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Microbial Diversity in the Forefield of a Receding Glacier

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Ciro Miniaci

Dipl.-Biologist University of Göttingen, Germany

born on August 13, 1971

citizen of Italy

accepted on the recommendation of

Prof. Dr. Josef Zeyer, examiner
Prof. Dr. Bernhard Schmid, co-examiner
Prof. Dr. Matthias Baltisberger, co-examiner

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To See a World in a Grain of Sand
And a Heaven in a Wild Flower,
Hold Infinity in the Palm of Your Hand
And Eternity in an Hour.

Auguries of Innocence
William Blake

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1. Summary

Since the end of the Little Ice Age in 1850, Swiss Alpine glaciers have retreated to approximately 50 % of their original extent and continued increases in global temperatures may cause the total loss of the Alpine glaciers by the year 2100. This retreat reveals a nutrient-poor and vegetation-free forefield. Glacier forefields provide an opportunity to explore ecosystem development and primary succession in a natural environment. Previous studies have investigated the succession of flora and fauna in such systems, however, knowledge about the microbiological processes occurring in these habitats remains incomplete. Therefore a more complete understanding about the dynamics of these developing ecosystems is required.

In this thesis, bacterial interactions with the pioneering plant *Leucanthemopsis alpina* (L.) Heywood (*L. alpina*) in the forefield of the Dammaglacier (central Switzerland) were investigated. *L. alpina* is one of the most abundant plants at this site and therefore it was used to compare plant-bacterial interactions at different successional stages of the forefield. The successional stages of the forefield were assigned according to the number of years since glacial retreat.

Four research questions were addressed: (i) Can the patchy occurrence of *L. alpina* plants during early succession lead to the establishment of small-scale spatial gradients of nutrients, microbial community structure and activity? (ii) Does microbial community structure within the glacier forefield change during the growing season (June to September)? (iii) Does the time since glacial retreat influence the microbial community structure along the forefield? (iv) How can DGGE (Denaturant Gradient Gel Electrophoresis), a common molecular tool for the assessment of soil microbial diversity, be optimized to obtain more consistent experimental results?

In the early successional stages of the glacier forefield, the plant influenced bacterial cell numbers, activities, and soil enzymatic properties within the root-zone as far as 20 cm from

the plant. Fingerprinting did not reveal a clear distance effect on the microbial communities around patches of *L. alpina* plants. The effect of seasonal change on bacterial communities was dependent on the successional stage. While bacterial communities from the earlier successional stages were influenced by seasonality, microbial communities from later successional stages remained unaffected. While environmental parameters such as foliage-cover and N-availability changed in early successional soils over the growing season, they remained consistent in late successional soils. Soil-age affected microbial community structure. In the early successional stages the microbial community patterns from rhizosphere and bulk soils were clearly distinguishable from each other, whereas at the later successional stages the microbial community patterns from these two soil compartments were not significantly different. It was shown that electrophoresis run times of DGGE gels should be minimized to provide optimal band resolution. Extended electrophoresis run times may lead to inconsistent fingerprinting results due to the instability of the denaturing gradient in the gel.

2. Zusammenfassung

Seit dem Ende der kleinen Eiszeit in der Mitte des 19. Jahrhunderts sind die Schweizer Gletscher auf annähernd 50% ihrer ursprünglichen Ausdehnung abgeschmolzen. Ein weiterer Anstieg der globalen Temperaturen wird voraussichtlich dazu führen, dass die alpinen Gletscher bis zum Jahre 2100 weitgehend verschwinden werden. Als Folge davon wird die Ausdehnung von Gletschervorfeldern erheblich zunehmen. Daher ist es von besonderem Interesse, ein besseres Verständnis über die Dynamik dieser sich entwickelnden Ökosysteme zu gewinnen. Darüber hinaus bieten Gletschervorfelder eine hervorragende Möglichkeit, Prozesse der primären Sukzession in einer natürlichen Umwelt zu untersuchen. Die Sukzession von Pflanzen und Tieren in Gletschervorfeldern wird seit Jahrzehnten ausgiebig untersucht. Demgegenüber ist aber das Verständnis der mikrobiellen Prozesse in diesen Habitaten nach wie vor sehr lückenhaft.

In dieser Dissertation wurden Wechselwirkungen zwischen Bakterien und der Pionierpflanze *Leucanthemopsis alpina* (L.) Heywood (*L. alpina*) entlang des Dammagletscher Vorfeldes in der Zentralschweiz untersucht. *L. alpina* ist eine der am häufigsten vorkommenden Pflanzen in diesem Untersuchungsgebiet. Daher wurde diese Pflanzenart ausgewählt, um die Interaktion zwischen Pflanzen und Bakterien in verschiedenen Sukzessionsstadien zu untersuchen.

Vier Hauptfragen wurden in dieser Arbeit behandelt: (i) Die Pionierpflanze *L. alpina* kommt in Form einzelner Pflanzengruppen in den frühen Sukzessionsstadien des Gletschervorfeldes vor. Können sich im Bereiche dieser Pflanzengruppen kleinräumige Nährstoff- und mikrobielle Aktivitäts- und Diversitäts-Gradienten ausbilden? (ii) Hat der saisonale Wechsel (während der Untersuchungsperiode Juni bis September) einen Einfluss auf die mikrobiellen Gemeinschaften im Gletschervorfeld? (iii) Wird das Muster der mikrobiellen Diversität entlang des Vorfeldes durch das Alter des Bodens beeinflusst? (iv) *Denaturant Gradient Gel Electrophoresis* (DGGE) wird für die Untersuchung der mikrobiellen Diversität im Boden

häufig genutzt. Wie kann diese Methode optimiert werden, um konsistente Versuchsergebnisse zu gewährleisten?

