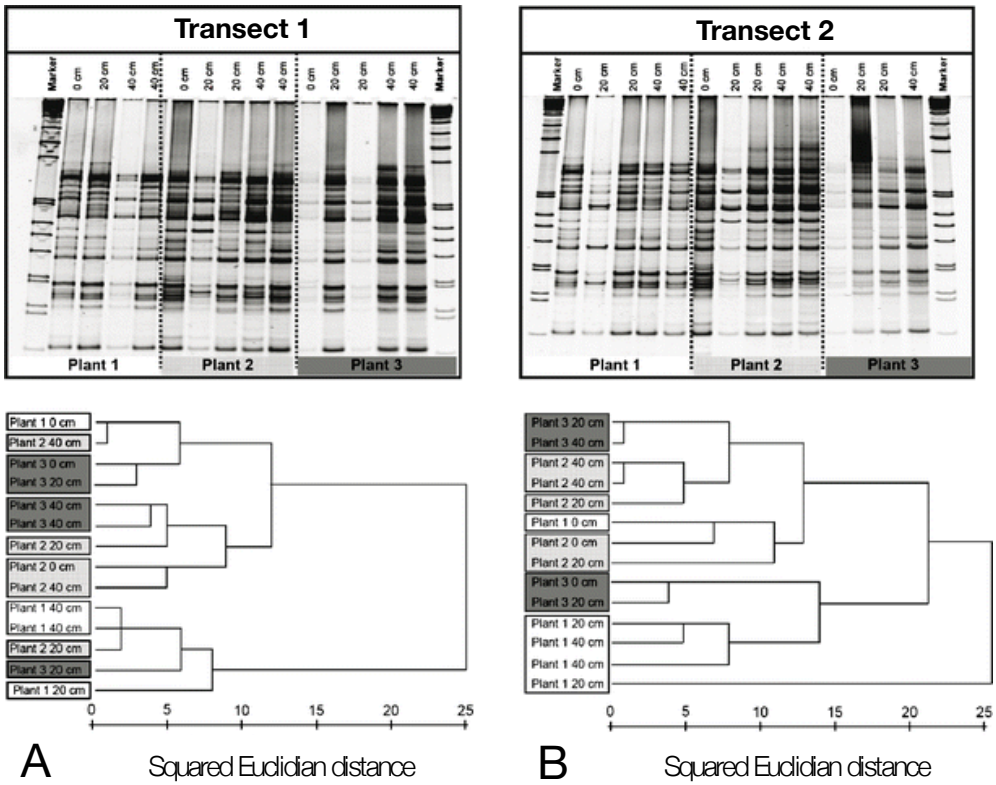


Figure 2-1 RFLP fingerprinting patterns of 16S rDNA fragments, originating from different distance classes (top panels) obtained from two transects (A and B) across L. alpina plants together with corresponding results obtained by hierarchical clustering using Ward's method (lower panels)

Fingerprinting by DGGE confirmed the diverse microbial community structure and lack of spatial effects (data not shown). However, in comparison to RFLP, the information obtained from DGGE profiling is more directly related to the microbial species level because, theoretically, each DGGE band could be related to a distinct bacterial phylotype (Muyzer et al., 1993). We therefore used DGGE banding information to investigate two different diversity parameters: richness (number of different phylotypes) and Shannon index (Table 2-3), which is a general diversity parameter.

Species richness, as indicated from DGGE band numbers, was highest in samples from the rhizosphere and decreased with distance (Table 2-3). Shannon diversity values near the plants were slightly higher than those obtained from profiles at 40 cm distance ($p = 0.06$; unpaired t test). Overall, the decrease was only marginally significant (Pearson correlation –0.352, significance 0.07).

Table 2-3 Summary of diversity parameters calculated from DGGE banding patterns for samples from three distance classes

	Distance from plant			PC	sig	*p* value[a]
	0 cm	20 cm	40 cm			
Shannon index	2.66 ± 0.07	2.51 ± 0.08	2.39 ± 0.11	–0.352	0.07	0.06
Richness	18.67 ± 1.20	17.55 ± 1.55	16.20 ± 1.69	–0.202	0.31	0.25

PC;Pearson correlation, sig; two-tailed significance
[a] Unpaired sample *t* test comparing 20-cm with 40-cm samples, significance threshold 0.05

Like cell counts, several enzyme activities were significantly related to distance (Table 2-2). Saccharase activity decreased significantly from the plant center soil of *L. alpina* [119.46 nmol glucose (g dry soil h)$^{-1}$], (ANOVA, $p < 0.01$) to 29.25 nmol glucose (g dry soil h)$^{-1}$ at 40 cm distance (Table 2-2). The ratio of saccharase activity divided by the number of FDA stained bacterial cells also decreased with distance (Table 2-2), but the trend was not significant ($p = 0.155$). β-Glucosidase activity was significantly higher in the plant center soil than in the soil sampled at 40 cm (ANOVA, $p < 0.001$). The ratio of β-glucosidase activity divided by the number of active (FDA stained) bacterial cells followed the same trend, which was also statistically significant, $p < 0.001$ (Table 2-2). The sugar-related enzyme activites showed no significant differences between 20 and 40 cm (*t* test). Alkaline phosphatase activity was highest in the plant center soil [57.80 nmol phenol (g dry soil h)$^{-1}$] and decreased with distance to 34.63 nmol phenol (g dry soil h)$^{-1}$ (Table 2-2). Acid phosphatase activity was about an order of magnitude higher [480 nmol phenol (g dry soil h)$^{-1}$ in plant center soil] and decreased significantly with distance (ANOVA, $p < 0.01$). Differences between 20 and 40 cm were again not significant. Urease activity fluctuated between 90 and 120 nmol N (g dry soil h)$^{-1}$ with no significant differences between distance classes (Table 2-2).

2.5 Discussion

L. alpina occurs together with other early pioneering plants such as *Agrostis*, *Cerastium*, or *Poa* species. One of the earliest and most abundant vascular plant species that we investigated at our field site is the perennial plant *L. alpina*. In general we observed single plants or small clusters of plants in the study area, with individual plants reaching up to 10 cm diameter and making a vigorous appearance, in contrast to the sampling of Edwards et al. (2006), when individual plants were very small, probably representing seedlings from the year. We therefore assume that the plants sampled in this study had established the previous year or earlier.

We found significantly increased total and active cell numbers and enzymatic activities and elevated soluble organic carbon concentrations in plant center soils, as would be expected based on numerous studies on the rhizosphere effect (e.g., Youssef et al., 1987; Badalucco et al., 1996; de Neergaard et al, 2001; Corgie et al., 2004). The root biomass measurements showed that the root zone of *L. alpina* did not extend beyond the 10 cm diameter sampled as plant center soil, indicating that the 40 cm soil was not in obvious contact with the *L. alpina* root system. The effect on the microbial biomass, however, clearly extended to the 20-cm samples, with both total and active cell counts significantly increased

relative to the 40-cm samples. It is noteworthy that previous studies in similar environments have not observed a significant difference in total cell counts or bacterial biomass between interspace and rhizosphere in young soils (Tscherko et al., 2004; Edwards et al., 2006). This may be related to the species and age of the sampled plants, but may also indicate that the sampled interspace/bulk soils could have originated from within the zone of influence of the plant. In the case of Edwards et al. (2006), interspace soil was taken from within 15 cm of the plant.

Similar to total and active cell counts, several enzymatic activities showed a significant decrease with distance, although differences between the 20 and 40 cm soil were no longer significant. For saccharase and glucosidase, this result may be related to easily available sugars that can readily be metabolized in the rhizosphere, but it may also reflect the ability of young mineral soil substrates to preserve organic matter and enzymes due to sorption processes on mineral surfaces (Burns and Dick, 2002; Guggenberger and Kaiser, 2003). Acid phosphatase, which also showed a significant trend with distance, may originate from the plant, as well as microbes (Dick et al., 1983). The soil we studied has been shown to become both P- and N-limited in the presence of sufficient carbon (Bleikolm and Bürgmann, unpublished data); however, we observed no effect on urease activity, possibly because urea may not be a significant source of nitrogen in this soil, where soluble nitrogen is mostly inorganic (Edwards et al., 2006). Unlike our results, Tscherko et al. (2004) did not observe enhanced enzymatic activities in the rhizosphere of the alpine grass *P. alpina* in pioneering successional stages, while such differences were occasionally observed in more developed soils. These contrasting results might reflect different underlying environmental parameters such as nutrient availability, organic matter content, and pH at the research sites, or the different plant species and associated root exudation patterns.

Enzyme assays measure both intracellular and extracellular activities (Nannipieri et al., 2003). In this study, we have applied a new approach that relates enzyme activities to the number of active (FDA-stained) bacteria. This ratio is an alternative to previously used ratios of enzymatic activity to total bacterial biomass measurements, which include dead and dormant cells (Landi et al., 2000). This provides the possibility to qualitatively link the observed enzyme activities to microbial processes. Our results showed that per-cell enzymatic activity generally (and for glucosidase significantly) decreased with distance (Table 2-2); e.g., the saccharase and glucosidase activities per FDA-stained cell in 40 cm soil were only 42 and 24% of values of plant center soil, respectively. This would indicate that the bacterial populations show lower levels in the microbial biomass and are increasingly nutrient- or energy-limited with distance from the plant and show lower levels of microbial activity. The overall decrease in activity is therefore due both to the decreasing trend in the biomass and decreasing levels of activity of individual cells.

How can the increased bacterial biomass and activity beyond the actual root zone be explained? It is possible that fine roots, which may not be detected by our root biomass measurements, may provide exudates to the 20 cm soil. Alternatively, in these young soils, low biomass and a predominantly mineral character (low organic matter and clay content) may allow diffusion or advection of dissolved organic carbon to a much greater extent than has been determined in developed

soils (Karthikeyan and Kulakow, 2003; Poll et al., 2006). The occurrence of isolated plant patches in young soils could thus promote the formation of strong gradients and lead to a spatially extended impact of plants on young soils. However, the lack of a clear trend in the extractable organic carbon and other chemical parameters rather supports the involvement of fine roots. Finally, the presence of isolated vegetation may have indirect effects that change the living conditions for the microbial biomass independent of the root zone, through effects such as increased aeolic sedimentation rates, decomposition of aboveground biomass, attraction of animals, shading, etc. (Matthews, 1992). These effects would have to be studied in more detail in future research.

We performed RFLP and DGGE analysis to characterize the soil microbial community structure at different distances from *L. alpina* plants. At the observed scale, the differences in microbial activities were not related to an obvious change of the microbial community structure.

Previous research suggests that rhizospheres can have a selective effect on soil bacteria (Marilley et al., 1998). Specific compounds in the root exudates might even selectively stimulate certain beneficial bacterial groups, which have mechanisms to potentially improve plant growth (Griffiths et al., 1999; Dobbelaere et al., 2003; Bürgmann et al., 2005b). Edwards et al. (2006) observed different microbial communities in rhizosphere of *L. alpina* and interspace soil also sampled in this study using DGGE. The different findings may be explained by the different sampling procedures because Edwards et al. (2006) sampled rhizosphere soil directly within the root zone. However, Tscherko et al. (2005) studied the microbial community directly in *P. alpina* rhizosphere (root-adhering soil) using phospholipid fatty acid analysis and found similar microbial community structure in bulk soil and rhizosphere in young soils. Our results support the conclusion of Tscherko et al. (2005) that in young glacial soils the rhizosphere community is mostly recruited from the bulk soil community. However, they also observed no significant differences in enzymatic activities and microbial biomass, unlike our results. Here we observed that despite a significant increase in numbers and activity of the microbial community in the vicinity of *L. alpina*, the community was not subject to selective pressure that affected the community composition at the coarse resolution that could be studied using RFLP and DGGE (Nocker et al., 2007). In conclusion, the currently available data remain inconclusive as to under which conditions shifts in microbial community structure in the rhizosphere of pioneer plants occur. This may depend on specific spatial, temporal, or plant species-related effects. The variation of rhizodepostion patterns might be a general mechanism regulating the growth of root-associated bacteria, depending on the environmental conditions and nutrient requirements. Recently, Edwards et al. (2006) reported that the rhizosphere of *L. alpina* at different successional stages exhibited distinct root-exudation patterns.

In conclusion, we have shown, in accordance to our hypothesis, that vegetational patches occurring in a glacial forefield affected microbial biomass and activity, and that this effect extended to 20 cm distance from the plant. This creation of relatively large zones of microbial activity may create islands with improved conditions for further biological colonization, e.g., by plants and animals. Similar scenarios have been reported for semiarid and desert zones where isolated patches of

shrub canopies represent areas of enhanced nutrient availability (“resource islands”) (Herman et al., 1995; Su et al., 2004). In contrast to our original hypothesis, the microbial community composition and soil chemistry did not reveal obvious gradients on the studied scale. The dominant members of the bacterial community remained the same despite a doubling of the bacterial cell count, indicating little selective pressure.

3 HIGH DIVERSITY OF DIAZOTROPHS IN THE FOREFIELD OF A RECEDING ALPINE GLACIER

Duc L., Noll M., Meier B.E., Bürgmann H., Zeyer J.

Microbial ecology (2009) 57:179-190

3.1 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.

3.2 Introduction

Most alpine glaciers have been retreating since the end of the little ice age around 1850 (Oerlemans, 1994; Fagan, 2000). This retreat exposed large areas to colonization by microorganisms, plants and animals (Chapin et al., 1994; Ohtonen et al., 1999; Schlag and Erschbamer, 2000; Kaufmann, 2001; Sigler et al., 2002). 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 (Marschner, 1995; Grayston et al., 1997). Moreover, root systems of pioneer plants contribute to slope stabilization (Korner, 2004). In recently deglaciated environments, nutrient shortage is the main limitation for plant growth (Heer and Körner, 2002). Organic acid anions (e.g. malate, citrate, oxalate) released in the rhizosphere can act as ligands, thus increasing the uptake of mineral nutrients (Marschner, 1995). Soil bacteria are also responsible for the release of organic acids (Jones, 1998) and chelators (e.g. siderophores) (Neilands and Leong, 1986), facilitating the mineral nutrient acquisition by plants. Moreover, some of them can suppress plant pathogens through their competitive and biocontrol abilities (Haas et al., 2000). Overall, plants receive considerable benefits from soil microbial communities (Glick, 1995) 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) (Ohtonen et al., 1999), and the Rotmoosferner and Ödenwinkelkees glaciers (Austria) (Tscherko et al., 2003a; Tscherko et al., 2004; Deiglmayr et al., 2006). In the Damma glacier forefield (Switzerland), two different scales have been explored. Edwards et al. (2006) observed a decrease of plant influence on soil-bacterial communities along the chronosequence, while Miniaci et al. (2007) 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 to more than 40 kg per hectare and year, as given by the Swiss Federal Office for the Environment (FOEN) (www.bafu.admin.ch). The study by Korner et al. (1997) (2470m, Swiss Central Alps), as well as a preliminary study on the Damma glacier (Bleikolm, 2005) 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 (Jensen and Hauggaard-Nielsen, 2003). However, only few studies have focused on nitrogen fixation in glacier forefields. For example, Kohls et al. (1994) studied the impact of symbiotic nitrogen fixation on plant succession and soil formation and Jacot et al. (2000b) 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 (Jacot et al., 2000a). 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 (Burris and Roberts, 1993a), and an extensive database of sequences retrieved from multiple environments is available (Zehr et al., 2003a). Comparison of 16S rRNA and *nifH* phylogenies gives no strong evidence for lateral *nifH*-gene transfer (Zehr et al., 2003a) although some anomalies were observed (Machado et al., 1996; Minerdi et al., 2001). For example, Dedysh et al. (2004) 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 (Cluster I-IV) (Chien and Zinder, 1996). This phylogenetic classification, based on amino-acid sequences, has proven to be reasonable (Kessler et al., 1998; Braun et al., 1999; Lilburn et al., 2001) and was updated and rearranged by Zehr et al. (2003b). 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. Widmer et al., 1999; Izquierdo and Nusslein, 2006), including arctic soil (Deslippe and Egger, 2006), 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. (2003b).

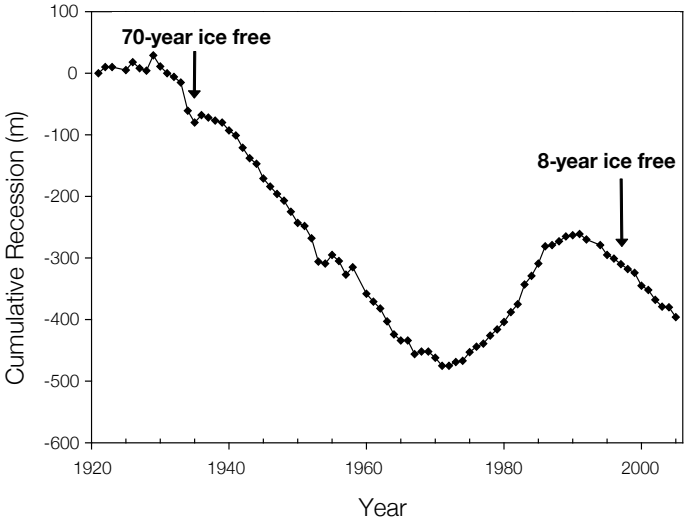
3.3 Material and methods

3.3.1 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 2000 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. 3-1). Since the beginning of systematic measurement in 1921 the glacier has been receding at a mean rate of 10 m yr^{-1} (http://glaciology.ethz.ch/swiss-glaciers/, (Bauder et al., 2001; Bauder and Hoelzle, 2002; Bauder et al., 2004; Bauder et al., 2006). 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 and 5°C, and a mean annual precipitation of 2400 mm (Spreafico et al., 1992). The parent rock of the forefield is granite.

3.3.2 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. (2007). The two sites corresponded to 8- and 70-year ice-free forefield (year of deglaciation 1997 and 1935) and were situated at 60 m 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. 3-1). 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 ca. 5 cm and transported in plastic bags on ice to the laboratory within eight hours. Pioneer plants, including root systems, were collected with a spade and then vigorously shaken to remove non-adherent 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.



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	Rhizosphere soil	15
2005	3	Bulk soil	3

Figure 3-1 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. (2006).

3.3.3 Chemical and physical characterization of soils

Soils ranged from coarse sand (8-year ice-free forefield) to well define 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 (Miniaci et al., 2007). 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 1200°C, using a Leco 2000 CNS device elementary analyser (Leco, Krefeld, Germany) following manufacturer's instructions. Available ammonium was determined photometrically after 2 M KCl soil extraction (Mulvaney, 1996). 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 x plant presence were examined through two-way analysis of variance (ANOVA) using the JMP software (Sall et al., 2001).

3.3.4 Acetylene reduction assay

Potential asymbiotic nitrogenase activity was estimated using the acetylene reduction assay (ARA) (Bürgmann et al., 2003). 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 hours of artificial light per day. Ethylene (C_2H_4) production was measured after 5 min, 24 h, 48 h 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 (GC/FID) 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.

3.3.5 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 (Noll et al., 2005). The extracted nucleic acids were dissolved in 50 µl TE buffer (10 mM Tris-HCl (pH 8), 1 mM EDTA). 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 PCR approach was performed according to Bürgmann et al. 2004 (Widmer et al., 1999; Bürgmann et al., 2004). 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.

3.3.6 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.

3.3.7 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 BLAST search (Altschul et al., 1990) at NCBI (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. 318 Damma glacier NifH sequences were merged with 3324 sequences retrieved from the Pfam Fer4_NifH database (2005) (http://www.sanger.ac.uk) (Bateman et al., 2004), and 4298 sequences 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 (Ludwig et al., 2004). A phylogenetic inference tree was calculated at the protein level using the neighbour joining algorithm (Saitou and Nei, 1987) and a Kimura correction (Kimura, 1980), 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. (2003b) was used as backbone for tree construction.

3.3.8 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) (Colwell, 2005) and total NifH phylotype richness of the four libraries was estimated using Chao1 (Chao, 1984) as a non-parametric 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 (Sall et al., 2001). Diversity and evenness are given by the Shannon-Wiener diversity index, $H'=-\sum(pi)(\ln pi)$, where pi is the proportion of i th 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.

3.3.9 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, EU305256-EU305295.

3.4 Results

3.4.1 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 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, Ctot and Ntot values were out of the validity range of the CNS elementary analyzer and therefore have to be interpreted with caution.

3.4.2 Nitrogenase activity

Acetylene reduction rates were low but above the detection limit in most samples, with a few outliers showing higher rates (Table 3-1). 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).

Table 3-1 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)$^{-1}$]	4.7±3.4	38.37±9.3	10.45±8.8	529.4±524.22	0.2459	0.1997	0.2563
Nitrate [µg N (g dry soil)$^{-1}$]	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)-1]	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)$^{-1}$]	155.1±203.6	668.2±50.1	233.7±179.0	10016.83± 10800.8	0.1248	0.0958[a]	0.1307
DNA [ng DNA (g dry soil)$^{-1}$]	2.6±0.4	156.4±46.4	5.9±3.2	4338.2±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 C2H4 (day g dry material)$^{-1}$]	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

3.4.3 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. 3-2). 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 were similar, while 8-year and 70-year bulk soils were significantly different.

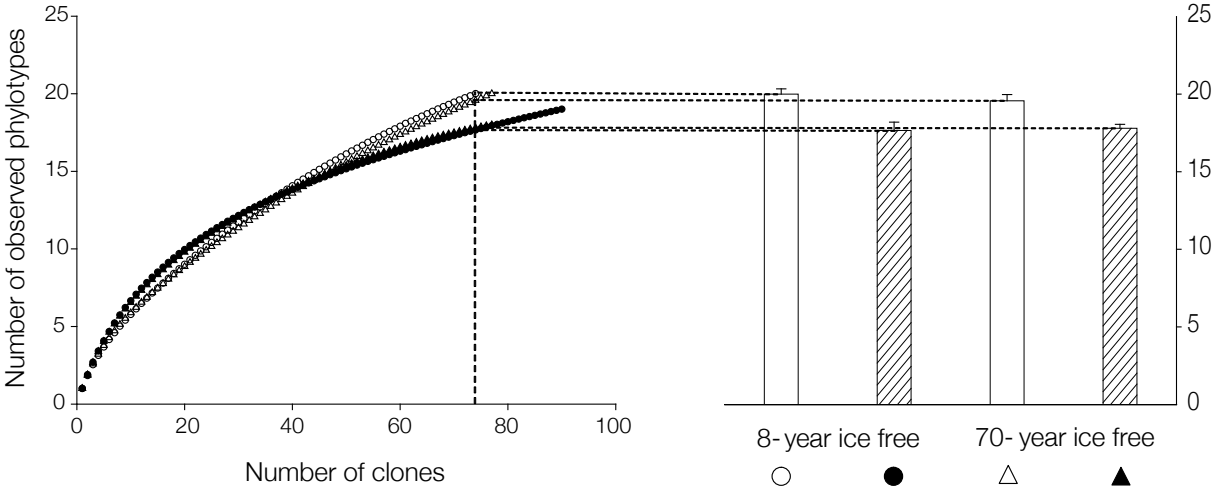


Figure 3-2 Species richness of the four NifH sequence libraries. Symbols: 8-year bulk soil (❍), 70-year bulk soil (Δ), 8-year rhizosphere soil (●) and 70-year rhizosphere soil (▲). Left: Rarefaction curves were constructed using EstimateS software package at 97% amino acid similarity level. Right: Species richness at n=74. Error bars correspond to Sobs standard error (Mao Tau) (Colwell et al., 2004).

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 3-2). 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 3-2).

Table 3-2 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.