Untersuchungen im Gletschervorfeld haben zu folgenden Ergebnissen geführt: Im Bereiche des Wurzelraumes von *L. alpina* wurden in den frühen Sukzessionsstadien bakterielle Zellzahlen, mikrobielle Aktivitäten und enzymatische Parameter von der Pflanze markant beeinflusst. Dieser Effekt erstreckte sich bis zu einem Radius von 20 cm um die Pflanzen. Die Daten zum genetischen Profil der mikrobiellen Gemeinschaft im Umfeld der Pflanzen zeigten aber, dass die Struktur der mikrobiellen Gemeinschaften nicht vom Abstand zu *L. alpina* abhängig war. Der saisonale Einfluss auf die mikrobielle Struktur war vom jeweiligen Sukzessionsstadium abhängig. Während die mikrobiellen Gemeinschaften in den frühen Sukzessionsstadien deutlich einem saisonalen Effekt unterlagen, war dieser bei den mikrobiellen Gemeinschaften der späten Sukzessionsstadien nicht erkennbar. In ähnlicher Weise zeigten verschiedene Umweltparameter, wie beispielsweise Stickstoffverfügbarkeit und Pflanzenbedeckungsgrad, unterschiedliche saisonale Muster. In den frühen Sukzessionsstadien nahm der Pflanzenbedeckungsgrad während der Sommermonate kontinuierlich zu. Parallel dazu nahm die Stickstoff-Verfügbarkeit ab. Dagegen war in den späten Sukzessionsstadien kein eindeutiger Trend erkennbar. Das Alter des Bodens hatte einen Einfluss auf die mikrobielle Populationsstruktur. In frühen Sukzessionsstadien zeigten die mikrobiellen Strukturen in rhizosphären- und nicht-rhizosphären Böden erhebliche Unterschiede. Im Gegensatz dazu war in späten Sukzessionsstadien kein Unterschied erkennbar. Es zeigte sich, dass die Laufzeit von DGGE Gelen minimiert werden muss, um eine optimale Auflösung des Bandenmusters zu gewährleisten. Zudem können erhöhte Gel-Laufzeiten durch Instabilität des denaturierenden Gradienten zu inkonsistenten *Fingerprints* führen.

The following chapters have been published or are accepted for publishing in refereed journals:

4.1 Effects of pioneering plants on microbial structures and functions in a glacier forefield

Miniaci, C., M. Bunge, L. Duc, I. Edwards, H. Bürgmann and J. Zeyer (2007) Biology and Fertility of Soils (DOI 10.1007/s00374-007-0203-0)

4.3 Variation in microbial community composition and culturability in the rhizosphere of Leucanthemopsis alpina (L.) Heywood and bare soil along an alpine chronosequence

Edwards, I. P., H. Bürgmann, C. Miniaci and J. Zeyer (2006) Microbial Ecology 52(4), 679–692

4.4 Electrophoresis time impacts the denaturing gradient gel electrophoresis-based assessment of bacterial community structure

Sigler, W.V., C. Miniaci and J. Zeyer (2004) Journal of Microbiological Methods 57(1), 17–22

In addition to the main topic of this thesis Ciro Miniaci also contributed to the following publications:

Local expansion and selection of soil bacteria in a glacier forefield

Hämmerli, A., Waldhuber, S., Miniaci, C., Zeyer, J. and Bunge M (2007) European Journal of Soil Science (DOI 10.1111/j.1365-2389.2007.00948.x)

Benzoate-driven dehalogenation of chlorinated ethenes in microbial cultures from a contaminated aquifer

Bunge, M., Kleikemper, J., Miniaci, C., Duc, L., Muusse, M. G., Hause, G., Zeyer, J. (2007) Applied Microbiology and Biotechnology (DOI 10.1007/s00253-007-1097-3)

3. Introduction

3.1 Why study glacier forefields?

Glacier forefield environments seem hostile and harsh to maintain life, yet they are fascinating as they raise the question of how organisms manage to establish and survive under extreme conditions. The challenge for alpine glacier forefield organisms generally lies in low mean annual temperatures, and the scarcity of nutrients in recently exposed sediments close to the glacier front. These sediments appear to carry no traces of life immediately after being released from the glacier ice. However, within a few meters from the terminus of the glacier, the bare rocks and sediments are colonized by a few pioneering life-forms such as microorganisms and plants. This study was motivated by the desire to understand how these pioneers interact to make a living in an environment which seems at least from a human perspective extreme.