3.4.4 Phylogenetic characterization

All NifH amino-acid sequences derived from the glacier forefield were assigned to the NifH phylogenetic clusters defined by Zehr et al. (2003b). Diazotrophs present in the Damma glacier forefield were highly diverse with respect to phylogeny (Fig. 3-3 and 3-4). All previously described NifH clusters (Chien and Zinder, 1996) 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. 3-3 and the low Sorensen similarity indices (Table 3-3). 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 3-3). Shannon evenness indices were smaller for the two bulk-soil libraries than for rhizosphere soil (Table 3-2). In both case, more than half of the NifH sequences belonged to few phylotypes that were almost exclusive to these two libraries.

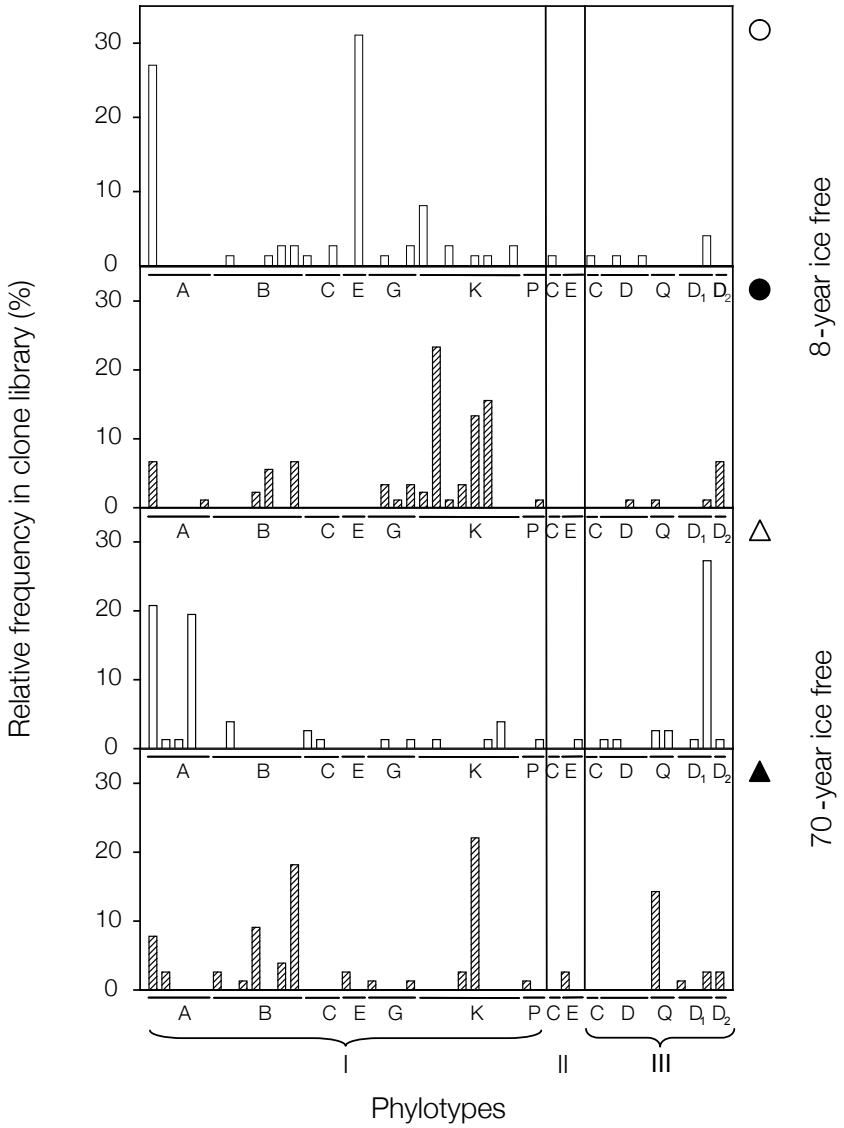


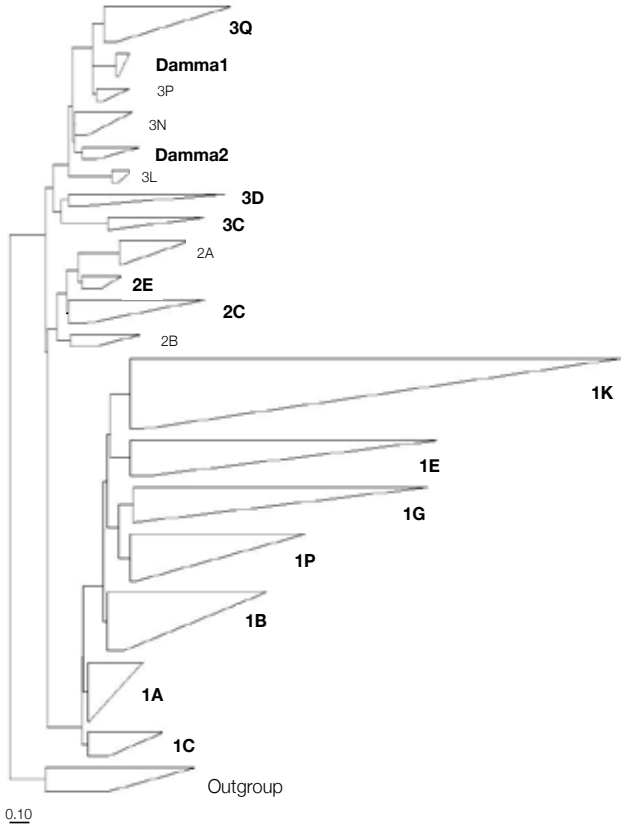
Figure 3-3 Relative frequency of phylotypes in individual NifH libraries, classified in phylogenetic clusters as defined by Zehr et al. (2003b)(82), based on the phylogenetic analysis shown in Fig. 3-4. Symbols: 8-year bulk soil (○), 70-year bulk soil (Δ), 8-year rhizosphere soil (●) and 70-year rhizosphere soil (▲).

Table 3-3 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	
		Rhizosphere 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

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*, *G. bemidjensis* or *G. 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 occured 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 3-4.). 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*).



Libraries ○ ● △ ▲	Closest cultivated relatives
4 1 11	
3 22 1 3	
1 6 2	
2 2 1	
1	*Clostridium pasteurianum*
	Methanosarcina mazei
1 2	*Heliobacterium* sp.
1	
12 5 53 19	***Ideonella* sp., *Methylococcus capsulatus*, *Methylococcus* sp., *Methylocystis* sp., *Methylocystis minimus*, *Azospirillum lipoferum*, *Azospirillum oryzae*, *Bradyrhizobium elkanii***
23 2	***Paenibacillus durus***
3 2 7 2	***Azotobacter vinelandii***
1 1 1	***Dechloromonas aromatica***
6 3 13 27	***Anabeana cylindrica*, *Aphanizomenon* sp., *Nostoc muscorum*, *Nostoc* sp., *Scytonema* sp., *Pseudanabeana* sp., *Oscillatorioa* sp.**
20 33 7 8	***Geobacter uraniumreducens*, *Geobacter bemidjensis*, *Geobacter lovleyi***
3 3	***Desulfitobacterium hafniense***

Figure 3-4: 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 1713 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.

3.5 Discussion

3.5.1 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 (Tscherko et al., 2004; Miniaci et al., 2007) 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 (eg. reduced temperature and moisture fluctuation) due to vegetation cover (Tscherko et al., 2004; Miniaci et al., 2007).

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 (Sigler et al., 2002; Edwards et al., 2006). 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. (1995) and Bengtson et al. (2005) 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.

3.5.2 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 (Widmer et al., 1999; Poly et al., 2001b; Rosch and Bothe, 2005; Deslippe and Egger, 2006). 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 (Nocker et al., 2007). 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 et al. (2006) 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)(Izquierdo and Nusslein, 2006). 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 (Edwards et al., 2006). Our results were in agreement with this observation, showing a significantly higher NifH phy-

lotype richness in bulk soil. It may result from a higher diversity of the microhabitat (Poly et al., 2001b; Gros et al., 2004), as well as more fluctuating growth conditions (Tscherko et al., 2003a). 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 (IDH) (Connell, 1978), 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 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) (Tscherko et al., 2003a). Similar findings are reported for a nutrient rich agricultural soil by Marilley et al. (1999) 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. (2005) observed a poor relationship between *nifH* diversity and nitrogenase activity in natural environment. In a laboratory pot experiment, Bürgmann et al. (2005a) 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.

3.5.3 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 (Widmer et al., 1999; Rosch and Bothe, 2005) and cultivated soil (Poly et al., 2001b), 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 et al. (2006), 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. (2003a), 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 (Figure 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, *Methylococcus 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 (Bender and Conrad, 1992; Kolb et al., 2005). 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. (2004) the affiliation of the NifH sequence from the ***γ**-Proteobacteria M. capsulatus* with ***α**-proteobacterial* sequences may results 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 (Hamelin et al., 2002,Yeager, 2005 #778). *Geobacter* species are able to gain energy from Fe(III) reduction using organic acids or acetate as electron donors (Lovley et al., 2004). Representatives of the *Geobacteraceae* are also able to use oxygen as alternative electron acceptor, giving them competitive advantages in oxic-anoxic boundaries (Lin et al., 2004). 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 favour 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 (Chapin et al., 1991; Liengen and Olsen, 1997) and alpine (Fritzsheridan, 1988) environments. Furthermore, they are known as primary colonizers of recently deglaciated soil (Davey and Clarke, 1991; Kastovska et al., 2005). Nevertheless, while *Cyanobacteria* are well studied in aquatic and symbiotic systems (Bergman et al., 1996; Parodi and de Cao, 2002), very little is known about *Cyanobacteria* under the soil surface (Whitton, 2000). 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 (Yeager et al., 2004), but crust formation was not evident at the sampling sites. *Cyanobacteria* are known to form symbioses with mosses (e.g. *Azolla*, *Hepatica*) (Solheim et al., 2004), 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 (Stal and Moezelaar, 1997), or chemoorganotrophs (Bottomley and Vanbaalen, 1978). Nine of our *cyanobacterial* sequences were related to *Nostoc* sp., a species able to

perform aerobic respiration (Stal and Moezelaar, 1997). Gantar et al. (1991) 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 (Ikeda et al., 2006) that were distantly affiliated with facultative fermentative *Oscillatoria* sp. (Moezelaar et al., 1996). 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.

3.6 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.

4 DEVELOPMENT AND EXPERIMENTAL VALIDATION OF A *NIFH* OLIGONUCLEOTIDE MICROARRAY TO STUDY DIAZOTROPHIC COMMUNITIES IN A GLACIER FORFIELD

Duc L., Neuenschwander S., Rehrauer H., Wagner U., Sobek J., Schlapbach R., Zeyer J.

Environmental Microbiology 2009, accepted

4.1 Abstract

Functional microarrays are powerful tools that allow the parallel detection of multiple strains at the species level and therefore to rapidly obtain information on microbial communities in the environment. However, the design of suitable probes is prone to uncertainties, as it is based so far on in silico predictions including weighted-mismatch number and Gibbs free-energy values. This study describes the experimental selection of probes targeting sub-sequences of the *nifH* gene to study the community structure of diazotrophic populations present in Damma glacier (Swiss Central Alps) forefield soils. Using the Geniom® One *in situ* synthesis technology (Febit, Germany), 2727 *in silico* designed candidate probes were tested. A total of 946 specific probes were selected and validated. This probe set covered a large diversity of the NifH phylotypes (35 out of the 45) found in the forefield. Hybridization predictors were tested statistically. Gibbs free-energy value for probe-target binding gave the best prediction for hybridization efficiency, while the weighted-mismatch number was not significantly associated to probe specificity. In this study, we demonstrate that extensive experimental tests of probe-hybridization behaviour against sequences present in the studied environment remains a prerequisite for meaningful probe selection.

4.2 Introduction

Microbial nitrogen fixation plays an important role in the nitrogen cycle and in sustaining fertility in nitrogen-poor systems like in oligotrophic oceans and lakes, and in alpine, arctic and aridic soils (Chapin et al., 1994). Diversity of nitrogen-fixing microorganisms (diazotrophs) has been widely studied in aquatic and terrestrial environments using molecular approaches based on the *nifH* marker gene (Zehr et al., 1995; Widmer et al., 1999; Zani et al., 2000; Steppe and Paerl, 2002; Steward et al., 2004b; Church et al., 2005; Langlois et al., 2005; Deslippe and Egger, 2006; Izquierdo and Nusslein, 2006; Bostrom et al., 2007). This gene encodes the iron protein of the nitrogenase complex (dinitrogenase reductase) (Raymond et al., 2004).

The Damma glacier (Swiss Central Alps) has been receding over decades creating a natural alpine soil chronosequence. In the forefield of this glacier, Duc et al. (2009a) observed a great diversity of free-living diazotrophic bacteria that still needs to be resolved spatially and temporarily in order to identify phylogenetic groups actively contributing to the soil nitrogen cycle along the chronosequence.

Recently, several environmental studies (Steward et al., 2004a; Moisander et al., 2006; Zhang et al., 2007) focused on the development of *nifH* oligonucleotide microarrays. These arrays facilitate the characterization of diazotrophic communities with a high throughput comparable to other fingerprinting methods (terminal restriction length polymorphism (T-RFLP), denaturing gradient gel electrophoresis (DGGE) and a phylogenetic resolution similar to the cloning-sequencing approach. The design of specific and sensitive probes constitutes one of the most challenging steps in the development of microarrays. Hierarchical design is commonly applied in environmental studies to enhance the specificity and the robustness of a probe set (Loy et al., 2002; Bodrossy et al., 2003a; Sanguin et al., 2006). However, the low conservation degree of the *nifH* gene, compared to other genes (e.g., 16S rRNA, *pmoA*), limits the use of this strategy. The reliance on *in silico* hybridization predictors, such as the amount, type and position of mismatches (MM) (Letowski et al., 2004), and Gibbs free energies (Matveeva et al., 2003; Weckx et al., 2007) for the optimization of probe selection is prevalent in microarray design. However, Pozhitkov et al. (2006) showed that none of the known predictors efficiently anticipates probe performance and therefore recommend to test each probe towards all targets included in one microarray experiment.

Experimental testing of probes becomes ambitious when working with environmental samples containing highly diverse microbial communities. Considerable costs and time should be invested for the individual synthesis of several thousands of candidate probes, their spotting on microarray glass-slides and their validation against a large amount of targets in order to realize an exhaustive screening. It might explain why only few studies, focused on restricted numbers of bacterial strains or specific environments, adopted this strategy (Harbers et al., 2007). In contrast, *in situ* synthesis technologies (e.g., synthesis using printing technique, physical masks, electrogenerated acid, photolithography) (Gao et al., 2004) represent highly flexible methods facilitating the modification of the probe set design for every single chip production run. The Geniom® One platform (Febit GmbH, Heidelberg, Germany)(Beier and Hoheisel, 2000; Beier et al., 2002; Guimil et al.,

2003) involves the photolithography based synthesis and allows the rapid screening of up to 6500 probes against eight different targets in one single run. Probes are synthesized *in situ* in a microfluidic biochip, using photolabile protected DNA building blocks and a high-resolution digital micromirror device. The probe set design can be modified for every single chip production run, making this technology highly flexible.

This study describes the development of a *nifH* oligonucleotide (20 nts) microarray for the monitoring of the NifH diversity in soil samples obtained from the Damma glacier forefield. We report the selection and the validation of 946 probes, out of 2727 candidates designed *in silico* with the ARB software package (Ludwig et al., 2004), using the *in situ* synthesis Geniom® One platform. A small number of probes were then tested using a second microarray platform to demonstrate the consistency of the *nifH* microarray. Finally, the applicability of the probe set to study NifH diversity in environmental samples was assessed by comparing microarray and cloning-sequencing data.

4.3 Material and methods

4.3.1 Target preparation

Thirty seven DNA clones containing *nifH* target sequences (Table 4-1) were grown overnight on culture plates containing imMediaTM Amp Blue medium (Invitrogen), and DNA was extracted using a protocol adopted from Bürgmann et al. (2003). Additionally, one bulk soil and four rhizosphere soils (1x *Agrostis* sp., 2x *Poa alpina*, 1x *Leucanthemopsis alpina*) were sampled in the Damma glacier forefield in summer 2004 as previously described (Duc et al., 2009a). DNA was extracted using a bead-beating method (Noll et al., 2005) from 0.7 g (fresh weight) of soil.

A nested PCR approach (Widmer et al., 1999; Bürgmann et al., 2004) was used to amplify the *nifH* gene. The initial PCR was performed with the forward primer *nifH*-forA (5'-GCIWTITAYGGNAARGGNGG-3') and the reverse primer *nifH*-rev (5'-GCRTAIABNGCCATCATYTC-3'), followed by a nested PCR reaction using the forward primer *nifH*-forB (5'-GGITGY-GAYCCNAAVGCNGA-3') and the same reverse primer. For each DNA sample, PCR was run in triplicate and the products were pooled and purified through a QIAquick PCR purification column (QIAGEN). Purified PCR products, corresponding to 2.4 µg DNA, were labelled using a BioPrime Array CGH Genomic Labeling System Kit (Invitrogen) and 3 µl DY-547-labelled dUTP (1 mM) (Dyomics GmbH). Unincorporated labelled dUTPs were removed using mini Quick Spin Columns (Roche Applied Science). Dye incorporation rates were measured by spectrophotometry at 550 nm.

4.3.2 Probe design

The design of probes was based on a *nifH* database containing 318 *nifH* sequences retrieved from Damma glacier forefield soil samples (Duc et al., 2009a) aligned with 3324 sequences retrieved from the Pfam Fer4_NifH database (2005) (Bateman et al., 2004) and 4298 sequences kindly provided by B. D. Jenkins (Department of Cell and Molecular Biology, University of Rhode Island, Kingston). The Probe Design function of the ARB software package was applied to develop PM probes targeting specifically NifH phylotypes defined by Duc et al. (2009a) (Fig. 4-1). ARB parameters were set as follows: 20 nts, 50 < GC% < 75, max hairpin bonds = 4, *E. coli* position = 0-10000, max non group hits = 0, min group hits = 100%. When this requirements could not be met the min group hits value was reduced. The matching properties, or WMM value, of the probe set against the entire *nifH* database were assessed using the ARB Probe Match function, taking into account the amount, type and position of MM. For this calculation, ARB default parameters were used. Finally, 81 negative control sequences (20 nts), presenting at least 5 MM with all *nifH* sequences comprised in the database, were generated randomly.

4.3.3 Geniom® One platform experiment

nifH candidate probes (n = 2727) were synthesized *in situ* using the Geniom® One microarray platform (Beier and Hoheisel, 2000). Duplicate of the entire probe set and 36 Geniom® One positive-control oligonucleotides were randomly and simultaneously synthesized in eight independent 3D micro-channels (arrays) per chip using a light modulator matrix. Standard DNA synthesis reagents were used in addition to phosphoramidites, carrying a 5'-photolabile protective group (5´-NPPOC (2-[2-nitrophenyl]-propoxycarbonyl) (Febit, Heidelberg, Germany). The array was activated by adding a linker molecule with a NPPOC protecting group to the tips of spacer molecules.

DY-547-labeled *nifH* PCR products (6 µl), equivalent to 150 pmol of dye, were dissolved in 18 µl hybridization buffer (54.8 mM MES (2-(N-morpholino)ethanesulfonic acid) free acid, 147.7 mM MES sodium salt, 1.8 M NaCl (total NaCl concentration 1.9 M), 40 mM Na_2EDTA, 0.2% Tween 20, 0.5 mg mL^{-1} BSA, 0.1 mg mL^{-1} Hering sperm DNA, 2 nM Cy3-labeled positive-control targets), and hybridized for 16 hours at 40ºC with active mixing by using a pressure driven hybridization device. Individual arrays were washed at low-stringency conditions (25°C, 6x SSPE buffer) and hereupon at stringent conditions (40°C, 2x SSPE buffer). Signals were recorded by the built-in CCD camera.

4.3.4 Data analysis, normalization

Intensities of replicated spots were averaged and normalized relative to the negative control probes. Specifically, negAVG was subtracted from each probe intensity and results were divided by the standard deviation (SD) of the negative controls.

4.3.5 Specific probes selection and phylotype detection

Results of hybridizations with labelled *nifH* PCR products, corresponding to single NifH phylotypes, served to select probes and processing parameters. The set of probes and processing parameters were optimized such that the PhyloDetect web application (Rehrauer et al., 2008) would optimally identify the hybridized products. Optimal detection was performed through the maximization of the Area-Under-Curve (AUC) value of the Receiver-Operator-Characteristic (ROC) curve that plots the detection sensitivity as a function of one minus the detection specificity. The iterative scheme was conducted as follow:

1. Computation of normalized signals from raw data.

2. Definition of probes as present if they were above the SIG_{thresh}.

3. For each hybridization, comparison of the presence call of each probe with the WMM between the probe and the hybridized PCR product, and assessment of the type of result displayed by the probe: true positive (signal > SIG_{thresh} and WMM < 0.1), false positive (signal > SIG_{thresh} and WMM ≥ 0.1), true negative (signal < SIG_{thresh} and WMM ≤ 0.1) or false negative (signal < SIG_{thresh} and WMM < 0.1).

4. Exclusion of probes that exceeded, in the set of hybridizations, the number of allowed false positives (FP_{thresh}) or false negatives (FN_{thresh}).

5. Using PhyloDetect and the remaining probes, identification of the hybridized phylotypes and computation of the sensitivity, the specificity and the resulting AUC of the phylotype detection.

6. Repetition of steps 1 to 5 with different thresholds and parameters in order to maximize the AUC.

The above optimization determined best values for the three following thresholds:

–SIG_{thresh}: mean intensity of negative controls plus one standard deviation of the negative controls

–FP_{thresh}: maximum number of acceptable false positive results in the trainings set: 5

–FN_{thresh}: maximum number of acceptable false negative results in the training set: 0

For the detection of phylotype in environmental samples the following three additional PhyloDetect parameters were optimized (for detailed definition of these parameters see Rehrauer et al. (2008)):

–Minimum number of specific probes in order to consider a phylotype as detectable

–Expected average probe-wise false negative rate in the hybridizations

–Threshold of the phylotype-presence likelihood (a phylotype is detected in a hybridization if its likelihood score exceeds this value)

4.3.6 Sensitivity and specificity prediction

Calculation of Gibbs free energies of probe-target binding ($\Delta G°_b$) and probe-probe dimerization ($\Delta G°_d$) at 40°C were calculated using the Excel macro constructed by Matveeva et al. (2003), while Gibbs free energies of self looping probe ($\Delta G°_p$), local target denaturation ($\Delta G°_t$) and melting temperature at 40°C were determined with DINAMelt Server (Markham and Zuker, 2005). $\Delta G°_t$ was defined as the difference between the free energy of the DNA target and the free energy of the target with bases at the binding site prevented from pairing. Finally, the overall Gibbs free energy of probe-target binding ($\Delta G°_{overall}$) at 37°C (default temperature) was computed with RNAstructure v. 4.5 (Mathews et al., 1999). All calculation were processed with ionic conditions corresponding to 1 M Na^+.

The minimal WMM value was defined as the lower WMM value encountered by a probe against the 37 NifH phylotypes used in this study and the discrimination signal of a probe was calculated as the difference between its PM's and next WMM's signal intensities.

Relationship between the thermodynamic variables and PM signal intensity values, and between minimal WMM and discrimination signal values were estimated using linear regression model. Correlation degree was assessed using the Pearson correlation coefficient. Statistical work was computed in Microsoft Excel.