Glacier forefields represent classical field sites for investigating ecological aspects of primary succession [1, 2] and for pedogenic processes [3]. Primary succession describes the development of biotic communities on entirely new substrates, with no biological legacy. Further typical examples of environments for the study of primary succession are the recently exposed surfaces after the eruption of volcanoes [4, 5] or newly formed sand dunes [6] . The earliest records that describe successional processes date back to the Greek naturalist and philosopher Theopharstus in 300 BC, who describes changes in vegetation of river floodplains [7]. However, the modern understanding of these concepts has been heavily influenced by the works of Clements [2, 8, 9] who developed a conceptual framework for the understanding of succession. The chronosequence of glacier forefields has been intensively investigated with regard to primary succession of plants (e.g [1, 10, 11]) and insects [12, 13]. The role of microorganisms in this context is still not fully understood although there is a general agreement that microorganisms play key roles with regard to nutrient cycling processes [14].

Previous studies on the succession of microorganisms in glacier forefields suggest, that microbial community structure is changing with distance from the glacier terminus (e.g. [15, 16]). Other studies indicate that plant-microbial interactions change along the chronosequence of glacier forefields [17]. A major challenge of current research is to identify key parameters that determine the diversity of microbial organisms in the developing landscape of glacier forefields and to identify their functional roles.

The glacier forefield which was investigated within this thesis is located in an alpine environment. The climate is fundamental to the understanding of the characteristic challenges that an environment poses to life. Since the climate of alpine environments is particularly different from the climate in lowland environments, this thesis will begin with a brief overview of the alpine climate.

3.2 The climate of alpine environments

The climate in alpine environments varies greatly with altitude. Climate itself, however, is a rather complex term referring to a wide range of atmospheric phenomena. The meteorological definition for the term climate describes the averaged weather events that have occurred over a longer period in a certain region or place [18]. Weather refers to parameters such as temperature, atmospheric pressure, precipitation or wind that can be measured in the atmosphere. Climate is the statistical description of weather events over a period of time ranging from months to thousands or millions of years. The general concept of climate is illustrated in Figure 1.

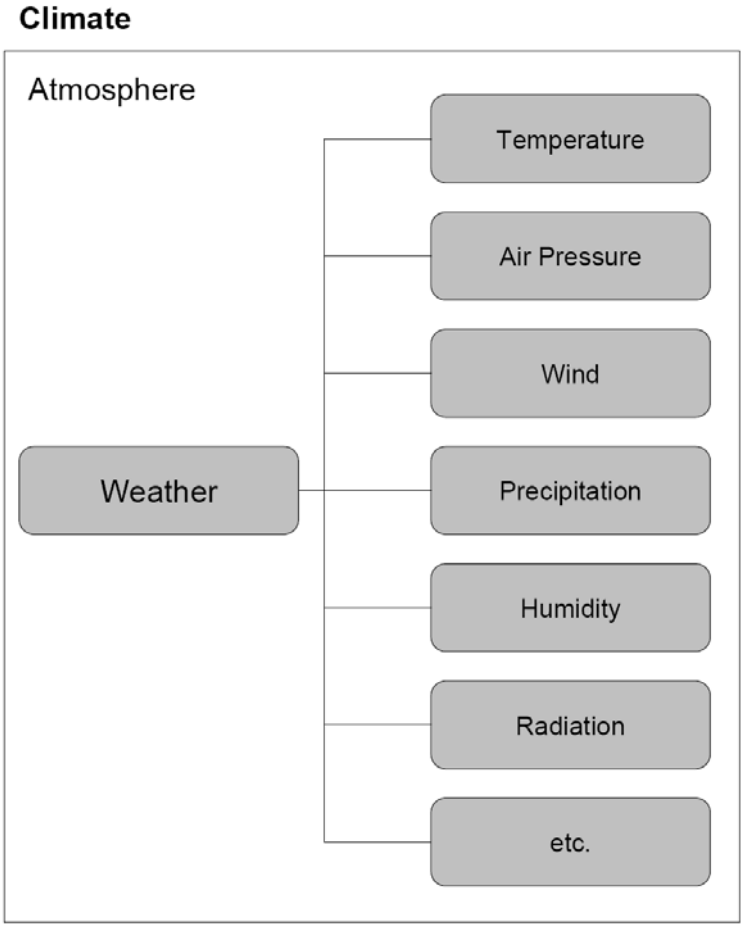

Figure 1: Climate describes the average of weather events over a defined period of time in a region. Weather events refer to the variability of parameters such as temperature, wind, air pressure, radiation, and so on, in the atmosphere.

3.2.1 Altitudinal effects of climate in alpine environments

The alpine climate is characterized by large altitudinal changes over short distances. Large altitudinal changes over short distances in combination with the particular meteorological circumstances can also lead to sharp gradients of weather parameters on the micro-scale (Figure 2). An overview showing the relationships of some important weather parameters with altitude is illustrated in Table 1.