4.3.7 Spotted microarray platform experiment

A selection of 14 probes (Sigma-Genosys, Steinheim, Germany) showing specific (7), unspecific (3) and false positive (4) signals on the Geniom® One platform were spotted onto Slide H (Harbers et al., 2007) (Schott, Jena, Germany) in four concentrations (20 µM, 10 µM, 5 µM, 2.5 µM) in 150 mM phosphate buffer (pH 8.5) using a sciFLEXARRAY S11 piezoelectric spotting device (Scienion, Berlin, Germany). Slide H has a poly-(ethylene glycol) based three-dimensional surface chemistry, which acts as a linker and thus supports mobility and accessibility of immobilized probe molecules. Three

droplets (about 1.2 nL per spot) were deposited at the slide surface in six replicates. Oligonucleotides were immobilized in a humidity chamber (23°C, 75% relative humidity) over night.

Hybridizations were performed in an automated hybridization station (HS4800, Tecan, Salzburg, Austria). Briefly, DY-547-labeled *nifH* PCR products equivalent to 150 pmol of dye were dissolved in 65 µl MES/SSC buffer and hybridized for 48 h at 45°C. Slides were washed with 0.5 × SSPE at 23°C, and dried in the hybridization station with a flow of nitrogen. Slides were scanned using a confocal microscope scanner (Agilent, Santa Clara, USA). Raw data were evaluated using ArrayPro 4.5 software (Media Cybernetics, Bethesda, MD). Trimmed mean (trim 10%) fluorescence intensities of six replicates were averaged. After subtraction of the background noise, probes with signal intensities higher or equal to 10% of the target specific signal were considered as positive.

Finally, correlation degree between Geniom® One and spotted microarray experiments was evaluated using the Pearson correlation coefficient. The latter was calculated with normalized data, including values under the 10% presence threshold.

4.4 Results and discussion

4.4.1 Probe design

Forty-five NifH phylotypes, defined as group of amino acid sequences sharing at least 97% similarity, were found in Damma glacier forefield soils (Duc et al., 2009a). Using the ARB software package, a total of 2727 perfect match (PM) probes (20 nts) were designed to target specifically 37 NifH phylotypes. Due to technical reasons (bad growth of DNA clones), eight NifH phylotypes were omitted from the design. The phylogenetic characterization of these 37 phylotypes is presented in Fig.4-1.

4.4.2 Selection and validation of optimal probes

The efficiency and specificity of the 2727 candidate probes were tested using clonal DNA targets. *nifH* PCR products of clones corresponding to the 37 NifH phylotypes (Table 4-1) were labelled and hybridized individually on one to two microarrays each, using the Geniom® One platform.

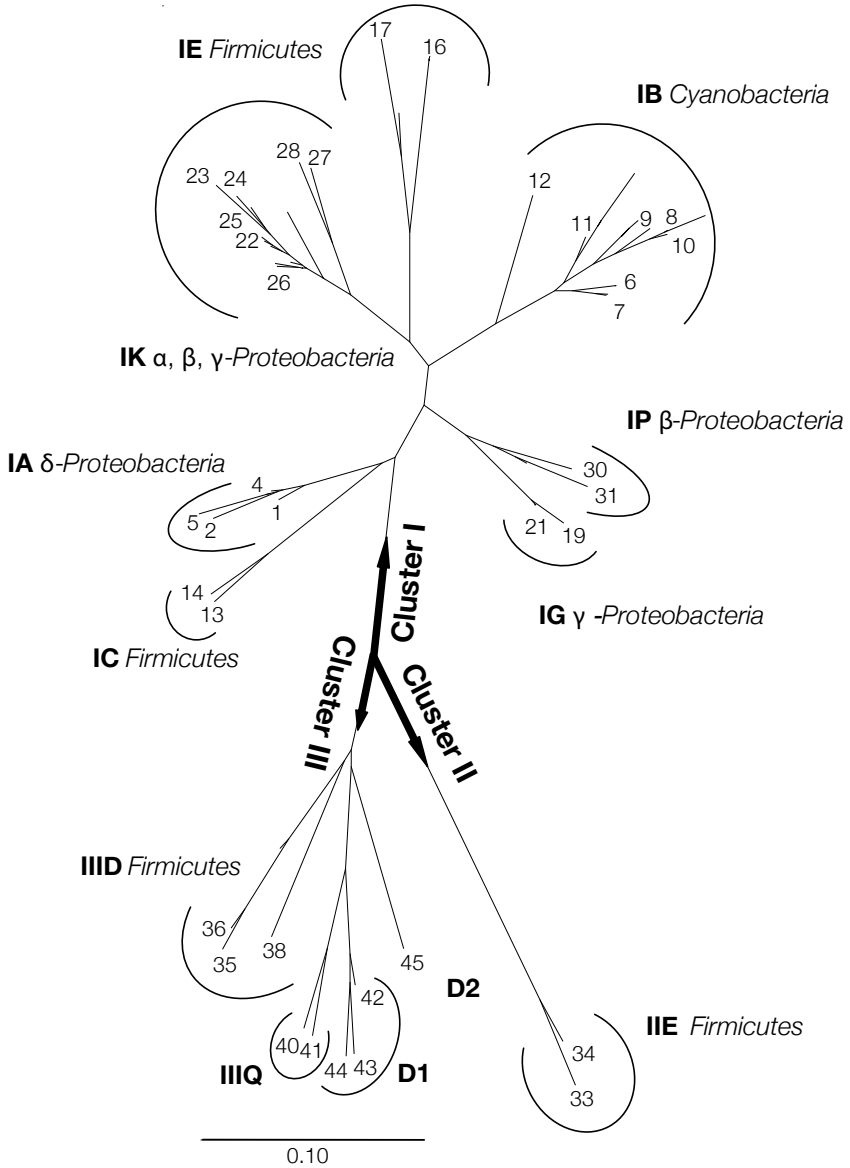


Figure 4-1 Unrooted phylogenetic NifH tree comprising 37 NifH protein sequences recovered from the Damma glacier forefield and corresponding to the phylotype listed in Table 4-1. Nineteen sequences from cultivated microorganisms showing more than 90% protein sequence similarity with one or more Damma phylotypes were included, but not numbered in the figure. The neighbour-joining tree was calculated using a Kimura correction (Kimura, 1980), considering 107 amino-acid positions and excluding highly variable regions within the sequences using a 30% amino acid frequency filter. Numbers correspond to the phylotype numeration.

Table 4-1 List of the 37 NifH targeted phylotypes and accession numbers of environmental clones used in this study

Targeted phylotype	NifH Cluster[a]	Bacterial group	Validation target	Closest cultivated species
1	IA	*δ-Proteobacteria*	EF988343	*Geobacter uraniumreducens*
2	IA	*δ-Proteobacteria*	EF988613	*Geobacter uraniumreducens*
4	IA	*δ-Proteobacteria*	EF988498	*Geobacter uraniumreducens*
5	IA	*δ-Proteobacteria*	EF988394	*Geobacter loveleyi*
6	IB	*Cyanobacteria*	EF988515	*Anabeana cylindrica*
7	IB	*Cyanobacteria*	EF988339	*Aphanizomenon* sp.
8	IB	*Cyanobacteria*	EF988413	*Nostoc muscorum*
9	IB	*Cyanobacteria*	EF988443	*Nostoc* sp.
10	IB	*Cyanobacteria*	EF988371	*Scytonema* sp.
11	IB	*Cyanobacteria*	EF988371	*Pseudanabaena* sp.
12	IB	*Cyanobacteria*	EF988431	*Oscillatoria* sp.
13	IC	*Firmicutes*	EF988543	*Desulfitobacterium hafniense*
14	IC	*Firmicutes*	EF988498	[c]
16	IE	*Firmicutes*	EF988392	[c]
17	IE	*Firmicutes*	EF988586	*Paenibacillus durus*
19	IG	*γ-Proteobacteria*	EF988362	*Azotobacter vinelandii*
21	IG	*γ-Proteobacteria*	EF988617	*Azotobacter vinelandii*
22	IK	*β-Proteobacteria*	EF988359	*Ideonella* sp.
23	IK	*γ-Proteobacteria*	EF988533	*Methylococcus capsulatus*
24	IK	*γ-Proteobacteria*	EF988461	*Methylococcus* sp.
25	IK	*β-Proteobacteria*	EF988522	*Ideonella* sp.
26	IK	*α-Proteobacteria*	EF988406	*Methylocystis* sp.
27	IK	*α-Proteobacteria*	EF988455	*Methylocystis minimus*
28	IK	*α-Proteobacteria*	EF988538	*Methylocystis* sp.
30	IP	*β-Proteobacteria*	EF988366	*Dechloromonas aromatica*
31	IP	*β-Proteobacteria*	EF988427	*Dechloromonas aromatica*
33	IIE	*Firmicutes*	EF988411	[c]
34	IIE	*Firmicutes*	EF988548	*Heliobacterium* sp.
35	IIID	*Firmicutes*	EF988547	*Clostridium pasteurianum*
36	IIID	*Firmicutes*	EF988537	*Clostridium pasteurianum*
38	IIID	*δ-Proteobacteria*	EF988621	[c]
40	IIIQ	[b]	EF988357	[c]
41	IIIQ	[b]	EF988495	[c]
42	D1	[b]	EF988465	[c]
43	D1	[b]	EF988525	[c]
44	D1	[b]	EF988609	[c]
45	D2	[b]	EF988384	[c]

[a] According to the *nifH* phylogeny defined by Zehr et al. (2003)
[b] Uncultured subcluster
[c] No cultivated species showing more than 90% similarity

Hybridizations, showing overall PM signals higher than MM signals, were used to check the quality of the probes. Phylotype detection was performed using the PhyloDetect web application (Rehrauer et al., 2008), and processing parameters were tested by a Receiver-Operator-Characteristic (ROC) curve analysis and optimized by the maximization of the Area-Under-Curve (AUC) value (see Material and methods). The optimized strategy is shown in Fig. 4-2 A. Shortly, the optimal signal intensity threshold (SIG_{tresh}) was equal to the mean intensity of negative controls (neg-AVG) plus one standard deviation (SD). Further, a probe was selected if it was showing a maximum of five false positive results and no false negative results.

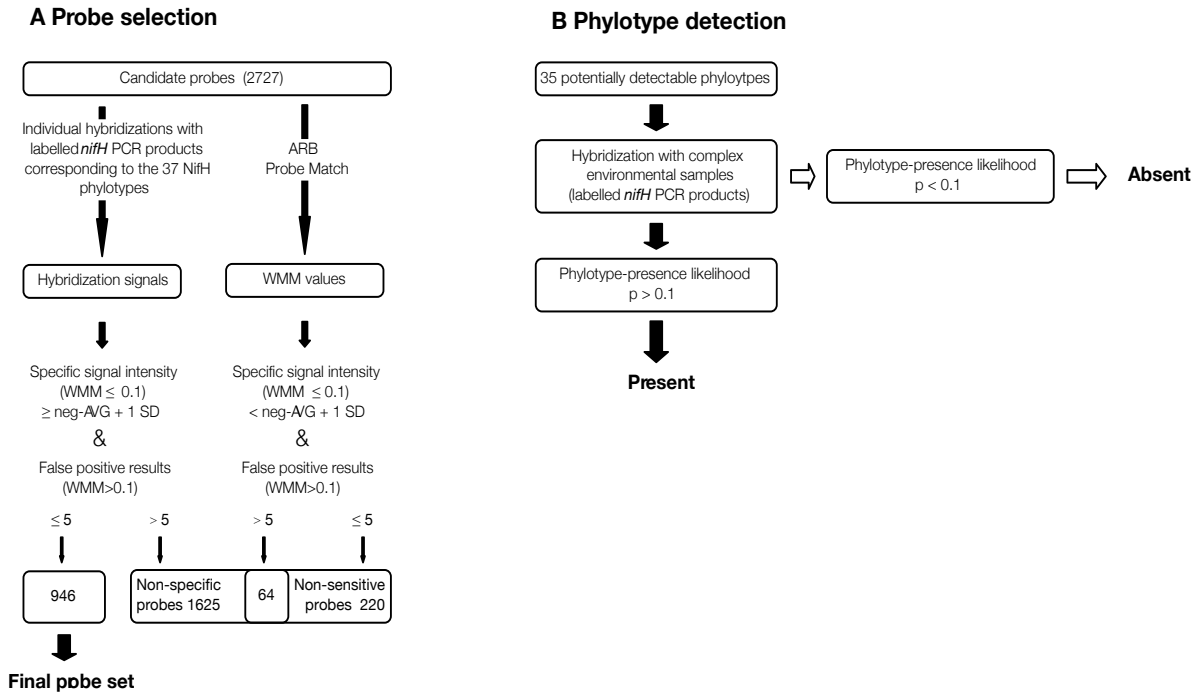


Figure 4-2 Schematic of probes selection (A) and phylotype detection (B) approaches. Abbreviations: WMM; weighted mismatch, neg-AVG; mean intensity of negative controls, SD; standard deviation, p; phylotype-presence likelihood.

Fig. 4-3 illustrates the performance of the microarray-based phylotype detection after the optimization of processing parameters. Considering this optimal configuration, the best ratio between sensitivity and specificity was reached using a phylotype-presence likelihood threshold of 0.3. Under these conditions, 946 probes (Table S1 available online) were qualified as sensitive and specific. Probes displaying low sensitivity or poor specificity are listed in Table S2.

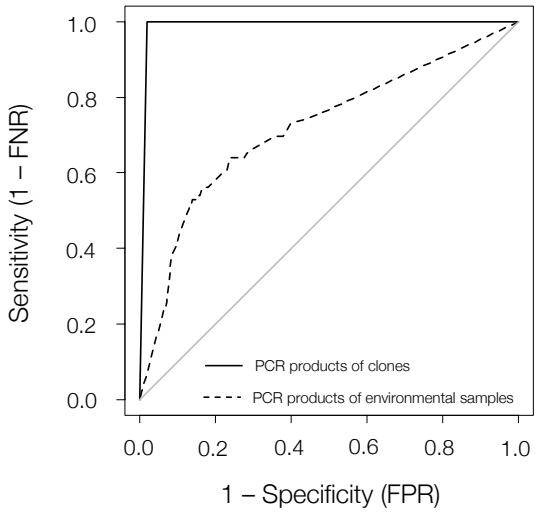


Figure 4-3 Optimized Receiver-Operator-Characteristic (ROC) diagrams for the clone experiment (solid line) and the environmental samples experiment (dashed line) after optimization of processing parameters. The plot shows the high identification of clonal DNA targets. The performance observed for the environmental samples was lower but well above a random identification (gray line). In both data sets the cloning-sequencing based identification was taken as a reference. Abbreviations: FPR; False Positive Rate, FNR; False Negative Rate.

The probe set yielded correct discrimination of 27 phylotypes. For technical reasons no hybridization data were produced for phylotypes 4, 5, 10, 14, 16, 24, 31 and 33. Probes corresponding to these eight phylotypes were therefore not validated experimentally. Nevertheless, they were kept in the probe set as long as they did not produce any false positive signal. For each of the 35 phylotypes, the best probe was selected regarding the discrimination signal (difference between PM and next MM signal intensities) and is listed in Table 4-2.

The final probe set covers 12 out of the 14 NifH subclusters found at the Damma glacier forefield site and represents an important tool to enhance the study of this environment. The high amount of specific probes per phylotype (up to 80) reduces the risk of false positive detection while keeping the possibility to enhance the sensitivity and lower the specificity by varying the phylotype-presence likelihood threshold in the PhyloDetect parameter configuration.

Table 4-2 Best probes a designed for each of the 35 NifH phylotypes discriminated by the nifH microarray

Name	Targeted phylotype	Sequence	Tm[a]	GC%	ΔG°_{b} [b] kcal/mol
Damma1-NifH-2	1	GTATTCTGCGCCTTTGCGTG	76.9	55	-29.3
Damma2-NifH-1	2	CCGTTGACTGTGCCTTTGCG	79.2	60	-30.2
Damma4-NifH-1	4	ACACCGGCATAACCGACCTT	79.3	55	-28.9
Damma5-NifH-1	5	CGGTGCCGCGATCACGCACC	85.9	75	-33.3
Damma6-NifH-1	6	ACATGACTTCGTGGAGTTCC	74.4	50	-26.4
Damma7-NifH-2	7	GGTTGTTTGAGCCTTGGAGT	75.4	50	-28.2
Damma8-NifH-1	8	CAGGGCCACCAGATTCTACG	77.3	60	-28.9
Damma9-NifH-1	9	GCTGCGAGGTGAAGAACGGT	79.9	60	-27.1
Damma10-NifH-1	10	CGCCCCGGAAGCCAGTCAAC	83.2	70	-32.3
Damma11-NifH-1	11	GTAGTTTGGGCTTTGCTGTG	74.2	50	-27.4
Damma12-NifH-1	12	ACTCCAGGCTCTGAACCGCC	81.8	65	-31.8
Damma13-NifH-1	13	ACAGTCCCTCTCTCGCGGGC	83.6	70	-33.8
Damma14-NifH-1	14	CGCGCCATGTCCATGACCGT	82.9	65	-32.5
Damma16-NifH-1	16	GCCGACAACATCTGCTAGCT	77.4	55	-28.4
Damma17-NifH-1	17	AGAGGATGTCGCCAAAGCCA	79.4	55	-28.0
Damma19-NifH-1	19	TTTCGCCGGAGCAGAACGAT	79.6	55	-27.3
Damma22-NifH-1	22	GGATCACGCGGGTCGAGTCG	81.9	70	-29.9
Damma23-NifH-8	23	CAGACGGGTCGAATCGGCGT	81.8	65	-28.7
Damma24-NifH-1	24	GGATATCCATGTAGCCCACT	73.7	50	-28.8
Damma25-NifH-1	25	GCTACCAGCTTCAGCAGCTA	78.3	55	-29.9
Damma26-NifH-1	26	CCTCGAGTTCGAGGTCCTCG	78.4	65	-30.4
Damma27-NifH-1	27	GAGGTAATCACACCACGTCC	74.7	55	-27.2
Damma28-NifH-1	28	ATCCTCAACGCTGCCAAGTT	77.5	50	-28.2
Damma31-NifH-1	31	GCGTGCAAGATCAAACGAGT	75.9	50	-24.3
Damma33-NifH-1[c]	33	ATTGGCATTGCAAATCCGCC	77.6	50	-27.6
Damma34-NifH-1	34	GTCAATGGCTGTGATGACTC	73.1	50	-25.3
Damma35-NifH-1	35	GTGCTTTTCCCTCACGCATT	76.3	50	-30.0
Damma36-NifH-1	36	AGCACCCAACGCTTCTAGCA	79.4	55	-29.7
Damma38-NifH-1	38	GTGTAGTCGAGATCGGAGGT	75.2	55	-26.9
Damma40-NifH-1	40	CTCCAGCAGGTTGATCGAAG	75.1	55	-26.4
Damma41-NifH-1	41	GTGTTGCCGTATCCGATTCT	74.9	50	-28.9
Damma42-NifH-1	42	AGTACGTCTCGCCATATCCT	75.0	50	-28.8
Damma43-NifH-1	43	TCTTCTGCACTAAGCCACCG	77.6	55	-28.5
Damma44-NifH-1	44	CCTCTGCGACGTACATCTTC	74.6	55	-27.8
Damma45-NifH-1	45	ACGCCTGACTCAGGGCCGCC	87.1	75	-34.9

[a] Probes were selected regarding the discrimination signal (difference between PM and next MM signal intensities) except for false negative phylotypes (4, 5, 10, 14, 16, 24, 31, 33) which were checked considering the minimal WMM (lower MM value encountered against the 37 NifH phylotypes).
[b] Melting temperature and ΔG°_{b} were calculated with temperature set at 40°C.
[c] Probe showing co-hybridization with phylotype 13.

No useful probes were found for phylotypes 21 and 30. The high nucleotide sequence similarity (90%) shared by phylotype 21 and 19 might explain the difficulty to discriminate them. Effectively, both phylotypes displayed a high proportion of unspecific probes (97% and 87%, respectively) and only two sensitive and specific probes were found for phylotype 19. Phylotype 30 was part of the few phylotypes for which only small amount of probes were designed. This resulted from the high similarity with *nifH* sequences that were not necessarily found in Damma glacier forefield soils, but were present in the *nifH* database.

4.4.3 Platform comparison

Cross-platform consistency is necessary to validate microarray technologies and to build universal probe databases (Draghici et al., 2006). Fourteen probes showing specific, unspecific and false-positive signals on the Geniom® One platform were tested against eight phylotypes, applying a spotted microarray approach with a 5'-end probe's immobilization similar to that produced with the Geniom® One device. This experiment demonstrated that the probe set selected by experimental testing was compatible with at least one other microarray platform.

Effectively, the large majority of the probes gave identical results on both platforms (Fig. 4-4). Only three probes (Damma7-NifH-33, Damma16-NifH-36, Damma45-NifH-63), showing weak unspecific signals (<15% of the target specific signal) on the Geniom® One platform, were negative or represented less than 10% of the target specific signal on the spotted microarray. Except for these three probes, correlations between both technologies were high, with Pearson correlation coefficients higher than 0.98 for all specific probes.

4.4.4 Detection of NifH phylotypes in environmental samples

The ability of the microarray to detect NifH phylotypes in complex environmental samples was assessed by comparing analyses of five DNA extracts using microarray (this study) and cloning-sequencing methodologies (Duc et al., 2009a). The SIG_{tresh} was kept the same as for the selection of optimal probes. As expected, hybridizations with environmental samples led to a less performing phylotype identification compared to the clone experiments (Fig. 4-3). This might be explained by the higher complexity of diazotrophic communities and the lower proportion of unique NifH phylotypes. Therefore, the stringency of the PhyloDetect parameters had to be reduced to optimize the matching with cloning-sequencing data. The optimal minimum number of specific probes needed to consider a phylotype as detectable and the optimal average probe-wise false negative rate were set to 2 and 0.3, respectively. Finally, a phylotype-presence likelihood threshold of 0.1, leading to the best ratio between sensitivity and specificity, was used for the phylotype detection (Fig. 4-2 B).

The five microarray experiments detected 79% of the NifH diversity found by cloning-sequencing. Thirty additional phylotypes (sum of the five hybridizations) were detected, pointing out that with a maximum of 40 *nifH* sequences, clone

libraries were far too small to profile completely diazotrophic communities in Damma glacier forefield soils. The sensitivity of the *nifH* microarray was slightly higher than that observed in other 20 nts microarray studies (Bodrossy et al., 2003a; Sanguin et al., 2006), with the detection of phylotypes accounting for 3.5% of a single clone library.

Finally, all undetected phylotypes had low abundances in clone libraries and represented 5% of all sequences retrieved from the five soil samples. The two methodologies were processed from different PCR amplification products. Consequently, PCR biases might partially explain the inconsistency of both methods with regard to these infrequent phylotypes. Overall, this experiment showed that the *nifH* microarray is a valuable tool to detect NifH phylotypes in environmental samples.