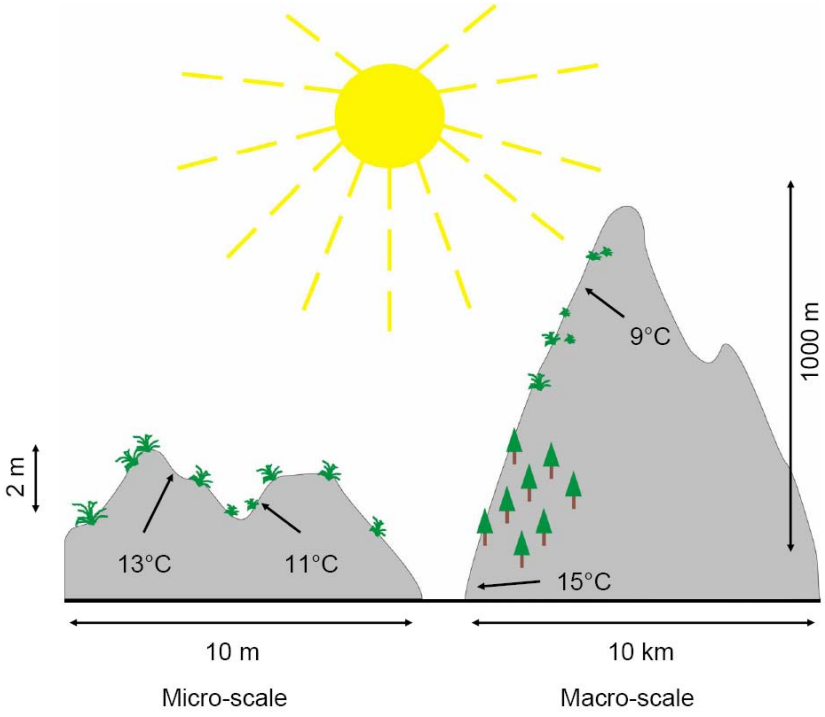

Figure 2: Thermal contrasts across the relief "stimulate" differences in temperature along elevation gradients. The arrows indicate how the relief can induce temperature differences within short distances (micro-scale) and how similar fluctuation can result from changes over wider distances or by changes of altitude of several hundred meters (macro-scale). (Modified after Körner, 1999).

Altitude [m]	P [mbar]	T [°C]	Air density [kg m^{-3}]	Saturation vapor pressure [mbar]
0	1013.25	15.0	1.2250	17.1
1000	898.8	8.5	1.1117	11.1
2000	795.0	2.0	1.0581	7.1
3000	701.2	-4.5	0.90925	4.1
4000	616.4	-11.0	0.81935	2.4
5000	540.5	-17.5	0.78643	1.3
6000	472.2	-24.0	0.66011	0.7

Table 1: The relationship between altitude, pressure, temperature, air density and saturation vapor pressure in the standard atmosphere from sea level to 6000 m above sea level (modified after Berry, 1981).

3.2.2 Altitudinal variation of air pressure, temperature, humidity, radiation, precipitation and wind

3.2.2.1 Air pressure

Climbing a mountain can be an exhausting activity not only because of physical exertion, but also because of the increasing scarcity of oxygen. With increasing altitude, the bulk of gases composing the atmosphere (mainly nitrogen, oxygen, argon and carbon dioxide) is continuously becoming thinner. While at sea level the atmospheric pressure is slightly more than 1000 mbar, at the peak of Mount Everest (8844 m) air pressure is not more than 314 mbar [18].

3.2.2.2 Temperature

Within the Troposphere, the decrease of atmospheric pressure with altitude is related to a decrease in temperature. This temperature decrease with altitude is occurring at an average rate of 6.5°C per 1000 m and depends on various factors, such as time of the day, general weather conditions as well as season [18]. This phenomenon is related to a physical process termed adiabatic cooling. As air rises, the atmospheric pressure is declining, consequently also the air expands. The expanding air has to exert work on its surroundings and therefore its temperature decreases, due to the conservation of energy.

3.2.2.3 Humidity

Humidity is a general term to describe the amount of water vapor in the atmosphere. The amount of water vapor to saturate a defined mass of air is essentially controlled by temperature [18]. Figure 3 illustrates the amount of water vapor required to saturate one kilogram of dry air at various temperatures. The graph indicates that at lower temperatures

1 kg of air is saturated with lower amounts of water vapor than at higher temperatures. Since temperature generally decreases with altitude (see chapter 3.2.2.2) at higher altitudes also the water vapor is generally decreased. The atmospheric water vapor at 3000 m altitude might be reduced by one third compared to that found at sea level [19]. As the water content of the atmosphere is of particular interest for the preservation of heat energy over a soil surface and is relevant for the ecology of glacier forefields it will be explained in more details in a later chapter.

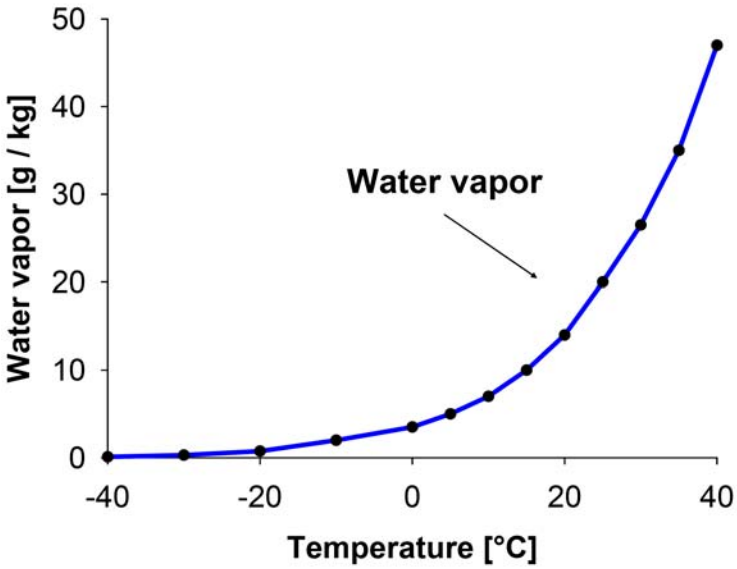

Figure 3: The amount of water vapor required to saturate 1 kg of dry air at various temperatures (after Lutgens and Tarbuck 2007).

3.2.2.4 Radiation

In the Alps, as well as at any other given position on the earth the radiation depends on the incidence angle of the sun towards the Earth. Radiation is most intense at a perpendicular incidence angle. The incidence angle α (Figure 4), determines the pathlength of a sun ray to reach the Earth's surface. The pathlength of a sunray through the atmosphere is minimal at an incidence angle of 90° and it increases at any shallower or steeper angle [18]. The absorbed solar radiation on land is reflected in the form of infrared (heat) radiation. Since air strata at

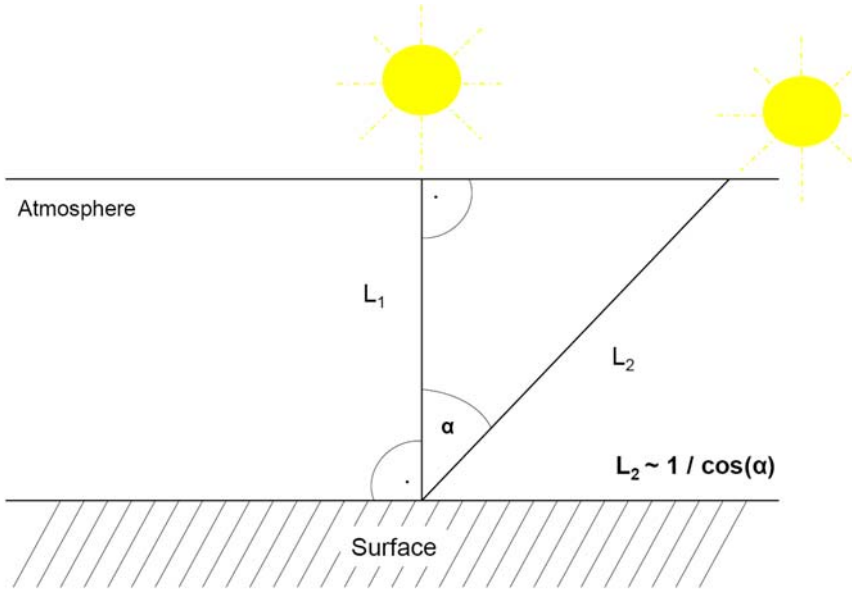

Figure 4: The path length of a sun ray through the atmosphere is dependent on the incidence angle α. At a perpendicular position of the sun towards the Earth's surface, the path length of the sun ray is minimal (L_1), whereas at any other angle, the path length increases ($L_2 > L_1$).

lower altitudes have a higher content of humidity (water vapour), CO_2 and particulate matter heat energy can be more effectively stored in the atmosphere at lower altitudes (Figure 5). Since at higher altitudes, the distance that a sun ray is traveling through the atmosphere is shorter, also the chance that sunlight will be absorbed, reflected or scattered is lower than at sea level. In the case of an ideal pure and dry atmosphere, direct solar radiation at 5500 m altitude is theoretically up to 12 % higher than at sea level [19]. About 20 % of solar radiation is absorbed by atmosphere and clouds. Another 50 % of the direct and diffused radiation is absorbed by land and sea [18]. As a result of reduced density of the alpine atmosphere, alpine environments can experience rather sharp differences of day-night temperatures. Especially on cloudless, windless nights, alpine soils lose much more heat by thermal radiation than soils

at lower altitudes. A soil surface at 3000 m altitude may radiate up to 40 % more heat energy during the night, than at 300 m altitude [20]. A further parameter related to radiation of the sun that correlates with altitude is light intensity. Light intensity may be twice as high at 1800 m altitude than at sea level [20]. Surfaces exposed to direct sunlight, may become extremely hot under alpine conditions. Thus on a clear summers day, the surface of a rock at 2000 m altitude, which corresponds to the altitude of the study site that was investigated within this thesis, may easily reach surface temperatures exceeding 50°C [20, 21] even though air temperature may only be 15°C.

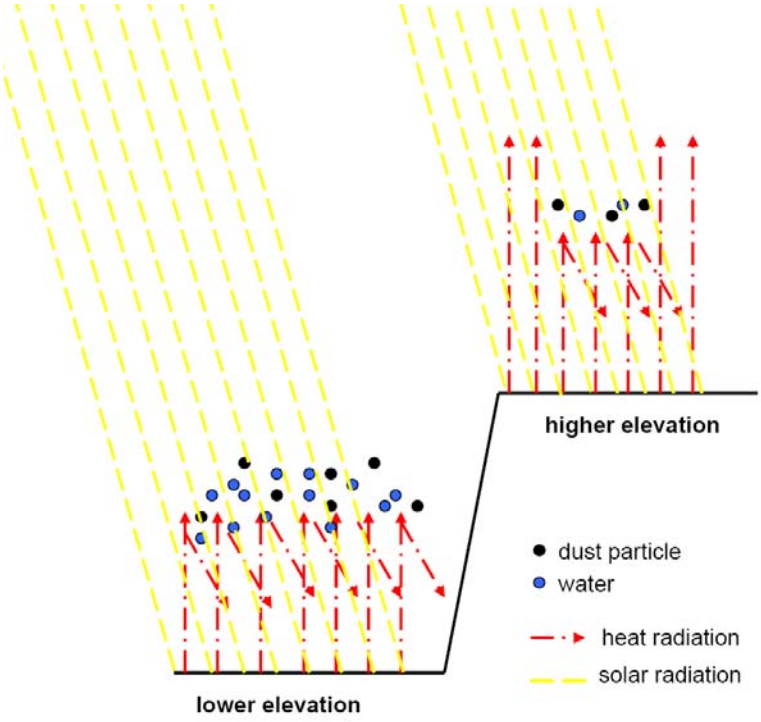

Figure 5: Solar radiation that is absorbed on the surface is radiated back in form of infrared (heat) energy. Lower atmospheric layers contain higher amounts of particulate matter and water, heat energy can be retained more effectively than at higher elevations.