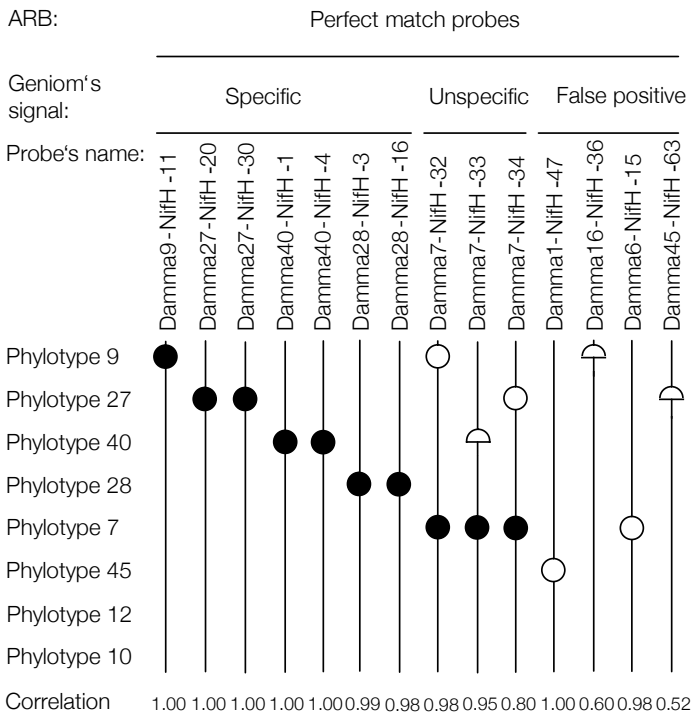


Figure 4-4 Comparison of Geniom® One and spotted microarray platforms' results for a selection of 14 probes showing specific, unspecific and false positive on the Geniom® One platform, for eight defined targets. Circles indicate phylotypes detected by both platforms and half circles, phylotypes detected only by the Geniom® One platform. Black fills stand for specific signals and white fills for unspecific signals. False positive probes are not listed in Table S1 and correspond to the following sequences: Damma7-NifH-32 AACGGTTGTTTGAGCCTTGG; Damma7-NifH-33 CGGGTAGAGTCAGCATTCGG; Damma7-NifH-34 CTTTCAGCAGCCAAGTGAAG; Damma1-NifH-47 CGTCCTGTATTTCCAGGTCC; Damma16-NifH-36 CACGGTTTGCTGGGCCTTCG; Damma6-NifH-15 CAGCATCAAACGGGTAGAGT; Damma45-NifH-63 TGGTAGAATCGGCATTGGGG. Correlation coefficients (n = 8) describe the degree of association between Geniom® One and spotted microarray results for each probe.

4.4.5 *In silico* design: relationship between hybridization predictors and experimental data

A major finding of this work was the extremely low yield of valuable probes designed *in silico* using ARB. Effectively, almost two thirds of the initial probe set were discarded due to low sensitivity (9%) or poor specificity (91%). This points out the concern of interpreting data generated with partially validated microarrays, and the necessity to test probes against all possible targets present in the environment. The analysis of the accuracy of seven hybridization predictors, using hybridization data of four phylotypes (2, 13, 27 and 45), confirmed this statement. For all PM probes present in the initial set (41 probes per phylotypes in average), relationships and correlations between melting temperature, Gibbs free energies ($\Delta G°_b$, $\Delta G°_d$, $\Delta G°_p$, $\Delta G°_t$, $\Delta G°_{overall}$) and signal intensity, and between minimal weighted mismatch (WMM) value and discrimination signal were tested.

Table 4-3 Pearson correlation coefficients of hybridization predictors relative to signal intensity and discrimination signal values

Predictor	Phylotype2	Phylotype 13	Phylotype 27	Phylotype 45
	Signal intensity			
$\Delta G°_b$ (40°C) Matveeva	0.66	0.71	-0.04	0.69
$\Delta G°_d$ (40°C) Matveeva	0.10	0.03	-0.04	-0.12
$\Delta G°_p$ (40°C) DINAMelt	0.13	0.39	-0.03	0.08
$\Delta G°_t$ (40°C) DINAMelt	0.52	0.65	-0.04	0.40
Tm (40°C) DINAMelt	0.24	-0.45	-0.33	0.57
$\Delta G°_{overall}$ (37°C) RNA structure	-0.47	0.39	0.01	0.34
	Discrimination signal			
Minimal position weighted mismatch	0.19	0.16	0.17	-0.16

In agreement with Pozhitkov et al. (2006), the analysis of the relationship between theoretical and experimental data revealed poor linearity. For five out of seven predictors, R^2 values were below 0.2 (data not shown). Only $\Delta G°_b$ and $\Delta G°_t$ might be useful, with R^2 values of 0.44 and 0.25 for phylotype 2, 0.51 and 0.32 for phylotype 13, and 0.47 and 0.16 for phylotype 45. Values for phylotype 27 were close to zero for both predictors. Correlation values for these two predictors and phylotypes 2, 13, and 45 (Table 4-3) were higher than that found by Matveeva et al. (2003) and seemed to agree with results by Weckx et al. (2007), giving $\Delta G°_b$ as a suitable predictor for signal intensity. Nevertheless the $\Delta G°_b$ predictor has to be used with caution as it was correlated only with three out of four experimental data sets. Moreover, high signal intensity is necessary but not sufficient requirement to define good probes.

We also studied the signal intensities of all candidate probes as a function of the WMM value (Fig. 4-5). The specificity of a probe against a defined target was not efficiently predicted by the WMM value. This is not surprising as several studies (Sugimoto et al., 2000; Pozhitkov et al., 2006) denoted high occurrence of brighter MM signal compared to PM signal, manifestly due to the gap created by a MM, presenting two purines, favouring the binding of target labelled with fluorescent pyrimidine (Naef and Magnasco, 2003). Nevertheless, the use of modified parameters, as described by Yilmaz et

al. (2008) to calculate the WMM value, might increased the strength of this predictor.

In conclusion, the experimental approach chosen in this study revealed to be highly valuable for the validation of the microarray probe set. It enabled the selection of 946 probes for the sensitive and specific detection of 35 NifH phylotypes present at the Damma glacier forefield. Additionally, we have shown that the final probe set is consistent and adequate to analyze environmental samples. It represents the first *nifH* microarray set experimentally validated and thus constitutes a promising tool to provide solid data on the diversity and activity of free-living diazotrophs in alpine environment.

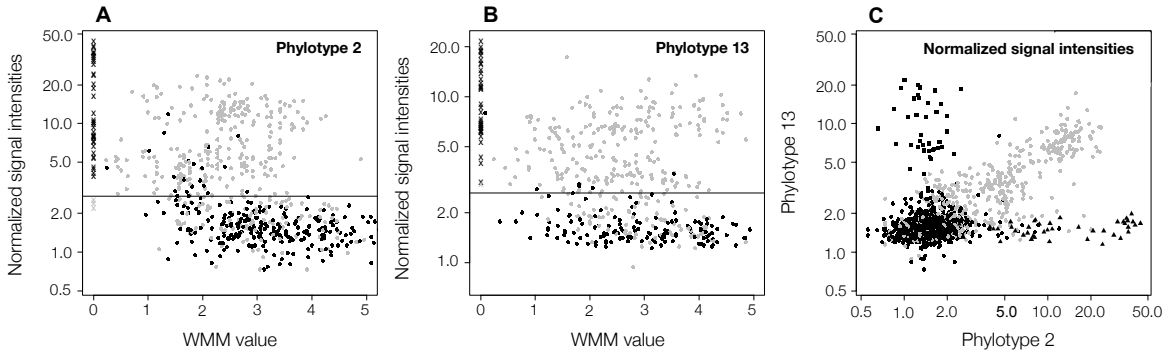


Figure 4-5 Hybridization signal intensities of the initial probe set (2727), arranged according to their WMM value for phylotypes 2 (A), 13 (B) and as a comparison of both phylotypes (C). WMM values were determined by taking into account the amount, the type and the position of MM and were calculated using ARB default parameters. Probes with more than 5 MM are not plotted. Probes in black are present in the final probe set, while probes in grey deleted because of bad sensitivity or bad specificity. Symbols: A and B: PM probe (x), MM probe (●), C: probes specific for Phylotype 13 (■), specific probes for Phylotype 2 (▲).

5 ASSESSING THE IMPACT OF ENVIRONMENTAL FACTORS ON FREE-LIVING DIAZOTROPHS IN A GLACIER FOREFIELD BY USE OF A *NIFH* MICROARRAY

Duc L., Neuenschwander S., Rehrauer H., Zeyer J.

FEMS Microbiology Ecology, submitted

5.1 Abstract

Glacier forefield environments are exposed to extreme and fluctuating climatic and nutritional conditions. The high diversity of free-living diazotrophic communities found in these environments indicates that nitrogen fixers are able to efficiently cope with such conditions. In this study, we examined the effect of environmental variations on the structure of free-living diazotrophic populations and identified microorganisms showing significant changes in their abundances under different environmental conditions. Impacts of plant presence, bedrock type (siliceous/calcareous), season, temperature (10°C, 15°C) and nitrogen deposition (0, 10, 40 kg N (ha year)$^{-1}$) were assessed using a diagnostic *nifH* microarray. Population structures were responding highly dynamically to environmental changes. Plant presence had the strongest impact, which was decreasing toward the end of the season and with high amount of nitrogen deposition. Temperature and nitrogen deposition increases indirectly affected diazotrophic communities through their positive impact on plant growth. These results indicate strong carbon limitation in young glacier forefield soils. Phylotypes related to the genus *Methylocystis* strongly responded to environmental variations. These methanotrophic microorganisms able to retrieve nitrogen and carbon from the atmospheric pool, are particularly adapted to extreme nutritional conditions found in glacier forefields and therefore might play key role in maintaining nitrogen fixation in this particular environments.

5.2 Introduction

Diazotrophs are microorganisms with the ability to fix atmospheric nitrogen. They have been described in diverse habitats like lakes, oceans, estuaries, marshes, microbial mats, sediment, soil, plants and invertebrates (Zehr et al., 1998; Braun et al., 1999; Jacot et al., 2000b; Lovell et al., 2000; Zani et al., 2000; Ohkuma et al., 2002; Jenkins et al., 2004; Omoregie et al., 2004; Izquierdo and Nusslein, 2006; Yannarell et al., 2006; Montoya et al., 2007; Langlois et al., 2008; Wartiainen et al., 2008). They belong to different trophic levels, ranging from phototrophs (anoxygenic phototrophic microorganisms, *Cyanobacteria*) to chemotrophs (Eady, 1992). The latter represent a large proportion of all diazotrophic species, and can be divided in symbiotic and free-living microorganisms. In soil ecosystems, symbiotic diazotrophs have been widely studied and are known to contribute significantly to the nitrogen input (Jensen and Haugaard-Nielsen, 2003). However, much less is known about the role of free-living diazotrophs and the factors controlling the composition of their populations, especially in nitrogen-poor pioneer sites (e. g., like glacier forefields, volcanoes, sand dunes, post mining sites).

Former work at the Damma glacier forefield (Central Switzerland) revealed a broad distribution and a high diversity of free-living diazotrophic soil communities (Duc et al., 2009a), supporting the idea that the ability to fix nitrogen provides a selective advantage in nitrogen-poor environments. In glacier forefields, pioneer plants represent an important source of carbon (e. g., root exudates, decaying biomass) which are expected to change dramatically the local soil nutritional status (Grayston et al., 1997). Plants and soil microorganisms are part of a dynamic equilibrium along the season, in respect to uptake and release of nitrogen (Bardgett et al., 2005). Besides these spatial and temporal nutritional variations, glacier forefield environments are characterized by extreme and highly variable climatic conditions, including daily temperature fluctuations of more than 30°C (Miniaci, 2007). Therefore, glacier forefields seem to be ideal ecosystems to study the response of free-living diazotrophs to environmental variation.

Impacts of environmental factors on soil free-living diazotrophs have been extensively studied at the level of the microbial nitrogen fixation activity (Chapin et al., 1991; Liengen, 1999; Söderberg and Bååth, 2004; Sorensen et al., 2006; Miniaci, 2007). For example, low temperature and low soil moisture were shown to be the major factors restricting the fixation activity, while impacts of phosphorus and nitrogen fertilization are less strong. However, only few studies examined such impacts on the structure and the diversity of free-living diazotrophic populations. Variations in fixation activity were shown to be independent of changes observed at the population level (Deslippe et al., 2005). Temperature increase seemed to have strong influences on the community structure (Deslippe et al., 2005; Sabacka and Elster, 2006), whereas the latter remained stable after short-term nitrogen amendment or plant exudates shift (Piceno and Lovell, 2000; Deslippe et al., 2005). Further, season and soil pH have been shown to induce strong shifts in total soil microbial communities (Bardgett et al., 2005; Fierer and Jackson, 2006), however their impacts on free-living diazotrophs have not been investigated.

A diagnostic *nifH* microarray was successfully designed to detect free-living diazotrophs in glacier forefield soil samples (Duc et al., 2009b). This tool displays a higher resolution than semi quantitative methods (denaturing gradient gel electro-

phoresis, terminal restriction length polymorphism) which were previously used to investigate the response of free-living diazotrophs to environmental variations. The application of this technology is therefore expected to provide more details on this response and to allow the identification of highly reactive species.

In this project we used the diagnostic *nifH* microarray to study the impact of changing environmental parameters including plant presence, season, bedrock type, air temperature and nitrogen deposition on free-living diazotrophic communities in two glacier forefields. The forefields were located on a siliceous (Damma glacier forfield, central part of Switzerland) and calcareous bedrock (Tsanfleuron glacier forefield, western part of Switzerland). Variations in air temperature and nitrogen deposition were simulated in the laboratory in a pot experiment using soil from the siliceous site.

5.3 Material and methods

5.3.1 Study sites

Two forefields of receding glaciers were investigated. The recession of the glaciers has been monitored for more than 80 years and the data are available on www. glaciology.ethz.ch/swiss-glaciers. The first site is located in the forefield of the Damma glacier (E 8° 27', N 46° 38') in the canton of Uri (Central Switzerland) at an altitude of 2060 m above sea level. The exposition of the forefield is North-East. The site is characterized by a mean annual air temperature of between -1°C and 6°C (1998-2008), a mean annual precipitation of 2400 mm (www.meteoswiss.admin.ch), and nitrogen deposition ranging between 0-10 kg N (ha year)$^{-1}$ (www.bafu.admin.ch). The parent rock of the forefield is granite. The second site is located in the forefield of the Tsanfleuron glacier (E 7° 15', N 46° 19.5') in the canton of Valais (West Switzerland) at an altitude of 2440 m above sea level. Characteristics of this site are different from that of the Damma glacier forefield. It has an east exposition, receives a higher amount of nitrogen deposition (10-15 kg N (ha year)$^{-1}$) and lies on carbonate rock. Mean annual temperatures range between -5°C and 0°C (1998-2008) and mean annual precipitation reaches 1800 mm.

5.3.2 Sample collection

In the Damma glacier forefield, 8-year ice-free bulk soils and rhizosphere soils from Leucanthemopsis alpina were sampled in 2005 as previously described (Duc et al., 2009a). In total, triplicates for each type of soil were collected at five time points (S1-S5) during the snow-free season (June 28th, July 18th, August 17th, September 5th, and September 28th). The soil used for the pot experiment was collected in September 2006, at eight sites along a transect perpendicular to the border moraines and which corresponded to 8-year ice-free glacier forefield. In the 8-year ice-free Tsanfleuron glacier forefield, five bulk soils were sampled at June 15th 2006 and September 14th 2007. Soil collection sites were localized using a Geographic Information System (ArcMap™) and Global Positioning System (GPS).

5.3.3 Pot experiment

Microcosms were set up with the soil collected in the 8-year ice-free Damma glacier forefield. The soil was mixed and sieved through a 2 mm mesh and distributed in 96 plastic pots (Ø: 7 cm, height: 8 cm) (120 g soil/pot). Seeds of Leucanthemopsis alpina (Sandeman Seeds, Lalongue, France) were placed on sterile humid cotton wool for germination. After an incubation of four days in the dark followed by four days in the light, plantlets without fungal contamination and displaying a root (> 2 cm) were planted in half of the pots (four plantlets/pot) and were directly watered with sterile nutrient solution (0.156 g L^{-1} $CaHPO_4$ $2H_2O$, 0.226 g L^{-1} KH_2PO_4; 0.72 g L^{-1} $MgSO_4$ $7H_2O$, 2.0 mg L^{-1} $FeSO_4$ $7H_2O$, 1.37 mg L^{-1} $MnSO_4$ H_2O, 2.86 mg L^{-1} H_3BO_3, 0.22 mg L^{-1} $ZnSO_4$ $7H_2O$, 0.08 mg L^{-1} $CuSO_4$ $5H_2O$, 0.094 mg L^{-1} Na_2MoO_4 $2H_2O$, 0.56 µg L^{-1} $CaCl_2$ $6H_2O$). Microcosms were maintained for seven months at 10°C or 15°C (24 pots with plants and 24 pots without plant per temperature) with a light regime of 12 hours per day (Nurturelite Purple 125W, Warrington, UK). Twice a month, pots received sterile NH_4NO_3 solution to mimic three different nitrogen deposition levels: 0, 10, 40 kg N $(ha\ year)^{-1}$ (treatments N00, N10 and N40). Additionally, 10 ml of nutrient solution was added every two weeks and microcosms were kept moist with sterile deionized water. In total, each treatment contained eight replicates. After seven months, rhizosphere soils adhering the roots as well as bulk soils from the microcosms without plant were sampled and used for further analysis.

5.3.4 Chemical and physical characterization of soils

Soils from both field sites were constituted of gravels and coarse sand. Soil nitrate and pH were measured in 0.01 M $CaCl_2$ soil extracts (2:5 soil/extractant ratio) (Duc et al., 2009a), using a Dionex DX-320 ion chromatograph (Dionex, Sunnyvale, CA, USA) and a Mettler-Toledo 409 pH electrode (Mettler-Toledo, Greifensee, Switzerland), respectively. Available ammonium was quantified photometrically in 2 M KCl soil extracts (1:5 soil/extractant ratio)(Mulvaney, 1996). Total carbon (C_{tot}) and nitrogen (N_{tot}) contents were determined by combustion of 1 g of ground soil at 1200°C, using a Leco 2000 CNS device elementary analyser (Leco, Krefeld, Germany) following manufacturer's instructions. Soil samples were dried at room temperature and weighted to assess soil moisture. Total dissolved organic carbon (DOC) was measured in 1:10 water extracts using a TOC-analyzer (TOC-5000, Shimadzu, Kyoto, Japan). Soil samples from pot experiment were subject to the same investigations. Effects of plant presence, nitrogen deposition, temperature, bedrock type, season and their interaction were examined for each soil properties by means of a multivariate analysis of variance (MANOVA), with season (field experiment) and nitrogen deposition (pot experiment) as the repeated measures variable, using JMP software (Sall et al., 2001).

5.3.5 Acetylene reduction assay

Potential asymbiotic nitrogenase activity was estimated using the acetylene reduction assay (ARA) (Bürgmann et al., 2003). Briefly, bulk-soil and rhizosphere-soil samples were weighed and placed in serum bottles, saturated or not with sterile water and sealed with gas-tight neoprene septum lids to facilitate the sampling of gas. The headspace was flushed with N_2, before the addition of 10% (v/v) acetylene (C_2H_2). The bottles were incubated at 15°C with 14 hours of artificial light per day. Ethylene (C_2H_4) production was measured once a week, by injecting 0.5 ml headspace into a 8000 GC gas chromatograph (Carlo Erba Instruments, Milan, Italy) equipped with a flame-ionization detector (GC/FID) and a Hayesep N capillary column (BGB Analytik, Auwil, Switzerland) at 75°C. Due to the slow nitrogenase activity of the samples, rates of ethylene production could not be monitored and ethylene concentrations were simply measured at the stationary phase. Effects of plant presence and season on the ethylene production were tested as described above.

5.3.6 Nucleic acid extraction and target preparation

DNA was extracted from 0.7 g of (fresh weight) soil using a PowerSoil™DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, USA). Replicates of the pot experiment were reduced to four by pooling DNA extracts two by two. DNA concentration and purity were checked spectrophotometrically using the NanoDrop ND-1000 device (Thermo Fisher Scientific, Waltham, USA).

Amplification of *nifH* genes was completed using the nested PCR approach defined by Bürgmann et al. (2004). PCR triplicates were pooled and purified with QIAquick PCR purification columns (Qiagen, Hombrechtikon, Switzerland). PCR products, corresponding to 2.4 µg DNA, were labelled with 3 µl DY-547- labelled dUTP (1 mM) (Dyomics GmbH, Jena, Germany) using the BioPrime Array CGH Genomic Labeling System Kit (Invitrogen, Basel, Switzerland) and purified with mini Quick Spin Columns (Roche Applied Science, Rotkreuz, Switzerland). Dye incorporation was measured spectrophotometrically at 550 nm.

5.3.7 Microarray experiment

Microarray analyses were performed on the Geniom® One platform (Febit GmbH, Heidelberg, Germany) (Beier and Hoheisel, 2000) using the *nifH* probe set (*n*=946) developed by Duc et al. (2009b) to detect NifH phylotypes present glacier forefield soils. Briefly, quadruplicates of the entire probe set, 81 negative-control and 36 Geniom® One positive-control oligonucleotides were randomly and simultaneously synthesized in situ in eight independent arrays (3D micro-channels) comprised in one chip, using a light modulator matrix. DY-547-labeled *nifH* PCR products, equivalent to 150 pmol of dye, were dissolved in 24 µl hybridization buffer (54.8 mM MES (morpholinoethansulfonic acid) free acid, 147.7 mM MES sodium salt, 1.8M NaCl (total NaCl concentration 1.9 M), 40 mM Na_2EDTA, 0.2% Tween 20, 0.5 mg mL^{-1} BSA, 0.1 mg mL^{-1} Hering sperm DNA, 2 nM Cy3-labeled positive control) and hybridized in the micro-channels for 16 hours at 40ºC

with active mixing. A first washing step at 25°C using 6x SSPE buffer was followed by two steps at increasing stringency conditions (25°C, 2x SSPE; 40°C 2x SSPE). Signal intensities were recorded after each washing step by the built-in CCD camera. DNA samples from sampling times S2 and S3 were not examined by microarray analysis.

5.3.8 Microarray data analysis

Hybridization intensities of replicated spots were averaged. Subsequently the mean of the negative control probes was subtracted from the intensity of each probe and results were divided by the standard deviation of the negative controls.

Changes in abundance of NifH phylotypes were computed with the PhyloDetect web application (Rehrauer et al., 2008; Duc et al., 2009b) through a comparative analysis of samples hybridized on the same chip. Within this analysis, a probe was considered to have different signal intensities between two hybridizations, if the intensities' $\log_2$ ratio was above 0.3 or below -0.3. For each phylotype, PhyloDetect was used to compute the likelihood that probes with higher signal intensities resulted from an increase in the phylotype abundance, and vice versa. Phylotypes were considered to have increased or decreased abundances in the presence of at least three matching probes showing higher or lower signal intensities and a likelihood value above 0.05.

Effects of environmental factors on the abundance of NifH phylotypes were tested using JMP software (Sall et al., 2001). A two-way analysis of variance (ANOVA) was applied in the field experiment, while the pot experiment was examined by means of MANOVA, with nitrogen deposition (15°C over 10°C and rhizosphere soil over bulk soil sub-experiments) and temperature (nitrogen deposition sub-experiment) as the repeated measures variables.

5.4 Results

5.4.1 Biogeochemistry of field samples

Biogeochemical properties of the field samples are listed in Table 5-1. For better visualization some of the data are also presented graphically (Fig. 5-1).Generally values for nitrogen and carbon compounds were low but above the detection limit except for N_{tot} and C_{tot} in S5 calcareous samples and S3 siliceous samples.

The presence of the plant was the strongest environmental factor, with six out of eight measured soil biogeochemical parameters discriminating significantly ($p<0.01$) bulk soil samples from rhizosphere soil samples (Table 5-2). DOC, C_{tot}, DNA and moisture were significantly increased in presence of the plant, while nitrate was negatively affected. The pH was significantly lower in rhizosphere soils than in bulk soils. Ammonium concentrations were similar in both types of soil.