3.2.2.5 Precipitation

The precipitation received in alpine environments is generally higher than at lowland sites of the same climatic region [22]. A general rule of thumb states that between 500 and 2500 m altitude, precipitation approximately increases by 100 mm per each 100 m increase in altitude [20]. This value, however, strongly depends on season and also on geographic location [19]. Figure 6 illustrates the altitudinal effect of precipitation with real-world data that have been recorded by SwissMeteo form various weather stations around Switzerland. When comparing the precipitation sums for Zurich at 556 m altitude and mount Pilatus at 2106 m altitude the data reveals, that during the observation period between 1961 and 1990, on the yearly average, the weather station on mount Pilatus recorded 867 mm more precipitation than the weather station in Zurich.

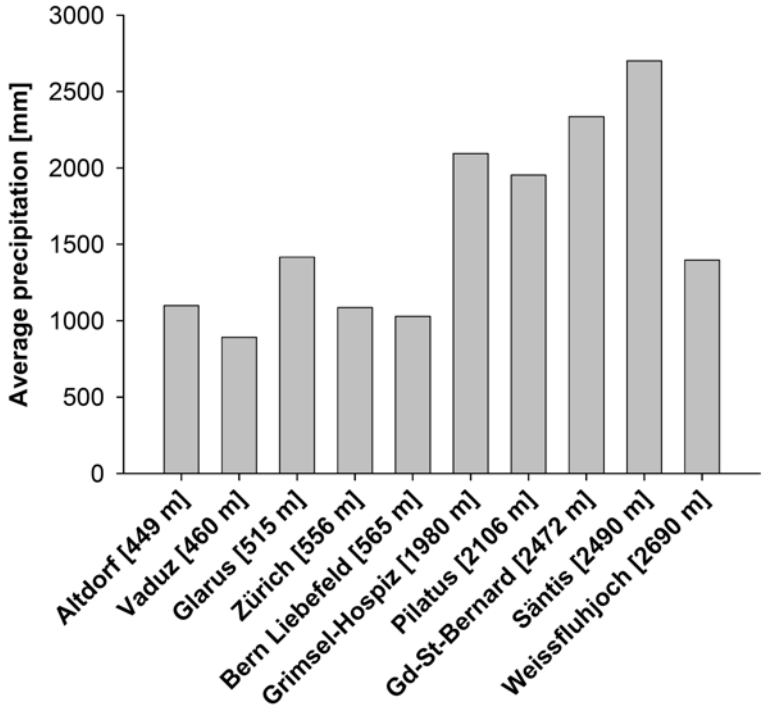

Figure 6: Altitudinal effect of precipitation. The figure illustrates average yearly precipitation sums that have been recorded by SwissMeteo between the years 1961 and 1990.

During the winter period, in middle altitudes, precipitation is frequently associated with the occurrence of wind, which may force the lifting of air over a mountain barrier. This process may lead to a lifting of the condensation level and intensify conditional instability and shower

activity [19]. The amount of precipitation received in mountain areas is, however, conditioned by different parameters, which predominantly depend on the characteristics of the air masses and the local pressure pattern, the local vertical motion of air which is affected by the property of the relief at the site, as well as microphysical processes in the clouds and the evaporation of falling drops [19].

3.2.2.6 Wind

Horizontal differences in air pressure are the driving force for the development of wind. Wind is the movement of air masses from areas of higher pressure to areas of lower pressure [18]. Pressure differences are ultimately generated by the unequal heating of the Earth's surface by the sun. An altitudinal relationship of wind speed can be observed (Figure 7), although the most important factors governing wind speed in mountain areas, are related to topographic rather than altitudinal effects [19].

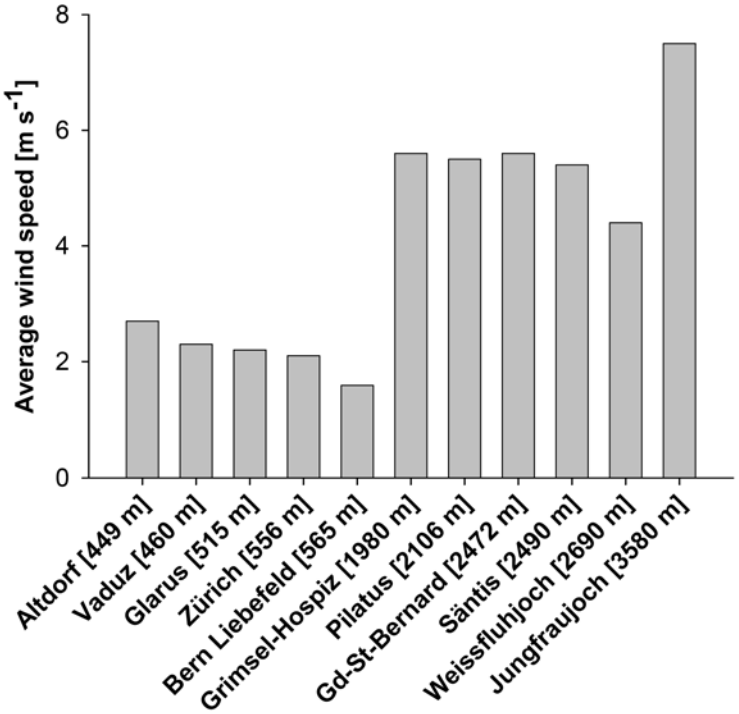

Figure 7: Increasing wind speed with increasing altitude. The diagram illustrates average yearly wind speeds recorded by SwissMeteo between the years 1981 and 2000.

Isolated and exposed ridges have higher average and extreme wind speeds as a result of the limited frictional effect of the terrain on the motion of the free air (Figure 8). As explained in the following, wind may have diverse effects on alpine life.

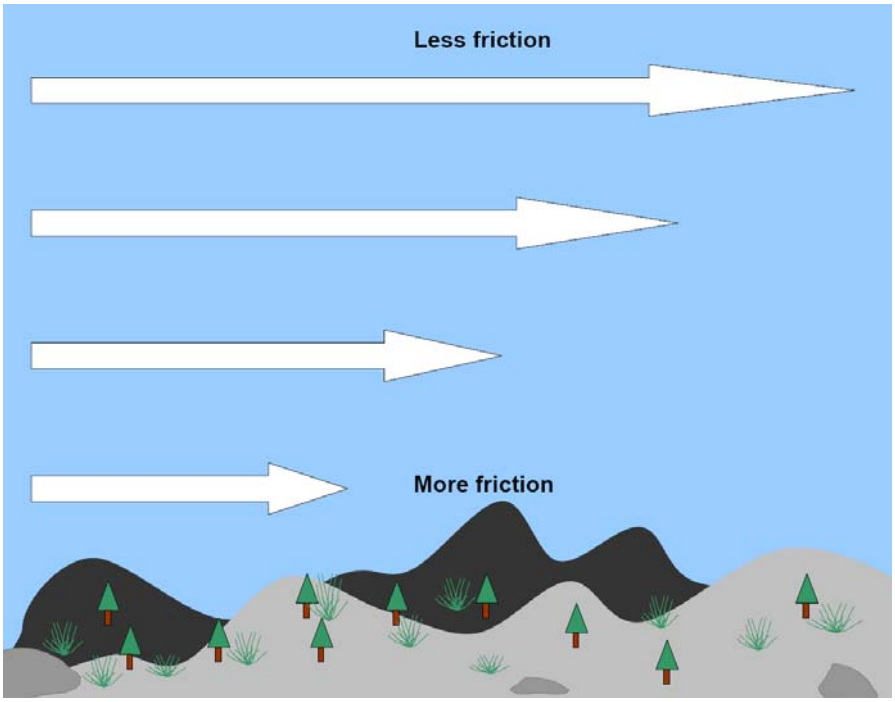

Figure 8: Wind increases in strength with increase in altitude because it is less affected by friction from objects near the Earth's surface. (Modified after Lutgens and Edward 2007).

The dispersal of plant seeds [23-25] or the inoculation of soils by microbial spores [26] in remote mountain areas may be particularly related to the action of wind. Moreover, wind may also play an important role for the transport of nutrients or pollutants in remote mountain areas. However, the action of strong winds may cause damage to plants or displace the snow cover during winter. Soils which are not densely covered by snow during winter may be subjected to strong frost, which may negatively affect or damage organisms that occur at such snow-free sites [21]. Furthermore, due to increased convection wind generally increases evaporation; therefore plants or other organisms that occur at highly wind exposed sites not only have to be well anchored into the soil, but they also need to have an efficient mechanism to reduce evaporation of water from their tissues [20].

3.2.3 Seasonal variability

3.2.3.1 Temperature variability

During the summer period in alpine environments increased radiation intensities during the day and increased reflection of heat energy during the night may lead to strong temperature variability between day and night. As a contrast during the winter periods under a deep stable snow cover soil surface temperatures may be kept at relatively constant temperatures around the melting point. This can be seen in Figure 9 where in the top panel a time series of diurnal mean temperatures ($T_{(S/A)}$) and in the bottom panel the corresponding standard deviations ($\sigma_{T\ (S/A)}$) are illustrated. The data shown in the top panel of Figure 9 were measured with a sensor installed 10 cm above ground. During the winter period when the temperature sensor was within the snow layer the standard deviation was marginal, suggesting a compact snow cover. By contrast when the sensor was not covered with snow, diurnal temperature fluctuations were high. From these temperature data it can be concluded that surface soil temperatures under a snow cover exhibit low diurnal fluctuation and are close to the melting point.