Table 5-1 Biogeochemical properties and nitrogenase activity for the soils used in the field experiment

	Nitrogen [µg N (g dry soil)$^{-1}$]			Carbon [µg C (g dry soil)$^{-1}$]		DNA [µg (g dry soil)$^{-1}$]	Moisture [%]	pH	ARA [µmol C_2H_4 (g dry soil)$^{-1}$]
	N_{tot}	NO_3^-	NH_4^+	C_{tot}	DOC				
Bulk soil									
Siliceous									
S1	17.9	0.11	8.3	286.3	0.02	0.28	8.7	4.7	n.d.
S2	16.2	0.09	6.3	289.0	0.02	0.30	4.2	4.8	4.0
S3	<10.0	0.08	6.6	<100.0	0.02	0.80	3.0	4.7	n.d.
S4	124.8	0.15	5.8	233.9	0.03	0.29	1.6	4.9	0.4
S5	84.4	0.11	6.4	173.5	0.02	0.46	2.7	4.6	0.4
Rhizosphere soil									
Siliceous									
S1	120.8	0.10	7.7	1603.3	0.12	0.99	14.6	4.5	n.d.
S2	76.5	0.04	6.4	971.3	0.08	0.89	7.6	4.3	19.9
S3	63.9	0.03	7.4	1063.0	0.04	0.31	13.5	4.5	n.d.
S4	48.9	0.01	6.1	717.3	0.07	0.96	3.7	4.8	3.1
S5	50.0	0.02	6.4	862.0	0.08	0.86	9.1	4.4	1.8
Bulk soil									
Calcareous									
S1	10.4	0.14	1.5	4056.0	0.49	0.43	0.1	7.6	n.d.
S5	<10.0	0.21	1.4	<100.0	0.82	0.44	0.6	7.6	n.d.

n.d. = not determined

The bedrock type had also a great impact, with five out of eight measured soil biogeochemical parameters differing significantly in siliceous and calcareous soil samples ($p<0.01$). Ammonium and moisture were higher in siliceous forefield soils, while N_{tot} and C_{tot} were higher in calcareous forefield soils. The latter had a pH of 7.6, while in siliceous soils pH varied between 4.5 and 4.9. Nitrate, DOC and DNA contents were not significantly affected.

The impact of the season was less pronounced. Ammonium concentrations decreased along the season. This trend was only significant ($p<0.1$) in calcareous soil. pH was significantly ($p<0.05$) affected in siliceous soils with the highest value in S4 samples. However, season and plant factors were interacting for this parameter. Soil moisture fluctuated along the season. These fluctuations fitted well with precipitation regimes observed just before sampling dates (data not shown).

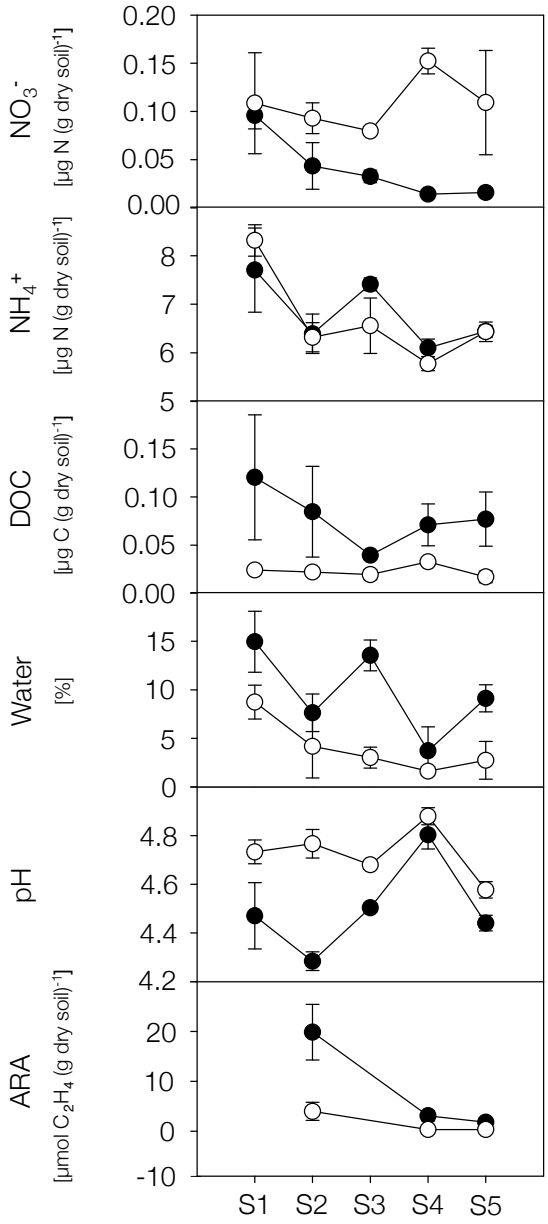


Figure 5-1 Seasonal trends in nitrate, ammonium, DOC, water content, pH and ARA. Symbols: Bulk soil (❍), rhizosphere soil (●). Error bars correspond to ± one standard error.

Table 5-2 MANOVA analysis of plant presence and bedrock effects on biogeochemical data and nitrogenase activity, with season as categorical variable (repetition)

		Nitrogen			Carbon		DNA	Moisture	pH	ARA
		N_{tot}	NO_3^-	NH_4^+	C_{tot}	DOC				
Plant										
Between treatments		*	***	n.s.	***	***	***	***	***	***
Within treatments	Season	n.s.	n.s.	n.s.	n.s.	n.d.	n.s.	n.s.	**	n.s.
	Season x plant	*	n.s.	n.s.	n.s.	n.d.	n.s.	n.s.	*	n.s.
Bedrock										
Between treatments		***	n.s.	***	***	n.s.	n.s.	***	***	n.d.
Within treatments	Season	n.d.	n.s.	*	n.d.	n.s.	n.s.	n.s.	n.s.	n.d.
	Season x bedrock	n.d.	n.s.	n.s.	n.d.	n.s.	n.s.	**	*	n.d.

n.s. = not significant (p>0.1); * significant at 0.1 level; **significant at 0.05 level; ***significant at 0.01 level
n.d. = not determined

5.4.2 Nitrogenase activity of field samples

Nitrogenase activity was only detected in water saturated samples. The plant presence enhanced significantly (p<0.01) the production of ethylene (Fig. 5-1), while the decrease of final ethylene concentrations toward the end of the season was not significant.

5.4.3 Biogeochemistry of pot samples

Biogeochemical properties of pot samples are listed in Table 5-3. Generally, plant growth was improved by increasing temperature and nitrogen deposition.

The presence of the plant had the strongest impact on biogeochemical parameters (Table 5-4). N_{tot}, nitrate, C_{tot} and DNA were significantly (p<0.01) higher in rhizosphere soils. Ammonium and DOC were not significantly affected by the presence of the plant. The temperature also influenced significantly soil properties. However, interactions of temperature with the plant presence factor were significant for six out of eight parameters. For example, the decrease of N_{tot} and the increase of DNA concentrations with rising temperature were much stronger in presence of the plant. Nitrogen deposition had a significant (p<0.05) impact on ammonium, DNA and pH. The highest ammonium concentration was found in N40 bulk soil samples at 10°C, while at 15°C the same samples displayed the lowest value. The opposite trend was observed in rhizosphere soils. DNA concentrations mirrored the values for ammonium concentration, showing exactly the opposite trend. pH was decreasing with increasing nitrogen deposition in both bulk and rhizosphere soil samples.

Table 5-3 Biogeochemical properties for the soils used in the pot experiment

		Nitrogen [µg N (g dry soil)$^{-1}$]			Carbon [µg C (g dry soil)$^{-1}$]		DNA [µg (g dry soil)$^{-1}$]	Moisture [%]	pH
		N_{tot}	NO_3^-	NH_4^+	C_{tot}	DOC			
Bulk soil									
10°C	N00	77.9	0.003	0.39	169.6	0.02	0.77	0.69	4.8
	N10	81.0	0.006	0.42	168.1	0.01	0.78	0.45	4.8
	N40	81.6	0.007	0.46	175.3	0.02	0.73	0.46	4.7
15°C	N00	76.4	0.010	0.53	157.1	0.02	0.59	1.49	4.7
	N10	77.5	0.011	0.53	149.6	0.04	0.52	3.06	4.7
	N40	78.2	0.010	0.50	315.6	0.02	0.92	0.35	4.6
Rhizosphere soil									
10°C	N00	100.0	0.015	0.66	262.3	0.01	0.62	0.24	4.9
	N10	103.3	0.018	0.46	286.1	0.02	0.65	0.18	4.6
	N40	98.3	0.220	0.59	239.2	0.02	0.92	0.17	45
15°C	N00	84.0	0.010	0.34	209.7	0.01	1.03	0.43	4.6
	N10	83.8	0.009	0.30	210.7	0.02	1.50	0.40	4.6
	N40	82.3	<0.001	0.42	218.6	0.01	1.01	0.38	4.4

n.d. = not determined

Table 5-4 MANOVA analysis of plant presence and temperature effects on biogeochemical data, with nitrogen deposition as categorical variable (repetition)

		Nitrogen			Carbon		DNA	Moisture	pH
		N_{tot}	NO^3_-	NH^4_+	C_{tot}	DOC			
Between treatments	Plant	***	***	n.s.	***	n.s.	***	**	*
	Temperature	***	n.s.	n.s.	*	n.s.	***	***	***
	Plant x temperature	***	***	*	*	n.s.	***	*	n.s.
Within treatments	Nitrogen	n.s.	*	**	n.s.	n.s.	**	n.s.	***
	Nitrogen x plant	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.
	Nitrogen x temperature	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Nitrogen x plant x temperature	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**	*

n.s. = not significant (p>0.1); * significant at 0.1 level; **significant at 0.05 level; ***significant at 0.01 level
n.d. = not determined

5.4.4 Microarray analysis of field samples

Effects of plant presence, bedrock type and season on free-living diazotrophic population structure were examined through a comparative analytical approach using the microarray technology. Results presented in Fig. 5-2 correspond to differences in phylotype abundance observed between samples that were distinguished only by one environmental factor. Phylotypes are sorted by their affiliation with NifH phylogenetic clusters as defined by Zehr et al. (2003a).

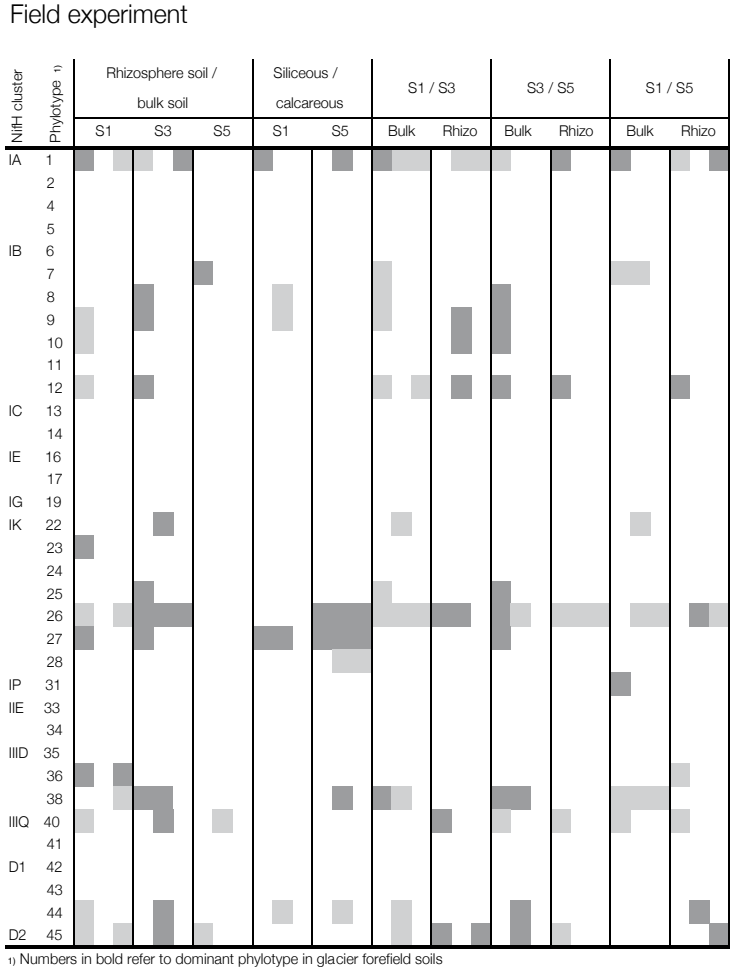


Figure 5-2 Characterization of the dynamic of diazotrophic populations in relation to plant presence, bedrock type and season, in the field experiment. Dark and light grey shadings indicate phylotypes with increased or decreased abundance, respectively.

Diazotrophic phylotypes that were shown to be dominant in glacier forefield soils (Duc et al. 2009a) were those displaying changes in their abundance in response to environmental variation (Fig. 5-2). Exceptions observed for phylotypes 5, 17, and 23 might be induced by the high environmental variability found in the glacier forefield. These frequent phylotypes were indeed observed in only one from several samples used to build one out of the four libraries constructed by Duc et al. (2009a). Phylotype 38 was the only responding phylotype that was formerly described as infrequent. All other phylotypes that were previously described as rare or unevenly distributed in glacier forefiled soil samples, did not present any significant differences in their abundance.

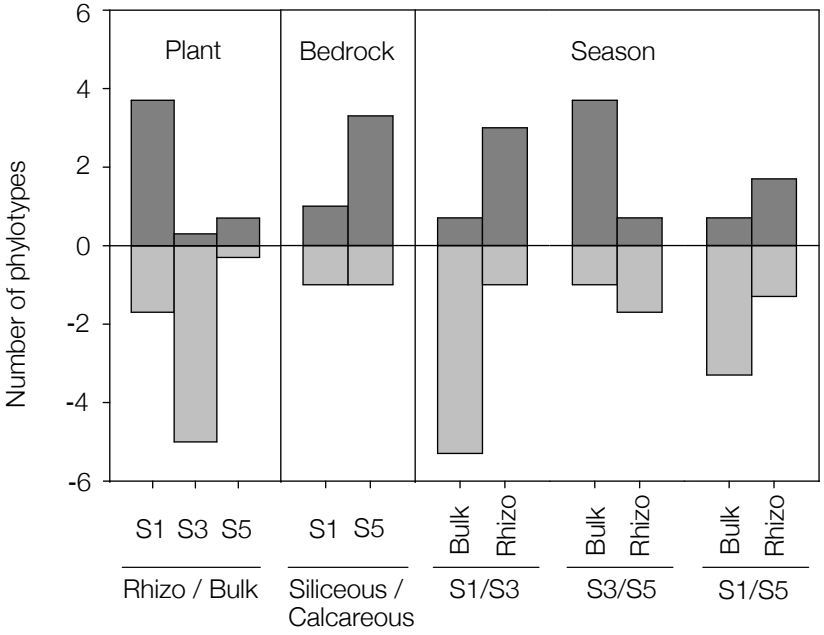


Figure 5-3 Average number of NifH phylotypes displaying differential abundance in response to plant presence, bedrock type and seasonal variation. Dark and light grey shadings indicate phylotypes with increased or decreased abundance, respectively.

Fig. 5-3 illustrates the impacts of plant presence, bedrock type and season on the structure of diazotrophic populations. All impacts were in the same range. The effect of plant presence was significantly greater in June ($p<0.01$) and August ($p<0.1$) compared to September, where bulk and rhizosphere soils were more comparable. Phylotypes that were promoted in rhizosphere soils at the beginning of the season, were in turn more abundant in bulk soils in August. The variability between triplicates was high (Fig. 5-2). The nature of the bedrock induced slightly less variation in population structure than the presence of plant, but the consistency of the triplicates was higher. The season also caused variations in NifH phylotype abundances. A similar and probably not independent phenomenon as that described for the plant presence

factor was observed. Namely diazotrophic population displayed opposite trend in bulk soils compared to rhizosphere soils. In bulk soil, 80% of the phylotypes were increasing along the season, with a peak in August for half of them, while around one fifth of the phylotypes were decreasing toward the end of the season. In rhizosphere soils the impact of the season was less pronounced. The few reactive phylotypes displayed the reverse dynamic as observed in bulk soils. Three quarter of them decreased and one quarter increased in the course of the season. Phylotype 26, 27 and 28 showed consistent responses among replicates and specific probes. Normalized signal intensities of all specific probes are shown in Fig. 5-4. These three phylotypes are affiliated with type II methanotrophs (Duc et al., 2009a) grouping within the NifH cluster IK (Zehr et al., 2003a) and they allowed to differentiate siliceous from calcareous soils. Phylotypes 26 and 27 were abundant in early (27) and late (26) season in siliceous bulk soils, while they seemed to be absent in calcareous bulk soils. Phylotype 28 was characteristic of calcareous soils, with a stronger abundance in September.

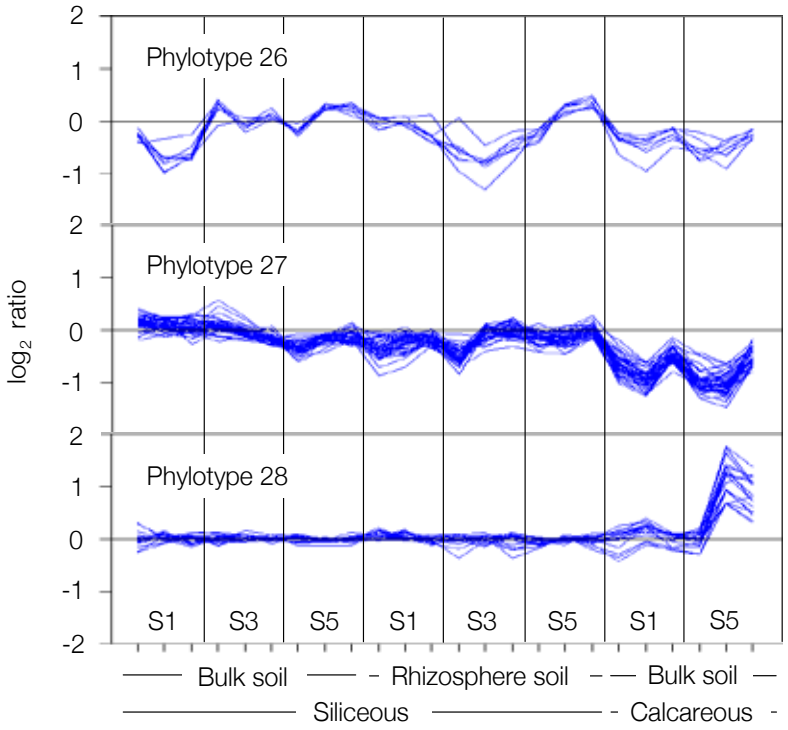


Figure 5-4 Log_2 ratios of probes targeting specifically phylotypes 26, 27 and 28. Log_2 ratios were computed with normalized signal intensities of each hybridization relative to the average signal of hybridizations performed with siliceous bulk soil samples.

Pot experiment

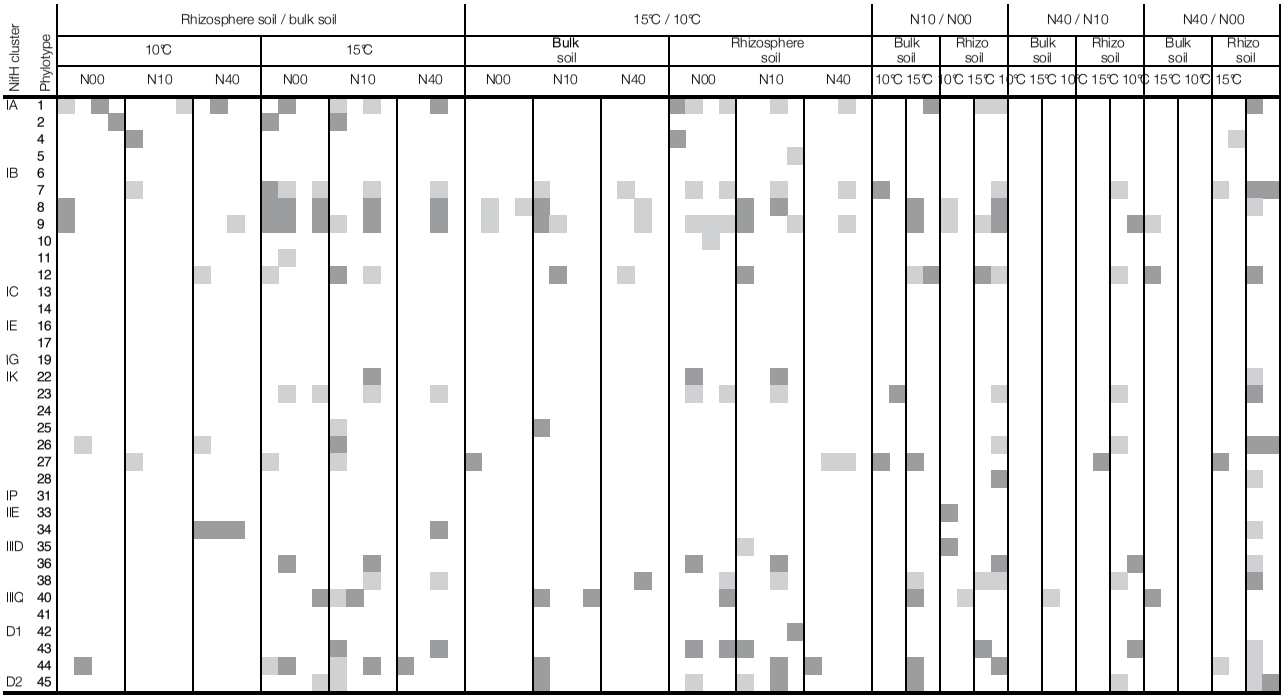


Figure 5-5 Characterization of the dynamic of diazotrophic populations in relation to plant presence, temperature increase and nitrogen deposition increase, in the pot experiment. Dark and light grey shadings indicate phylotypes with increased or decreased abundance, respectively. Due to restrictions in the experimental design only two instead of four comparisons per nitrogen treatment were feasible.

5.4.5 Microarray analysis of pot samples

The high variability among replicates did not allow to detect any phylotype showing consistent response. Nevertheless, the phylum of *Cyanobacteria* (IB) responded particularly strongly to environmental changes. Variation in the abundance of cyanobacterial phylotypes stood for 39.4% of the total variation (Fig. 5-5). All three factors (plant presence, temperature and nitrogen deposition) caused changes in the presence pattern of NifH phylotypes. Considering nitrogen deposition, greatest differences were found between N00 and N10 treatments and the lowest between N40 and N10 (Fig. 5-5). Nevertheless, this trend was not significant and data of the three comparisons were averaged in Fig. 5-6. Variations in the population structure induced by temperature and nitrogen deposition were significantly higher in rhizosphere soils compared to bulk soils ($p<0.05$). Plant presence and temperature had greater impact at low nitrogen deposition (N00 and N10) compared to high nitrogen deposition treatment (N40). Both trends were not significant. Finally, nitrogen deposition and plant presence effects were significantly higher at 15°C than at 10°C ($p<0.05$).

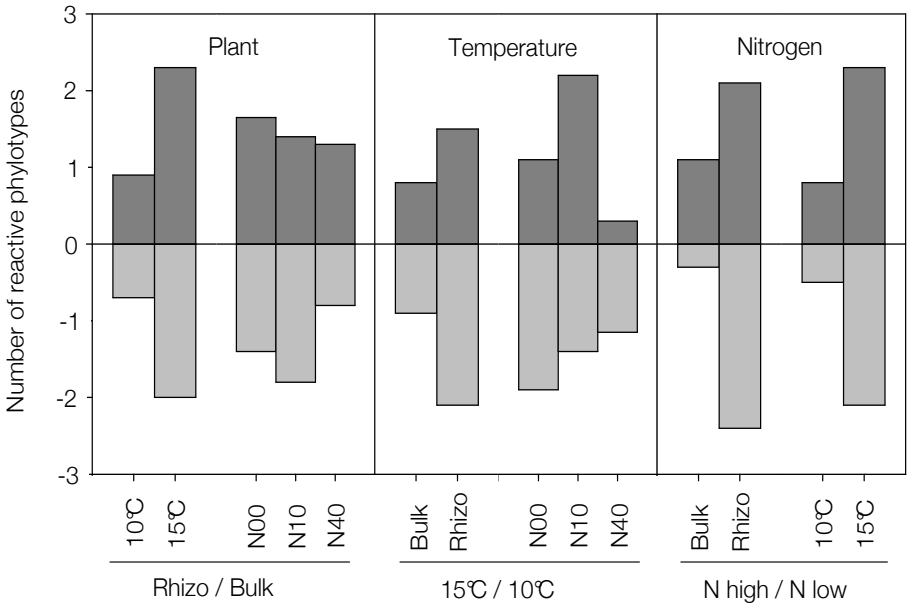


Figure 5-6 Average number of NifH phylotypes displaying differential abundance in response to plant presence, temperature increase and nitrogen deposition increase. Dark and light grey shadings indicate phylotypes with increased or decreased abundance, respectively. For nitrogen deposition, results of the three treatments were averaged.

5.5 Discussion

5.5.1 Analysis of environmental samples using microarrays

Inaccuracy and inconsistencies observed in microarray measurements (Draghici et al., 2006; Pozhitkov et al., 2007) is of major concern. The poor understanding of the relationship between probe sequences, target concentration and probe intensity complicates the interpretation of microarray data. In order to strengthen our results, we chose a comparative approach to examine DNA samples rather than an absolute NifH phylotypes detection. Actually, the precise discrimination obtained during the validation of the *nifH* microarray (Duc et al., 2009b) with pure culture experiments, might be difficult to repeat with complex environmental samples. The latter might contain unspecific co-amplified PCR products, all contributing to the increase of non-specific signal. Internal controls are necessary to eliminate unspecific noise. To make it possible, we compared two by two environmental samples that were processed identically (same chip) and were differing by one factor only.

5.5.2 Impacts of environmental factors

In the field, the presence of the plant strongly influenced biogeochemical soil properties, nitrogen fixation activity and diazotrophic population structure. This was in accordance with the common knowledge that pioneer plants represent the main source of carbon in glacier forefields (Bardgett and Walker, 2004), as well as a strong driver of microbial activity (Curl and Truelove, 1986) and diversity (Tscherko et al., 2005; Edwards et al., 2006). The higher effect of the presence of the plant in early and mid seasons compared to September was most probably induced by the dynamic of root exudates, which were shown to decrease towards the end of the season (Bardgett et al., 2005). Likewise the plant presence altered the effect of the season. Indeed population variation related to the season prevailed in absence of vegetation cover. The latter is known to create favorable and more stable conditions by decreasing heat stress and soil drying (Tscherko et al., 2005). Interestingly, diazotrophic population dynamics observed in the course of the season were opposite in bulk soils and rhizosphere soils. This trend remained unexplained after considering biogeochemical parameters measured in this study and was probably introduced by unmeasured environmental variability. This emphasizes the difficulty to study such heterogeneous soil ecosystems and the need of enlarging the range of observation.

Seasonal variation in the bulk soil population structure was somehow related to the precipitation regime. Most of the phylotypes had an increased abundance in August compared to June and September. This might be related to the double amount of precipitation observed 30 days before the sampling of the mid season. A comparable observation was made for eubacterial communities (DGGE analysis of the 16SrRNA gene) (Miniaci, 2007), with the differentiation of June and September populations from those observed in July and August, as well as higher precipitation during the mid season. It is not surprising as soil moisture is considered to be a major factor influencing differentially microbial growth and activity (Kowalenko and Cameron, 1976; Tiwari et al., 1987) depending on the microbial species. ARA measurements illustrated

further the importance of soil moisture. In fact, the elevation of soil water content was a prerequisite for nitrogen fixation activity. The high amount of added water might led to soil waterlogging, and therefore to lower oxygen concentration and enhanced dinitrogenase activity.

The pot experiment highlighted the strong carbon limitation in the young glacier forefield soil (Korner et al., 1997). Indeed, nitrogen deposition had only marginal impact on bulk soils diazotrophic populations, while it seemed to indirectly influence rhizospheric diazotrophs through the promotion of plant growth, resulting in an increased production root exudates and the local alleviation of carbon limitation. Similarly, the temperature increase affected both bulk and rhizosphere soil diazotrophic communities but this effect was amplified by the presence of the plant. This experiment also illustrated clearly the negative impact of nitrogen deposition on diazotrophic populations. In fact, effects of plant presence and temperature increase were lowered in presence of high nitrogen concentration (N40 treatment), suggesting a reduced competitivity of diazotrophs.

5.5.3 Identification of reactive phylotypes

In the field experiment, the variability among replicates was high, but clear trends in diazotrophic populations dynamic were recognizable. Especially, two phylotypes all related to the genus *Methylocystis* (type II methanotrophs) showed strong and consistent response to environmental variations among replicates. This genus represented 15% of the NifH diversity found in young Damma Glacier forefied soils (Duc et al., 2009a) and was shown to be particularly adapted to nutrient-limited environment (Bender and Conrad, 1992; Graham et al., 1993). Distinct but closely related methanotrophic phylotype was also displaying strong and consistent response to seasonal variation in the calcareous site. This observation indicates that the of dominance *Methylocystis*-like microorganisms might result from efficient adaptation to biotic and abiotic conditions found in glacier forefield environments, like for example their ability to get their carbon (CH_4) from the atmospheric pool, rather than from neutral ecological drift like for example spatial distance (Ramette and Tiedje, 2007). Therefore even if many NifH phylotypes might contribute to the increase of the soil nitrogen pool through microbial nitrogen fixation in glacier forefield environments, our results suggest that phylotypes related to the genus *Methylocystis* are relevant to maintain the function. Nevertheless, much effort is still needed to study the active NifH diversity, in order to examine the level of redundancy for nitrogen fixation in glacier forefields.

In the pot experiment, the variability among replicates was even higher. This suggests that diazotrophs were responding strongly to environmental changes but that the time of the experiment was not long enough to allow the establishment of a stable community reflecting environmental growth conditions. In this experiment, *Cyanobacteria* were the most reactive microorganisms. They were shown to be frequent in glacier forefield soils (Duc et al., 2009a). Additionally, they have been observed to be highly competitive in extreme and oligotrophic environments due to their resistance to temperature stress (Sabacka and Elster, 2006), desiccation (Potts, 1994) and high UV intensities (Xue et al., 2005), as well as their ability to

cope with nutrient stress (Bhaya, 1996). In both experiments, most responding NifH phylotypes coincided to dominant diazotrophs that were able to acquire carbon from the atmospheric pool (CO_2, CH_4). Similarly to the analysis of the impacts of environmental factors, this result stresses the strong carbon limitation found in glacier forefields and therefore the selective advantage of bacteria relying on inorganic carbon pool.

6 GENERAL DISCUSSION

6.1 Study of diazotrophic diversity in glacier forefield environments: methodological considerations

6.1.1 Sampling strategies in heterogeneous glacier forefield environments

Microbial diversity is highly influenced by environmental heterogeneity (niches diversification) (McArthur et al., 1988) and spatial distance (historical events and disturbance) (Borcard and Legendre, 1994). Both factors should be considered in the design of sampling strategies, as they might strongly influence the outcomes of diversity studies.

Environmental heterogeneity

Forefield landscapes are characterized by the alternation of vegetation patches, rocks, gravels, sand banks and glacier streams on uneven surfaces. This causes differential exposition to wind and solar radiation, as well as the formation of pools after rain events. Soil moisture regimes found within a glacier forefield are therefore highly diverse. The size of the microbial habitat also contributes to the increase of spatial heterogeneity, as soil microbial communities have been shown to be significantly different depending on the aggregate size fraction that is considered (Izquierdo and Nusslein, 2006). Additionally, climatic conditions in high alpine areas are variable and might strongly fluctuate, even within a day.

This spatial and temporal heterogeneity complicates sampling strategies and force investigators to adapt the scale of the examination to the research question. In this study, we applied several strategies. In chapter 3, we gather the largest number of unique *nifH* sequences for the development of the *nifH* microarray by sampling consciously different micro-environments within two soil-age classes. In chapter 5, in contrast, we focused our sampling effort on one small area in order to decrease the heterogeneity of samples and be able to assess the impact of defined parameters on diazotrophic communities. The limitation of the latter strategy is the difficulty to extrapolate results to the whole glacier forefield environment. Finally, the soil used in the pot experiment (chapter 5) was collected in eight sites randomly chosen within one soil-age class and previously localized using geostatistic technology (GIS, GPS). This methodology allowed us to avoid biases introduced by the investigator subjectivity.

Spatial distance

In addition to environmental heterogeneity, spatial distance is considered as a major factor of influence in microbial diversification. Therefore, it has to be identified and taken into account within sampling strategies. In the Damma glacier forefield, a clear example of the influence of spatial distance is the intermediate moraine that introduced a gap of 40 years in the chronosequence (see section 3.3.1 and Fig. 3-1).

However, Ramette and Tiedje (2007) showed that environmental heterogeneity and spatial distance explain only a part of the microbial genetic variation. Unexplained variation might be then attributed to unknown environmental variability, sampling effects and neutral ecological drift. Downsizing the studied environment is an option to decrease unexplained variation, leading unfortunately to results that can hardly be understood within the global scale of the glacier forefield environment. Additionally, if one considers the vast amount of environmental parameters influencing microbial populations, and that might be affected by environmental heterogeneity and spatial distance, the study of microbial ecology, at the macro scale of ecosystems like glacier forefields, represents a veritable challenge.

6.1.2 Detection of free-living diazotrophs in glacier forefied soils

Low abundance of nifH gene copies

An important factor limiting the detection of microorganisms in glacier forfield soil samples is the low quantity of genetic material. Additionally, soil DNA extracts contained high amounts of humic acids, known to inhibit PCR amplification (Wilson, 1997). Soil DNA extraction methods usually include steps to get rid of these inhibitors. In the phenol-chloroform method used in chapters 3 and 4 (Noll et al., 2005), the addition of polyvinylpyrrolidon in the extraction buffer allows to bind phenyl groups present in humic acids and consequently to reduce their amount in the purified DNA (Berthelet et al., 1996). Nevertheless, extracts still had to be diluted in order to get reliable PCR amplifications. The use of a commercially available soil DNA extraction kit (chapter 5) led to pure extracts, but final DNA concentrations were generally lower than those obtained with the phenol-chloroform extraction. In both situations, strategies to alleviate PCR inhibition are followed by loss of genetic material. Considering the already scarce microbial community in glacier forefield soils, this might lead to the loss of low-abundant phylotypes and consequently to the underestimation of microbial diversity. Moreover, PCR amplification performed with low amounts of DNA template are known to be more subject to PCR biases and unspecific amplifications (Mutter and Boynton, 1995; Chandler et al., 1997). Biases are caused by PCR drifts either introduced by the user (pipetting errors, variation of thermal profiles of the PCR machine wells) or resulting from different sequence characteristics (Polz and Cavanaugh, 1998). In fact, GC complements present a higher melting temperature (+2°C) than AT complements. Therefore, different GC content at degenerate position of primers results in variable primer hybridizations and preferential amplifications. In addition, the GC content of double strain DNA templates modifies their denaturation temperature and consequently their amplification. Recommendations to reduce PCR biases are the reduction of the PCR cycle number, and the use of undegenerated primers and high template concentrations (Polz and Cavanaugh, 1998). Template concentration can be increased by the pooling of DNA extracts. If the latter strategy might be true for bulk soil samples, it is inconceivable for rhizosphere soil samples, as it would imply the massive and destructive sampling of sparse pioneer plants. In this study we chose to apply the nested PCR method known to increase the sensitivity of the detection (Widmer et al., 1999). The nested PCR corresponds to a second amplification round of an already PCR-amplified sequence using a new pair of primers, which are internal to the original primers. Unfortunately, the use of two amplification steps augments the

number of cycles and consequently elevates the risk of bias. Therefore the study of diazotrophic communities in glacier forefield soils needs further optimization of soil DNA extraction and PCR amplification methods.

Low conservation of the nifH gene

The ability to fix nitrogen is found in very diverse microbial phylogenetical groups (Zehr et al., 2003b). The divergent evolution of these groups led to vast differences among *nifH* gene sequences pool (Zehr and McReynolds, 1989). Even DNA sequences coding for conserved protein regions show a high degree of variability (Widmer et al., 1999). Therefore, the amplification of the largest number of different *nifH* sequences necessitates the use of "universal" primers presenting a high amount of degeneracies (Zehr and McReynolds, 1989; Ueda et al., 1995). The specificity of the amplification is reduced by a high amount of degeneracies (Widmer et al., 1999). If unspecific sequences might be removed after identification through a cloning-sequencing approach, fingerprinting methods like DGGE and T-RFLP hardly allow to do the same and might produce misleading results. Unspecific amplification might be minimized by the use of nested PCR. The second amplification step results in a reduced background from products amplified during the initial PCR due to the additional specificity of the nested primers. Unfortunately, as mentioned above, the high number of cycles of nested PCR increases amplification biases. The isolation of specific PCR products through a separation on agarose gel followed by the incision and purification of the band presenting the right length remains therefore a prerequisite to any further molecular analysis.

The *nifH* database is growing rapidly and constitutes currently one of the largest non-ribosomal gene database (Zehr et al., 2003a). In 1994, Ueda et al. (1995) mentioned 37 publicly available sequences, while in 2003 already 1500 sequences composed the *nifH* database (Zehr et al., 2003a). Nowadays, 19192 *nifH* sequences can be downloaded from GenBank. Considering this fast expansion, the relevance of *nifH* "universal" primers to detect all known *nifH* sequences might be questioned. The coverage of primers should be regularly checked, and their sequence and position readjusted. This work is time-consuming and is unfortunately often omitted. Another issue is the use of different "universal" primer pairs in different studies. This makes the comparison between studies difficult. In fact, different primer pairs might be expected to detect variable portion of the *nifH* sequences pool and to be differently subject to PCR biases. Additionally, the incomplete automation of sequence analysis (e. g. alignment) as well as the multiple models used in phylogenetical studies increase the divergence of results. Therefore, one has to be cautious not to amalgam differences observed in community composition due to PCR biases, uniqueness of researchers or microbial habitats.

6.2 Use of oligonucleotide functional microarray to study microbial diversity

6.2.1 Reliability and reproducibility of microarray experiments

The microarray technology is a powerful tool to study microbial diversity and functions (Schadt et al., 2005). The development of a microarray is a time consuming process, however the application of this technology allows the rapid detection of unique microbial strains in numerous samples. Nevertheless, despite the wide use of microarrays, mechanisms behind this technology are still incompletely understood. The specificity of target-probe hybridization is influenced by many factors relative to probe and target sequences, as well as hybridization kinetics (Fig. 6-1). Considering only one or very few of these factors seems to deliver only a partial prediction of target-probe hybridization behavior (Pozhitkov et al., 2007). Koltai and Weingarten-Baror (2008) hypothesized that a coherent and complete prediction might only be obtained if all factors present in Fig. 6-1 are taken into account in a single complex model. As long as such a model does not exist, the empirical testing of each specific and unspecific probe-target duplex that might be encountered in a particular study is indispensable. Filtration of hybridization data might also be used to improve the specificity of microarray experiments. For example, valuable data can be selected by setting an arbitrary threshold for the signal intensity (Hammond et al., 2005). The increment of this threshold typically increases the specificity. Similarly, in presence of multiple probes targeting unique phylogenetical groups, the minimal proportion of positive probes needed to declare a phylogenetic group present might be elevated (Rehrauer et al., 2008). Finally, the use of different microarray technologies and sample preparation protocols has been shown to lead to low reproducibility between microarray platforms (Frantz, 2005; Koltai and Weingarten-Baror, 2008). Therefore, comparison of DNA samples within one microarray experiment (one chip) remains the best procedure to assess differences in microbial communities.

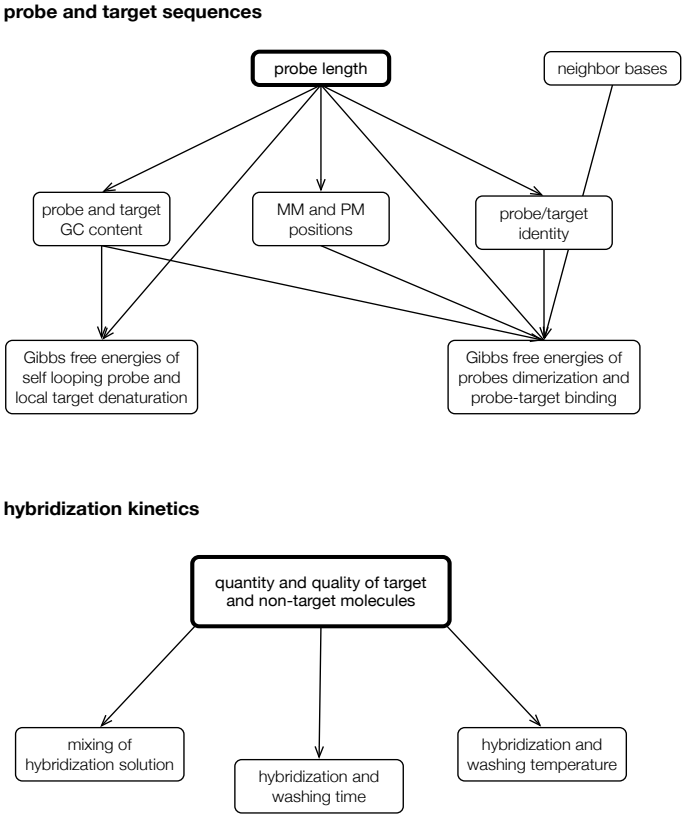


Figure 6-1 Factors influencing the specificity of microarray hybridizations and sorted according to their relatedness to sequence characteristics or hybridization kinetics. Adapted from Koltai and Weingarten-Baror (2008).

6.2.2 Quantitative potential of oligonucleotide functional microarrays

The estimation of absolute concentrations requires a detailed understanding of hybridization processes, careful calibrations with known amount of specific targets and a strong normalization of data. Biases added at each step of the preparation and the processing of samples, as well as the insufficient knowledge on target-probe interactions hinder the use of the microarray technology for absolute quantification of gene copies. Relative concentration measurements through the comparison of hybridizations within one microarray experiment (one chip) produce more accurate results (Draghici et al., 2006). In order to obtain robust comparison, samples should be prepared in parallel, without pre-amplification in order to avoid PCR biases (Polz and Cavanaugh, 1998). For functional oligonucleotide microarray experiments, the latter requirement is highly problematic. Functional genes are indeed present in microbial cells in much lower concentration than

the 16S rRNA gene and the actual sensitivity of the microarray technology might not be sufficient to detect them without any preliminary amplification. In our work the problem was even bigger, as we were working with environmental samples containing only low numbers of microbial cells and high amounts of inhibitory substances. In addition, the high diversity of diazotrophs in glacier forefield soils and the consequent low proportion of distinct diazotrophic strains (chapter 3) render a direct detection hardly possible. The application of whole-genome DNA amplification (Wu et al., 2006) represents a promising tool to compare quantifically microbial communities.

6.2.3 Feasibility and relevance of mRNA microarray experiments to detect and quantify active microorganisms

One challenging objective of modern microbiology is to monitor microbial activities using molecular methods. RNA microarray experiments are considered promising tools to study abundance, diversity and population structure of active microorganisms. Whole-community RNA amplification, based on the amplification of the T7 promoter (Moreno-Paz and Parro, 2006) allows to amplify genetic material avoiding PCR biases, and consequently to detect and quantify unique RNA molecules using the microarray technology. The relevance of mRNA microarray analysis to study functions of microorganisms in the environment is nevertheless questionable. First, mRNA molecules are known to be highly unstable and subject to degradation as soon as they are released from the cell (Evans and Kamdar, 1990). This complicates the extraction and the conservation of mRNA molecules, especially when working with environmental samples containing very low amount of genetic material. This also means that the sampling and the preparation of samples might alter the mRNA composition of samples. A second issue is the fast transcriptional response to environmental changes (Mager and Dekruijff, 1995; Bodrossy et al., 2006). The transcriptional status of a microbial cell is therefore highly correlated to the sampling time and might strongly vary within a day. Third, if the presence of mRNA can be directly linked to the expression of genes this does not necessarily imply RNA translation, protein synthesis and induction of related activities (Madigan and Martinko, 2006). Therefore, the detection of mRNA molecules only corresponds to potential activities. These three considerations highly restrain the potential of mRNA microarray experiments to study microbial functions.

6.3 Conclusions and outlook

This thesis provides a survey on the diversity, the structure and functions of microbial populations in glacier forefield soils, as well as their interactions with pioneer plants. Special attention was given to free-living diazotrophs due to their expected importance in nitrogen poor environments. Indeed, the cloning-sequencing approach revealed a large diversity and a broad distribution of NifH sequences, confirming the hypothesis that biological nitrogen fixation (BNF) confers selective advantage under nitrogen-limited conditions.

This work showed that microarray technology is suitable to study free-living diazotrophic populations in soil ecosystems. Nevertheless, the limit of this tool was obviously the high intensity of the detected background noise, constraining its

use to assess changes or differences between samples. Therefore, further studies should deal with the optimization of the target labeling. Increased signal intensities would allow to reduce the exposition time during signal acquisition and consequently to decrease the background noise level. This would enable the use of the *nifH* microarray to detect present NifH phylotypes in unique samples.

The *nifH* microarray was successfully applied to monitor the response of free-living diazotrophs to environmental variations, revealing that the presence of pioneer plants constitutes the environmental factor that mostly influences free-living diazotrophic population structure. Additionally, Cyanobacteria and type II methanotrophs were observed to be dominant and highly reactive to environmental changes. Future analyses could investigate the importance of these two phylogenetical groups to maintain BNF in glacier forefield soils. This might be realized through cultivation experiments under gnotobiotic conditions. The high NifH diversity suggests high functional redundancy for BNF, while the dominance of two reactive phylogenetical groups indicates the opposite trend. This could be clarified by assessing the potential active diversity through microarray experiments using soil RNA extracts. Finally, free-living diazotrophic populations might be further characterized by assessing their resistance and their resilience to environmental stress. The contribution of different phylogenetic groups and the influence of population diversity on of these both features could be further detailed.

REFERENCES

Alef, K. (1995) Estimation of the hydrolysis of fluorescein diacetate. In Methods in applied soil microbiology and biochemistry. K. Alef and P. Nanipieri, E. (ed). London: Academic Press, pp. 232-233.

Allison, S.D., and Martiny, J.B.H. (2008) Resistance, resilience, and redundancy in microbial communities. Proceedings of the National Academy of Sciences of the United States of America 105: 11512-11519.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990) Basic local alignment search tool. Journal of Molecular Biology 215: 403-410.

Amann, R., Fuchs, B.M., and Behrens, S. (2001) The identification of microorganisms by fluorescence *in situ* hybridisation. Current Opinion in Biotechnology 12: 231-236.

Amann, R.I., Ludwig, W., and Schleifer, K.H. (1995) Phylogenetic identification and *in-situ* detection of individual microbial-cells without cultivation. Microbiological Reviews 59: 143-169.

Arcondeguy, T., Jack, R., and Merrick, M. (2001) P_{II} signal transduction proteins, pivotal players in microbial nitrogen control. Microbiology and Molecular Biology Reviews 65: 80-105.

Astrand, M., Mostad, P., and Rudemo, M. (2008) Empirical Bayes models for multiple probe type microarrays at the probe level. BMC Bioinformatics 9: Article No. 156.

Bally, M., Halter, M., Voros, J., and Grandin, H.M. (2006) Optical microarray biosensing techniques. Surface and Interface Analysis 38: 1442-1458.

Barbulovic-Nad, I., Lucente, M., Sun, Y., Zhang, M.J., Wheeler, A.R., and Bussmann, M. (2006) Bio-microarray fabrication techniques - A review. Critical Reviews in Biotechnology 26: 237-259.

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

Bardgett, R.D., Bowman, W.D., Kaufmann, R., and Schmidt, S.K. (2005) A temporal approach to linking aboveground and belowground ecology. Trends in Ecology & Evolution 20: 634-641.

Bardgett, R.D., Richter, A., Bol, R., Garnett, M.H., Baumler, R., Xu, X.L. et al. (2007) Heterotrophic microbial communities use ancient carbon following glacial retreat. Biology Letters 3: 487-490.

Barry, R.G. (1992) Mountain weather and climate. New York: Routledge.

Bateman, A., Coin, L., Durbin, R., Finn, R.D., Hollich, V., Griffiths-Jones, S. et al. (2004) The Pfam protein families database. Nucleic Acids Research 32: D138-D141.

Bauder, A., and Hoelzle, M. (2002) Die Gletscher der schweizer Alpen. Die Alpen 10: 24-29.

Bauder, A., Vieli, A., and Hoelzle, M. (2001) Die Gletscher der schweizer Alpen 1999/2000. Die Alpen 10: 22-32.

Bauder, A., Schär, C., and Blatter, H. (2004) Die Gletscher der schweizer Alpen 2001/02 und 2002/03. Die Alpen 10: 30-39.

Bauder, A., Marty, C., and Nötzli, J. (2008) Schnee, Gletscher und permafrost 2005/06 und 2006/07. Die Alpen 9: 47-56.

Bauder, A., Pralong, A., Funk, M., and Failletaz, J. (2006) Die Gletscher der schweizer Alpen 2003/04 und 2004/05. Die Alpen 10: 34-43.

Beier, M., and Hoheisel, J.D. (2000) Production by quantitative photolithographic synthesis of individually quality checked DNA microarrays. Nucleic Acids Research 28: e11.

Beier, M., Baum, M., Rebscher, H., Mauritz, R., Wixmerten, A., Stahler, C.F. et al. (2002) Exploring nature‘s plasticity with a flexible probing tool, and finding new ways for its electronic distribution. Biochemical Society Transactions 30: 78-82.

Bender, M., and Conrad, R. (1992) Kinetics of CH_4 oxidation in oxic soils exposed to ambient air or high CH_4 mixing ratios. FEMS Microbiology Ecology 101: 261-270.

Bengtson, P., and Bengtsson, G. (2005) Bacterial immobilization and remineralization of N at different growth rates and N concentrations. FEMS Microbiology Ecology 54: 13-19.

Bergman, B., Matveyev, A., and Rasmussen, U. (1996) Chemical signalling in cyanobacterial-plant symbioses. Trends in Plant Science 1: 191-197.

Berthelet, M., Whyte, L.G., and Greer, C.W. (1996) Rapid, direct extraction of DNA from soils for PCR analysis using polyvinylpolypyrrolidone spin columns. FEMS Microbiology Letters 138: 17-22.

Bhaya, D. (1996) Molecular responses of *Cyanobacteria* to macronutrient limitation. Journal of Scientific & Industrial Research 55: 630-637.

Bleikolm, G. (2005) Evolution of microbial nutrient limitation along the forefield of the Dammaglacier, Canton Uri, Switzerland. Master thesis, ETH Zurich, Zurich.

Bodrossy, L., and Sessitsch, A. (2004) Oligonucleotide microarrays in microbial diagnostics. Current Opinion in Microbiology 7: 245-254.

Bodrossy, L., Strali-Pavese, N., Murell, J.C., Radajewski, S., Weilharter, A., and Sessitsch, A. (2003a) Development and validation of a diagnostic microbial microarray for methanotrophs. Environmental Microbiology 5: 566-582.

Bodrossy, L., Stralis-Pavese, N., Murrell, J.C., Radajewski, S., Weilharter, A., and Sessitsch, A. (2003b) Development and validation of a diagnostic microbial microarray for methanotrophs. Environmental Microbiology 5: 566-582.

Bodrossy, L., Stralis-Pavese, N., Konrad-Koszler, M., Weilharter, A., Reichenauer, T.G., Schofer, D., and Sessitsch, A. (2006) mRNA-based parallel detection of active methanotroph populations by use of a diagnostic microarray. Applied and Environmental Microbiology 72: 1672-1676.

Borcard, D., and Legendre, P. (1994) Environmental control and spatial structure in ecological communities: an example using oribatid mites (Acari, Oribatei). Environmental and Ecological Statistics 1: 37-61.

Bostrom, K.H., Riemann, L., Zweifel, U.L., and Hagstrom, A. (2007) *Nodularia* sp *nifH* gene transcripts in the Baltic Sea proper. Journal of Plankton Research 29: 391-399.

Bottomley, P.J., and Vanbaalen, C. (1978) Characteristics of heterotrophic growth in blue-green-alga *Nostoc* sp Strain Mac. Journal of General Microbiology 107: 309-318.

Bowman, W.D. (1992) Inputs and storage of nitrogen in winter snowpack in an alpine ecosystem. Arctic and Alpine Research 24: 211-215.

Braun, S.T., Proctor, L.M., Zani, S., Mellon, M.T., and Zehr, J.P. (1999) Molecular evidence for zooplankton-associated nitrogen-fixing anaerobes based on amplification of the *nifH* gene. FEMS Microbiology Ecology 28: 273-279.

Brunetti, M., Maugeri, M., Nanni, T., Auer, I., Bohm, R., and Schoner, W. (2006) Precipitation variability and changes in the greater Alpine region over the 1800-2003 period. Journal of Geophysical Research-Part D-Atmospheres|Journal of Geophysical Research-Part D-Atmospheres 111: D11107.

Bürgmann, H., Widmer, F., Sigler, W.V., and Zeyer, J. (2003) mRNA extraction and reverse transcription-PCR protocol for detection of *nifH* gene expression by *Azotobacter vinelandii* in soil. Applied And Environmental Microbiolgy 69: 1928-1935.

Bürgmann, H., Widmer, F., Von Sigler, W., and Zeyer, J. (2004) New molecular screening tools for analysis of free-living diazotrophs in soil. Applied And Environmental Microbiolgy 70: 240-247.

Bürgmann, H., Meier, S., Bunge, M., Widmer, F., and Zeyer, J. (2005) Effects of model root exudates on structure and activity of a soil diazotroph community. Environmental Microbiology. 7:1711-1724.

Burns, R.G., and Dick, R.P. (2002) Enzymes in The Environment: activity, ecology, and applications. New york.

Burris, R.H., and Roberts, G.P. (1993a) Biological Nitrogen Fixation. Annual Review of Nutrition 13: 317-335.

Burris, R.H., and Roberts, G.P. (1993b) Biological nitrogen-fixation. Annual Review of Nutrition 13: 317-335.

Caccianiga, M., and Andreis, C. (2004) Pioneer herbaceous vegetation on glacier forelands in the Italian Alps. Phytocoenologia 34: 55-89.

Chabrerie, O., Laval, K., Puget, P., Desaire, S., and Alard, D. (2003) Relationship between plant and soil microbial communities along a successional gradient in a chalk grassland in north-western France. Applied Soil Ecology 24: 43-56.

Chandler, D.P., Fredrickson, J.K., and Brockman, F.J. (1997) Effect of PCR template concentration on the composition and distribution of total community 16S rDNA clone libraries. Molecular Ecology 6: 475-482.

Chao, A. (1984) Nonparametric-estimation of the number of classes in a population. Scandinavian Journal of Statistics 11: 265-270.

Chapin, D.M., Bliss, L.C., and Bledsoe, L.J. (1991) Environmental-regulation of nitrogen-fixation in a high arctic lowland ecosystem. Canadian Journal of Botany-Revue Canadienne De Botanique 69: 2744-2755.

Chapin, F.S., Walker, L.R., Fastie, C.L., and Sharman, L.C. (1994) Mechanisms of primary succession following deglaciation at Glacier Bay, Alaska. Ecological Monographs 64: 149-175.

Chien, Y.-T., and Zinder, S.H. (1996) Cloning, functional organization, transcript studies, and phylogenetic analysis of the complete nitrogenase structural genes (*nifHDK2*) and associated genes in the archaeon *Methanosarcina barkeri* 227. Journal of Bacteriology 178: 143-148.

Church, M.J., Short, C.M., Jenkins, B.D., Karl, D.M., and Zehr, J.P. (2005) Temporal patterns of nitrogenase gene (*nifH*) expression in the oligotrophic North Pacific Ocean. Applied and Environmental Microbiology 71: 5362-5370.

Churchill, G.A. (2002) Fundamentals of experimental design for cDNA microarrays. Nature Genetics 32: 490-495.

Colwell, R.K. (2005) EstimateS: Statistical estimation of species richness and shared species from samples. Version 7.5.: Persistent URL <purl.oclc.org/estimates>.

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

Connell, J.H. (1978) Diversity in tropical rain forests and coral reefs. Science 199: 1302-13010.

Curl, E.A., and Truelove, B. (1986) The Rhizosphere. Berlin: Springer.

Darmody, R.G., Allen, C.E., and Thorn, C.E. (2005) Soil topochronosequences at Storbreen, Jotunheimen, Norway. Soil Science Society of America Journal 69: 1275-1287.

Davey, M.C., and Clarke, K.J. (1991) The spatial-distribution of microalgae on antarctic fell-field soils. Antarctic Science 3: 257-263.

Dedysh, S.N., Ricke, P., and Liesack, W. (2004) *NifH* and *NifD* phylogenies: an evolutionary basis for understanding nitrogen fixation capabilities of methanotrophic bacteria. Microbiology-Sgm 150: 1301-1313.

Deiglmayr, K., Philippot, L., Tscherko, D., and Kandeler, E. (2006) Microbial succession of nitrate-reducing bacteria in the rhizosphere of *Poa alpina* across a glacier foreland in the Central Alps. Environmental Microbiology 8: 1600-1612.

Deslippe, J.R., and Egger, K.N. (2006) Molecular diversity of *nifH* genes from bacteria associated with high arctic dwarf shrubs. Microbial Ecology 51: 516-525.

Deslippe, J.R., Egger, K.N., and Henry, G.H.R. (2005) Impacts of warming and fertilization on nitrogen-fixing microbial communities in the Canadian High Arctic. FEMS Microbiology Ecology 53: 41-50.

Dick, W.A., Juma, N.G., and Tabatabai, M.A. (1983) Effects of soils on acid-phosphatase and inorganic pyrophosphatase of corn roots. Soil Science 136: 19-25.

Dobbelaere, S., Vanderleyden, J., and Okon, Y. (2003) Plant growth-promoting effects of diazotrophs in the rhizosphere. Critical Reviews in Plant Science 22: 107-149.

Doolittle, W.F., and Papke, R.T. (2006) Genomics and the bacterial species problem. Genome Biology 7: 116.

Draghici, S., Khatri, P., Eklund, A.C., and Szallasi, Z. (2006) Reliability and reproducibility issues in DNA microarray measurements. Trends in Genetics 22: 101-109.

Duc, L., Noll, M., Meier, B.E., Bürgmann, H., and Zeyer, J. (2009a) High diversity of diazotrophs in the forefield of a receding alpine glacier. Microbial Ecology 57:179-190.

Duc, L., Neuenschwander, S., Rehrauer, H., Wagner, U., Sobek, J., Schlapbach, R., and Zeyer, J. (2009b) Development and experimental validation of a *nifH* oligonucleotide microarray to study diazotrophic communities in a glacier forefield. Environmental Microbiology, accepted.

Duc, L., Neuenschwander, S., Rehrauer, H., and Zeyer, J. (2009c) Assessing the impact of environmental factors on free-living diazotrophs in a glacier forefield by use of a *nifH* microarray. FEMS Microbiology Ecology, submitted.

Eady, R.R. (1992) The dinitrogen-fixing bacteria. In The Procaryotes. Balows, A., Truper, H.G., Dworkin, M., Harder, W., and Schleifer, K.H. (eds). New-York: Springer-Verlag, pp. 534-553.

Edwards, I.P., Bürgmann, H., Miniaci, C., and Zeyer, J. (2006) Variation in microbial community composition and culturability in the rhizosphere of *Leucanthemopsis alpina* (L.) heywood and adjacent bare soil along an alpine chronosequence. Microbial Ecology 52: 679-692.

Ekschmitt, K., and Griffiths, B.S. (1998) Soil biodiversity and its implications for ecosystem functioning in a heterogeneous and variable environment. Applied Soil Ecology 10: 201-215.

Evans, R., and Kamdar, S.J. (1990) Stability of RNA isolated from macrophages depends on the removal of an RNA-degrading activity early in the extraction procedure. Biotechniques 8: 357-&.

Fagan, B.M. (2000) the little Ice Age: how climate made history, 1300-1850. New York: Basic Books.

Ferrantini, A., and Carlon, E. (2008) On the relationship between perfect matches and mismatches in Affymetrix Genechips. Gene 422: 1-6.

Fierer, N., and Jackson, R.B. (2006) The diversity and biogeography of soil bacterial communities. Proceedings of the National Academy of Sciences of the United States of America 103: 626-631.

Franke-Whittle, I.H., Klammer, S.H., and Insam, H. (2005) Design and application of an oligonucleotide microarray for the investigation of compost microbial communities. Journal of Microbiological Methods 62: 37-56.

Frantz, S. (2005) An array of problems. Nature Reviews Drug Discovery 4: 362-363.

Fritzsheridan, R.P. (1988) Physiological ecology of nitrogen-fixing blue-green-algal crusts in the upper-subalpine life zone. Journal of Phycology 24: 302-309.

Frostegard, A., and Baath, E. (1996) The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. Biology and Fertility of Soils 22: 59-65.

Gantar, M., Kerby, N.W., Rowell, P., and Obreht, Z. (1991) Colonization of wheat (*Triticum vulgare* L.) by N_2 fixing cyanobacteria .1. A survey of soil cyanobacterial isolates forming associations with roots. New Phytologist 118: 477-483.

Gao, X.L., Gulari, E., and Zhou, X.C. (2004) *In situ* synthesis of oligonucleotide microarrays. Biopolymers 73: 579-596.

Glick, B.R. (1995) The enhancement of plant-growth by free-living bacteria. Canadian Journal of Microbiology 41: 109-117.

Graham, D.W., Chaudhary, J.A., Hanson, R.S., and Arnold, R.G. (1993) Factors affecting competition between Type I and Type II methanotrophs in two-organism, continuous-flow reactors. Microbial Ecology 25: 1-17.

Grayston, S.J., Vaughan, D., and Jones, D. (1997) Rhizosphere carbon flow in trees, in comparison with annual plants: The importance of root exudation and its impact on microbial activity and nutrient availability. Applied Soil Ecology 5: 29-56.

Griffiths, B.S., Ritz, K., Ebblewhite, N., and Dobson, G. (1999) Soil microbial community structure: Effects of substrate loading rates. Soil Biology & Biochemistry 31: 145-153.

Gros, R., Jocteur Monrozier, L., Bartoli, F., Chotte, J.L., and Faivre, P. (2004) Relationships between soil physico-chemical properties and microbial activity along a restoration chronosequence of alpine grasslands following ski run construction. Applied Soil Ecology 27: 7-22.

Guggenberger, G., and Kaiser, K. (2003) Dissolved organic matter in soil: challenging the paradigm of sorptive preservation. Geoderma 113: 293-310.

Guimil, R., Beier, M., Scheffler, M., Rebscher, H., Funk, J., Wixmerten, A. et al. (2003) Geniom (R) technology - The benchtop array facility. Nucleosides Nucleotides & Nucleic Acids 22: 1721-1723.

Haas, D., Blumer, C., and Keel, C. (2000) Biocontrol ability of fluorescent pseudomonads genetically dissected: importance of positive feedback regulation. Current Opinion in Biotechnology 11: 290-297.

Haeberli, W., and Beniston, M. (1998) Climate change and its impacts on glaciers and permafrost in the Alps. Ambio 27: 258-265.

Halbleib, C.M., and Ludden, P.W. (2000) Regulation of biological nitrogen fixation. American Society for nutritional Sciences: 1081-1084.

Halperin, A., Buhot, A., and Zhulina, E.B. (2006) Hybridization at a surface: The role of spacers in DNA microarrays. Langmuir 22: 11290-11304.

Hamelin, J., Fromin, N., Tarnawski, S., Teyssier-Cuvelle, S., and Aragno, M. (2002) *nifH* gene diversity in the bacterial community associated with the rhizosphere of *Molinia coerulea*, an oligonitrophilic perennial grass. Environmental Microbiology 4: 477-481.

Hämmerli, A., Waldhuber, S., Miniaci, C., Zeyer, J., and Bunge, M. (2007) Local expansion and selection of soil bacteria in a glacier forefield. European Journal of Soil Science 58: 1437-1445.

Hammond, J.P., Broadley, M.R., Craigon, D.J., Higgins, J., Emmerson, Z.F., Townsend, H.J. et al. (2005) Using genomic DNA-based probe-selection to improve the sensitivity of high density oligonucleotide arrays when applied to heterologous species. Plant Methods 1: 10.

Harbers, G.M., Emoto, K., Greef, C., Metzger, S.W., Woodward, H.N., Mascali, J.J. et al. (2007) Functionalized poly(ethylene glycol)-based bioassay surface chemistry that facilitates bio-immobilization and inhibits nonspecific protein, bacterial, and mammalian cell adhesion. Chemistry of Materials 19: 4405-4414.

Hardin, G. (1960) Competitive exclusion principle. Science 131: 1292-1297.

Heer, C., and Körner, C. (2002) High elevation pioneer plants are sensitive to mineral nutrient addition. Basic and Applied Ecology 3: 39-47.

Heller, M.J., Tu, E., Holmsen, A., Sosnowski, R.G., and O'Connell, J. (1999) Active microelectronic arrays for DNA hybridization analysis. DNA Microarrays 205: 167-185.

Herman, R.P., Provencio, K.R., Herreramatos, J., and Torrez, R.J. (1995) Resource islands predict the distribution of heterotrophic bacteria in Chihuahuan desert soils. Applied and Environmental Microbiology 61: 1816-1821.

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

Hodkinson, I.D., Coulson, S.J., and Webb, N.R. (2004) Invertebrate community assembly along proglacial chronosequences in the high Arctic. Journal of Animal Ecology 73: 556-568.

Horton, P., Schaefli, B., Mezghani, A., Hingray, B., and Musy, A. (2006) Assessment of climate-change impacts on alpine discharge regimes with climate model uncertainty. Hydrological Processes 20: 2091-2109.

Ikeda, S., Omura, T., Ytow, N., Komaki, H., Minamisawa, K., Ezura, H., and Fujimura, T. (2006) Microbial community analysis in the rhizosphere of a transgenic tomato that overexpresses 3-hydroxy-3-methylglutaryl coenzyme A reductase. Microbes and Environments 21: 261-271.

Izquierdo, J.A., and Nusslein, K. (2006) Distribution of extensive *nifH* gene diversity across physical soil microenvironments. Microbial Ecology 51: 441-452.

Jacot, K.A., Lüscher, A., Nösberger, J., and Hartwig, U.A. (2000a) The relative contribution of symbiotic N_2 fixation and other nitrogen sources to grassland ecosystems along an altitudinal gradient in the Alps. Plant and Soil 225: 201-211.

Jacot, K.A., Lüscher, A., Nosberger, J., and Hartwig, U.A. (2000b) Symbiotic N_2 fixation of various legume species along an altitudinal gradient in the Swiss Alps. Soil Biology & Biochemistry 32: 1043-1052.

Jenkins, B., Steward, G.F., Short, S.M., Ward, B.B., and Zehr, J.P. (2004) Fingerprinting diazotroph communities in the Chesapeake Bay by using a DNA macroarray. Applied And Environmental Microbiolgy 70: 1767-1776.

Jensen, E.S., and Haugaard-Nielsen, H. (2003) How can increased use of biological N_2 fixation in agriculture benefit the environment? Plant and Soil 252: 1777-1186.

Jones, D.L. (1998) Organic acids in the rhizosphere - a critical review. Plant and Soil 205: 25-44.

Kandeler, E., and Gerber, H. (1988) Short-term assay of soil urease activity using colorimetric determination of ammonium. Biology and Fertility of Soils 6: 68-72.

Karthikeyan, R., and Kulakow, P.A. (2003) Soil plant microbe interactions in phytoremediation. Phytoremediation 78: 51-74.

Kassen, R., and Rainey, P.B. (2004) The ecology and genetics of microbial diversity. Annual Review of Microbiology 58: 207-231.

Kastovska, K., Elster, J., Stibal, M., and Santruckova, H. (2005) Microbial assemblages in soil microbial succession after glacial retreat in Svalbard (high Arctic). Microbial Ecology 50: 396-407.

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

Kessler, P.S., Blank, C., and Leigh, J.A. (1998) The *nif* gene operon of the methanogenic archaeon *Methanococcus maripaludis*. Journal of Bacteriology 180: 1504-1511.

Kimura, M. (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide-sequences. Journal of Molecular Evolution 16: 111-120.

Kohls, S.J., Vankessel, C., Baker, D.D., Grigal, D.F., and Lawrence, D.B. (1994) Assessment of N_2 fixation and N cycling by *Dryas* along a chronosequence within the forelands of the Athabasca glacier, Canada. Soil Biology & Biochemistry 26: 623-632.

Kolb, S., Knief, C., Dunfield, P.F., and Conrad, R. (2005) Abundance and activity of uncultured methanotrophic bacteria involved in the consumption of atmospheric methane in two forest soils. Environmental Microbiology 7: 1150-1161.

Koltai, H., and Weingarten-Baror, C. (2008) Specificity of DNA microarray hybridization: characterization, effectors and approaches for data correction. Nucleic Acids Research 36: 2395-2405.

Korner, C. (2004) Mountain biodiversity, its causes and function. Ambio Suppl: 11-17.

Korner, C., Diemer, M., Schappi, B., Niklaus, P., and Arnone, J. (1997) The responses of alpine grassland to four seasons of CO_2 enrichment: a synthesis. Acta Oecologica-International Journal of Ecology 18: 165-175.

Kowalenko, C.G., and Cameron, D.R. (1976) Nitrogen transformations in an incubated soil as affected by combinations of moisture-content and temperature and adsorption-fixation of ammonium. Canadian Journal of Soil Science 56: 63-70.

Landi, L., Renella, G., Moreno, J.L., Falchini, L., and Nannipieri, P. (2000) Influence of cadmium on the metabolic quotient, L-: D-glutamic acid respiration ratio and enzyme activity : microbial biomass ratio under laboratory conditions. Biology and Fertility of Soils 32: 8-16.

Landolt, E. (1992) Unsere Alpenflora. Stuttgard, Jena: Gustav Fischer Verlag.

Langlois, R.J., LaRoche, J., and Raab, P.A. (2005) Diazotrophic diversity and distribution in the tropical and subtropical Atlantic ocean. Applied and Environmental Microbiology 71: 7910-7919.

Langlois, R.J., Hummer, D., and LaRoche, J. (2008) Abundances and distributions of the dominant *nifH* phylotypes in the Northern Atlantic Ocean. Applied and Environmental Microbiology 74: 1922-1931.

Letowski, J., Brousseau, R., and Masson, L. (2004) Designing better probes: effect of probe size, mismatch position and number on hybridization in DNA oligonucleotide microarrays. Journal of Microbiological Methods 57: 269-278.

Liengen, T. (1999) Environmental factors influencing the nitrogen fixation activity of free-living terrestrial cyanobacteria from a high arctic area, Spitsbergen. Canadian Journal of Microbiology 45: 573-581.

Liengen, T., and Olsen, R.A. (1997) Nitrogen fixation by free-living cyanobacteria from different coastal sites in a high arctic tundra, Spitsbergen. Arctic and Alpine Research 29: 470-477.

Lilburn, T.C., Kim, K.S., Ostrom, N.E., Byzek, K.R., Leadbetter, J.R., and Breznak, J.A. (2001) Nitrogen fixation by symbiotic and free-living spirochetes. Science 292: 2495-2498.

Lin, W.C., Coppi, M.V., and Lovley, D.R. (2004) *Geobacter sulfurreducens* can grow with oxygen as a terminal electron acceptor. Applied and Environmental Microbiology 70: 2525-2528.

Lovell, C.R., Piceno, Y.M., Quattro, J.M., and Bagwell, C.E. (2000) Molecular analysis of diazotroph diversity in the rhizosphere of the smooth cordgrass, *Spartina alterniflora.* Applied and Environmental Microbiology 66: 3814-3822.

Lovley, D.R., Holmes, D.E., and Nevin, K.P. (2004) Dissimilatory Fe(III) and Mn(IV) reduction. Advances in Microbial Physiology 49: 219-286.

Loy, A., Lehner, A., Lee, N., Adamczyk, J., Meier, H., Ernst, J. et al. (2002) Oligonucleotide microarray for 16S rRNA gene-based detection of all recognized lineages of sulfate-reducing prokaryotes in the environment. Applied and Environmental Microbiology 68: 5064-5081.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar et al. (2004) ARB: a software environment for sequence data. Nucleic Acids Research 32: 1363-1371.

Machado, I.M.P., Yates, M.G., Machado, H.B., Souza, E.M., and Pedrosa, F.O. (1996) Cloning and sequencing of the nitrogenase structural genes *nifHDK* of *Herbaspirillum seropedicae.* Brazilian Journal of Medical and Biological Research 29: 1599-1602.

Madigan, M.T., and Martinko, J.M. (2006) Brock biology of microorganisms. Upper Saddle River: Pearson Education.

Mager, W.H., and Dekruijff, A.J.J. (1995) Stress-induced transcriptional activation. Microbiological Reviews 59: 506-&.

Marchal, K., and Vanderleyden, J. (2000) The „oxygen paradox“ of dinitrogen-fixing bacteria. Biology and Fertility of Soils 30.

Marilley, L., and Aragno, M. (1999) Phylogenetic diversity of bacterial communities differing in degree of proximity of *Lolium perenne* and *Trifolium repens* roots. Applied Soil Ecology 13: 127-136.

Marilley, L., Vogt, G., Blanc, M., and Aragno, M. (1998) Bacterial diversity in the bulk soil and rhizosphere fractions of *Lolium perenne* and *Trifolium repens* as revealed by PCR restriction analysis of 16S rDNA. Plant and Soil 198: 219-224.

Markham, N.R., and Zuker, M. (2005) DINAMelt web server for nucleic acid melting prediction. Nucleic Acids Research 33: W557-W581.

Marschner, H. (1995) Mineral nutrition of higher plants. London: Academic Press.

Mathews, D.H., Burkard, M.E., Freier, S.M., Wyatt, J.R., and Turner, D.H. (1999) Predicting oligonucleotide affinity to nucleic acid targets. RNA Publication of the RNA Society 5: 1458-1469.

Matthews, J.A. (1992) Cambridge Studies in Ecology: The ecology of recently-deglaciated terrain: A geoecological approach to glacier forelands and primary succession. Cambridge Studies in Ecology: xvii+386.

Matveeva, O.V., Shabalina, S.A., Nemtsov, V.A., Tsodikov, A.D., Gesteland, R.F., and Atkins, J.F. (2003) Thermodynamic calculations and statistical correlations for oligo-probes design. Nucleic Acids Research 31: 4211-4217.

McArthur, J.V., Kovacic, D.A., and Smith, M.H. (1988) Genetic diversity in natural-populations of a soil bacterium across a landscape gradient. Proceedings of the National Academy of Sciences of the United States of America 85: 9621-9624.

Minerdi, D., Fani, R., Gallo, R., Boarino, A., and Bonfante, P. (2001) Nitrogen fixation genes in an endosymbiotic *Burkholderia* strain. Applied and Environmental Microbiology 67: 725-732.

Miniaci, C. (2007) Microbial diversity in the forfield of a receding glacier. PhD thesis, ETH Zurich, Zurich.

Miniaci, C., Bunge, M., Duc, L., Edwards, I., Bürgmann, H., and Zeyer, J. (2007) Effects of pioneering plants on microbial structures and functions in a glacier forefield. Biology and Fertility of Soils 44: 289-297.

Moezelaar, R., Bijvank, S.M., and Stal, L.J. (1996) Fermentation and sulfur reduction in the mat-building cyanobacterium *Microcoleus chthonoplastes*. Applied and Environmental Microbiology 62: 1752-1758.

Moisander, P.H., Shiue, L., Steward, G.F., Jenkins, B.D., Bebout, B.M., and Zehr, J.P. (2006) Application of a *nifH* oligonucleotide microarray for profiling diversity of N_2-fixing microorganisms in marine microbial mats. Environmental Microbiology 8: 1721-1735.

Montoya, J.P., Voss, M., and Capone, D.G. (2007) Spatial variation in N_2 fixation rate and diazotroph activity in the Tropical Atlantic. Biogeosciences 4: 369-376.

Moreno-Paz, M., and Parro, V. (2006) Amplification of low quantity bacterial RNA for microarray studies: time-course analysis of *Leptospirillum ferrooxidans* under nitrogen-fixing conditions. Environmental Microbiology 8: 1064-1073.

Mulvaney, R.L. (1996) Nitrogen-inorganic forms. In Methods of soil Analysis. Agronomy, S.S.S.o.A.a.A.S.o. (ed). Madison, WI, pp. 1123-1184.

Mutter, G.L., and Boynton, K.A. (1995) PCR bias in amplification of androgen receptor alleles, a trinucleotide repeat marker used in clonality studies. Nucleic Acids Research 23: 1411-1418.

Muyzer, G., and Smalla, K. (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology 73: 127-141.

Muyzer, G., Dewaal, E.C., and Uitterlinden, A.G. (1993) Profiling of complex microbial-populations by denaturing gradient gel-electrophoresis analysis of polymerase chain reaction-amplified genes-coding for 16s ribosomal-RNA. Applied and Environmental Microbiology 59: 695-700.

Naef, F., and Magnasco, M.O. (2003) Solving the riddle of the bright mismatches: Labeling and effective binding in oligonucleotide arrays. Physical Review E 68: 011906-011901-011904.

Nannipieri, P., Ascher, J., Ceccherini, M.T., Landi, L., Pietramellara, G., and Renella, G. (2003) Microbial diversity and soil functions. European Journal of Soil Science 54: 655-670.

Neilands, J.B., and Leong, S.A. (1986) Siderophores in relation to plant-growth and disease. Annual Review of Plant Physiology and Plant Molecular Biology 37: 187-208.

Nocker, A., Burr, M., and Camper, A. (2007) Genotypic microbial community profiling: A critical technical review. Microbial Ecology 54: 276-289.

Noll, M., Matthies, D., Frenzel, P., Derakshani, M., and Liesack, W. (2005) Succession of bacterial community structure and diversity in a paddy soil oxygen gradient. Environmental Microbiology 7: 382-395.

Nübel, U., Engelen, B., Felske, A., Snaidr, J., Wieshuber, A., Amann, R.I. et al. (1996) Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. Journal of Bacteriology 178: 5636-5643.

Oerlemans, J. (1994) Quantifying global warming from the retreat of glaciers. Science 264: 243-245.

Ohkuma, M., Noda, S., Hongoh, Y., and Kudo, T. (2002) Diverse bacteria related to the bacteroides subgroup of the CFB phylum within the gut symbiotic communities of various termites. Bioscience Biotechnology and Biochemistry 66: 78-84.

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

Omoregie, E.O., Crumbliss, L.L., Bebout, B.M., and Zehr, J.P. (2004) Determination of nitrogen-fixing phylotypes in *Lyngbya* sp and *Microcoleus chthonoplastes* cyanobacterial mats from Guerrero Negro, Baja California, Mexico. Applied and Environmental Microbiology 70: 2119-2128.

Osborn, A.M., Moore, E.R.B., and Timmis, K.N. (2000) An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. Environmental Microbiology 2: 39-50.

Parodi, E.R., and de Cao, S.B. (2002) Benthic microalgal communities in the inner part of the Bahia Blanca estuary (Argentina): a preliminary qualitative study. Oceanologica Acta 25: 279-284.

Piceno, Y.M., and Lovell, C.R. (2000) Stability in natural bacterial communities: I. Nutrient addition effects on rhizosphere diazotroph assemblage composition. Microbial Ecology 39: 32-40.

Poll, C., Ingwersen, J., Stemmer, M., Gerzabek, M.H., and Kandeler, E. (2006) Mechanisms of solute transport affect small-scale abundance and function of soil microorganisms in the detritusphere. European Journal of Soil Science 57: 583-595.

Poly, F., Monrozier, L.J., and Bally, R. (2001a) Improvement in the RFLP procedure for studying the diversity of *nifH* genes in communities of nitrogen fixers in soil. Research in Microbiology 152: 95-103.

Poly, F., Ranjard, L., Nazaret, S., Gourbière, F., and Jocteur Monrozier, L. (2001b) Comparison of *nifH* gene pools in soils and soil microenvironments with contrasting properties. Applied And Environmental Microbiolgy 67: 2255-2262.

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

Postgate, J.R., and Eady, R.R. (1988) The evolution of biological nitrogen fixation. Bothe, H., F. J. De Bruijn and W. E. Newton (Ed.).

Potts, M. (1994) Desiccation tolerance of prokaryotes. Microbiological Reviews 58: 755-805.

Pozhitkov, A., Noble, P.A., Domazet-Loso, T., Nolte, A.W., Sonnenberg, R., Staehler, P. et al. (2006) Tests of rRNA hybridization to microarrays suggest that hybridization characteristics of oligonucleotide probes for species discrimination cannot be predicted. Nucleic Acids Research 34.

Pozhitkov, A.E., Tautz, D., and Noble, P.A. (2007) Oligonucleotide microarrays: widely applied-poorly understood. Briefings in Functional Genomics & Proteomics 6: 141-148.

Rainey, P.B., Buckling, A., Kassen, R., and Travisano, M. (2000) The emergence and maintenance of diversity: insights from experimental bacterial populations. Trends in Ecology & Evolution 15: 243-247.

Ramette, A., and Tiedje, J.M. (2007) Multiscale responses of microbial life to spatial distance and environmental heterogeneity in a patchy ecosystem. Proceedings of the National Academy of Sciences of the United States of America 104: 2761-2766.

Raymond, J., Siefert, J.L., Staples, C.R., and Blankenship, R.E. (2004) The natural history of nitrogen fixation. Molecular Biology and Evolution 21: 541-554.

Rehrauer, H., Schönmann, S., Eberl, L., and Schlapbach, R. (2008) PhyloDetect: a likelihood-based strategy for detecting microorganisms with diagnostic microarrays. Bioinformatics 24: i83-i89.

Rosch, C., and Bothe, H. (2005) Improved assessment of denitrifying, N_2-fixing, and total-community bacteria by terminal restriction fragment length polymorphism analysis using multiple restriction enzymes. Applied and Environmental Microbiology 71: 2026-2035.

Sabacka, M., and Elster, J. (2006) Response of cyanobacteria and algae from Antarctic wetland habitats to freezing and desiccation stress. Polar Biology 30: 31-37.

Saitou, N., and Nei, M. (1987) The neighbor-joining method - a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4: 406-425.

Sall, J., Lehman, A., and Creighton, L. (2001) JMP Start Statistics. Pacific Grove: CA: Duxbury Press.

Sanguin, H., Herrera, A., Oger-Desfeux, C., Dechesne, A., Simonet, P., Navarro, E. et al. (2006) Development and validation of a prototype 16S rRNA-based taxonomic microarray for *Alphaproteobacteria*. Environmental Microbiology 8: 289-307.

Schadt, C.W., Martin, A.P., Lipson, D.A., and Schmidt, S.K. (2003) Seasonal dynamics of previously unknown fungal lineages in tundra soils. Science 301: 1359-1361.

Schadt, C.W., Liebich, J., Chong, S.C., Gentry, T.J., He, Z., Pan, H., and Zhou, J. (2005) Design and use of functional gene microarrays (FGAs) for the characterization of microbial communities. Methods in Microbiology: 331-368.

Schinner, F., and Vonmersi, W. (1990) Xylanase-activity, CM-cellulase-activity and invertase activity in soil - an improved method. Soil Biology & Biochemistry 22: 511-515.

Schlag, R.N., and Erschbamer, B. (2000) Germination and establishment of seedlings on a glacier foreland in the central Alps, Austria. Arctic Antarctic and Alpine Research 32: 270-277.

Shamir, R., Maron-Katz, A., Tanay, A., Linhart, C., Steinfeld, I., Sharan, R. et al. (2005) EXPANDER - An integrative program suite for microarray data analysis. BMC Bioinformatics 6.

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

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

Smith, B.E., and Eady, R.R. (1992) Metalloclusters of the nitrogenases. European Journal of Biochemistry 205: 1-15.

Söderberg, K.H., and Bååth, E. (2004) The influence of nitrogen fertilisation on bacterial activity in the rhizosphere of barley. Soil Biology & Biochemistery 36: 195-198.

Solheim, B., Wiggen, H., Roberg, S., and Spaink, H.P. (2004) Associations between arctic cyanobacteria and mosses. Symbiosis 37: 169-187.

Sorensen, P.L., Jonasson, S., and Michelsen, A. (2006) Nitrogen fixation, denitrification, and ecosystem nitrogen pools in relation to vegetation development in the subarctic. Arctic Antarctic and Alpine Research 38: 263-272.

Spreafico, M., Weingartner, R., Leibundgut, C. (1992) Hydrological atlas of Switzerland. Landeshydrologie und Geologie, Bern.

Stal, L.J., and Moezelaar, R. (1997) Fermentation in cyanobacteria. FEMS Microbiology Reviews 21: 179-211.

Steppe, T.F., and Paerl, H.W. (2002) Potential N_2 fixation by sulfate-reducing bacteria in a marine intertidal microbial mat. Aquatic Microbial Ecology 28: 1-12.

Steward, G.F., Jenkins, B., Ward, B.B., and Zehr, J.P. (2004a) Development and testing of a DNA macroarray to asses nitrogenase (*nifH*) gene diversity. Applied And Environmental Microbiolgy 70: 1455-1465.

Steward, G.F., Zehr, J.P., Jellison, R., Montoya, J.P., and Hollibaugh, J.T. (2004b) Vertical distribution of nitrogen-fixing phylotypes in a meromictic, hypersaline lake. Microbial Ecology 47: 30-40.

Su, Y.Z., Zhao, H.L., Li, Y.L., and Cui, J.Y. (2004) Influencing mechanisms of several shrubs on soil chemical properties in semiarid Horqin Sandy Land, China. Arid Land Research and Management 18: 251-263.

Sugimoto, N., Nakano, M., and Nakano, S. (2000) Thermodynamics-structure relationship of single mismatches in RNA/DNA duplexes. Biochemistry 39: 11270-11281.

Tabatabai, A. (1994) Soil enzymes. In Methods of soil analysis. Inc., S.S.S.o.A. (ed), pp. 775-833.

Tarraga, J., Medina, I., Carbonell, J., Huerta-Cepas, J., Minguez, P., Alloza, E. et al. (2008) GEPAS, a web-based tool for microarray data analysis and interpretation. Nucleic Acids Research 36: W308-W314.

Tilman, D. (2000) Causes, consequences and ethics of biodiversity. Nature 405: 208-211.

Tiwari, S.C., Tiwari, B.K., and Mishra, R.R. (1987) The influence of moisture regimes on the population and activity of soil-microorganisms. Plant and Soil 101: 133-136.

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

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

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

Tscherko, D., Bötler, M., Beyer, L., Chen, J., Elster, J., Kandeler, E. et al. (2003b) Biomass and enzyme activity of two soil transects at King George Island, maritime Antarctica. Arctic, antarctic, and alpine research 35: 34-47.

Ueda, T., Suga, Y., Yahiro, N., and Matsuguchi, T. (1995) Remarkable N_2-fixing bacterial diversity detected in rice roots by molecular evolutionary analysis of *nifH* gene sequences. Applied And Environmental Microbiolgy 177: 1414-1417.

Urisman, A., Fischer, K.F., Chiu, C.Y., Kistler, A.L., Beck, S., Wang, D., and DeRisi, J.L. (2005) E-Predict: a computational strategy for species identification based on observed DNA microarray hybridization patterns. Genome Biology 6.

Verhagen, F.J.M., Laanbroek, H.J., and Woldendorp, J.W. (1995) Competition for ammonium between plant-roots and nitrifying and heterotrophic bacteria and the effects of protozoan grazing. Plant and Soil 170: 241-250.

Verpoorte, E. (2003) Beads and chips: new recipes for analysis. Lab on a Chip 3: 60N-68N.

Walker, T.S., Bais, H.P., Grotewold, E., and Vivanco, J.M. (2003) Root exudation and rhizosphere biology. Plant Physiology 132: 44-51.

Wartiainen, I., Eriksson, T., Zheng, W.W., and Rasmussen, U. (2008) Variation in the active diazotrophic community in rice paddy - *nifH* PCR-DGGE analysis of rhizosphere and bulk soil. Applied Soil Ecology 39: 65-75.

Watson, M., Dukes, J., Abu-Median, A.B., King, D.P., and Britton, P. (2007) DetectiV: visualization, normalization and significance testing for pathogen-detection microarray data. Genome Biology 8.

Weckx, S., Carlon, E., De Vuyst, L., and Van Hummelen, P. (2007) Thermodynamic behavior of short oligonucleotides in microarray hybridizations can be described using Gibbs free energy in a nearest-neighbor model. Journal of Physical Chemistry B 111: 13583-13590.

Whitton, B.A. (2000) Chapter 8: Soils and Rice-Fields. In The ecology of *Cyanobacteria*. Whitton, B.A., and Potts, M. (eds). Dordrecht, The Netherlands: Kluwer Academic Publishers.

Widmer, F., Shaffer, B.T., Porteous, L.A., and Seidler, R.J. (1999) Analysis of *nifH* gene pool complexity in soil and litter at a douglas fir forest site in the Oregon cascade mountain range. Applied And Environmental Microbiolgy 65: 374-380.

Williams, M.W., and Melack, J.M. (1991) Solute chemistry of snowmelt and runoff in an alpine basin, Sierra-Nevada. Water Resources Research 27: 1575-1588.

Wilson, I.G. (1997) Inhibition and facilitation of nucleic acid amplification. Applied and Environmental Microbiology 63: 3741-3751.

Wong, C.W., Heng, C.L.W., Yee, L.W., Soh, S.W.L., Kartasasmita, C.B., Simoes, E.A.F. et al. (2007) Optimization and clinical validation of a pathogen detection microarray. Genome Biology 8.

Wu, L.Y., Liu, X., Schadt, C.W., and Zhou, J.Z. (2006) Microarray-based analysis of subnanogram quantities of microbial community DNAs by using whole-community genome amplification. Applied and Environmental Microbiology 72: 4931-4941.

Xue, L.G., Zhang, Y., Zhang, T.G., An, L.Z., and Wang, X.L. (2005) Effects of enhanced ultraviolet-B radiation on algae and cyanobacteria. Critical Reviews in Microbiology 31: 79-89.

Yannarell, A.C., Steppe, T.F., and Paerl, H.W. (2006) Genetic variance in the composition of two functional groups (diazotrophs and cyanobacteria) from a hypersaline microbial mat. Applied and Environmental Microbiology 72: 1207-1217.

Yilmaz, L.S., Bergsven, L.I., and Noguera, D.R (2008) Systematic evaluation of single mismatch stability predictors for fluorescence in situ hybrifization. Environmental Microbiology 10:2872-2885.

Yeager, C.M., Kornosky, J.L., Housman, D.C., Grote, E.E., Belnap, J., and Kuske, C.R. (2004) Diazotrophic community structure and function in two successional stages of biological soil crusts from the Colorado plateau and Chihuahuan desert. Applied and Environmental Microbiology 70: 973-983.

Zani, S., Mellon, M.T., Collier, J.L., and Zehr, J.P. (2000) Expression of *nifH* genes in natural microbial assemblages in Lake George, New York, detected by reverse transcriptase PCR. Applied and Environmental Microbiology 66: 3119-3124.

Zarda, B., Hahn, D., Chatzinotas, A., Schonhuber, W., Neef, A., Amann, R.I., and Zeyer, J. (1997) Analysis of bacterial community structure in bulk soil by *in situ* hybridization. Archives of Microbiology 168: 185-192.

Zehr, J.P., and McReynolds, L.A. (1989) Use of degenerate oligonucleotides for amplification of the *nifH* gene from the marine cyanobacterium *Trichodesmium thiebautii*. Applied and Environmental Microbiology 55: 2522-2526.

Zehr, J.P., Mellon, M.T., and Zani, S. (1998) New nitrogen-fixing microorganisms detected in oligotrophic oceans by amplification of nitrogenase (*nifH*) genes. Applied and Environmental Microbiology 64: 5067-5067.

Zehr, J.P., Jenkins, B.D., Short, S.M., and Steward, G.F. (2003a) Nitrogenase gene diversity and microbial community structure: a cross-system comparison. Environmental Microbiology 5: 539-554.

Zehr, J.P., Crumbliss, L.L., Church, M.J., Omoregie, E.O., and Jenkins, B.D. (2003b) Nitrogenase genes in PCR and RT-PCR reagents: implications for studies of diversity of functional genes. BioTechniques 35: 996-1005.

Zehr, J.P., Mellon, M., Braun, S., Litaker, W., Steppe, T., and Paerl, H.W. (1995) Diversity of heterotrophic nitrogen-fixation genes in a marine cyanobacterial mat. Applied and Environmental Microbiology 61: 2527-2532.

Zelles, L. (1999) Fatty acid patterns of phospholipids and lipopolysaccharides in the characterisation of microbial communities in soil: a review. Biology and Fertility of Soils 29: 111-129.

Zemp, M., Haeberli, W., Hoelzle, M., and Paul, F. (2006) Alpine glaciers to disappear within decades? Geophysical Research Letters 33.

Zhang, L., Hurek, T., and Reinhold-Hurek, B. (2007) A *nifH*-based oligonucleotide microarray for functional diagnostics of nitrogen-fixing microorganisms. Microbial Ecology 53: 456-470.