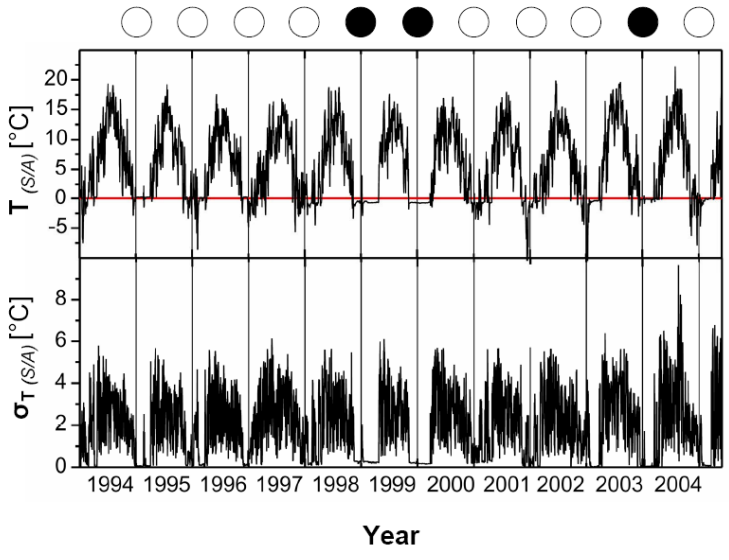

Figure 9: Diurnal mean temperatures (top panel) and diurnal standard deviations (lower panel) between 1994 and 2005, recorded by a temperature sensor installed 10 cm above soil surface. ● Winters with a compact snow cover; ○ winters with little or now snow. (Data was provided by Patrick Schleppi and originates from measurements for the NITREX project in the Alptal, Switzerland by WSL.)

3.2.3.2 Precipitation variability

In the Alps a strong seasonal and geographic variability of the annual precipitation regime is observed, which refers to the total annual precipitation amounts, but also to a distribution of the precipitation over the year [27]. Whereas for southern regions the greatest contribution to the total precipitation is made during spring and autumn, in the northern regions of the Alps precipitation may occur primarily during the winter period. The alpine region is covered with ice and snow for prolonged periods of the year. The hydrological regimes of mountain areas are strongly influenced by the water accumulated during winter in the from of snow, which is consecutively discharged during the subsequent spring period in a pronounced annual peak [28]. This accentuated discharge of water during the melting period strongly affects associated hydrological systems such as rivers and lakes. As the snow cover persists for prolonged periods during the year, it also acts as a receptor surface and an accumulation compartment for nutrients that are delivered by precipitation of dry or wet origin. Within the snowpack freeze-thaw cycles induce the fractionation of nutrients and leads to a concentration of nutrients in the deeper snow layers. As has been comprehensively reviewed [29] at the beginning of the melting period in spring, a concentrated discharge of nutrients may be released to the alpine environment, which can cover a considerable amount of the annual nitrogen demand of the alpine vegetation [30].

3.3 Glacier retreat

3.3.1 Distribution of European glaciers

In Europe, without considering Greenland, an area of approximately 54 000 km^2 is covered by glaciers. 68 % of this surface is located on the Svalbard islands (Spitzbergen), 20.9 % in Iceland, 5.5 % in Scandinavia and about 4.4 % in the Alps and the Pyrenees. A further 1.2 % is located in the Western Caucasus and the Azov sea basin area [31] (Figure 10).

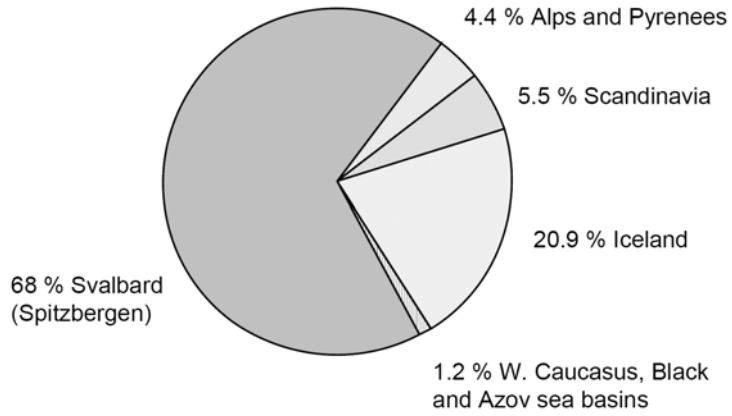

Figure 10: Distribution of European glaciers without Greenland (Data compiled by Dyurgerov and Meier 2005).

Currently most glaciated regions in Europe have revealed a retreating trend. The only areas in Europe, from where current substantial glacier advances have been reported are located along the Norwegian coast.

3.3.2 Mass dynamics

Glaciers represent bodies of ice that have developed on land and move, due to gravity, like rivers of ice. The formation of glaciers is conditioned by snow fall. Snow undergoes metamorphic changes induced by repeated sequences of freezing and thawing. Furthermore, the action of pressure as a result of newly accumulating snow and ice layers above the

previously fallen snow sheets, lead to the formation of glacial ice [32]. Physical processes in the ice allow the glacial body to plastically deform and slowly move into the landscape [33]. The mass balance of glaciers is determined by the equilibrium of newly accumulating snow within the upper part of the glacier (accumulation zone) and the melting of ice masses at the terminus of the glacier (ablation zone) [33]. Generally, the mass dynamics of glaciers is a complex process, dependent for example on the local climate and topography within the glaciated landscape. European Alpine glaciers have lost approximately 50 % of their original surface area, since the end of the Little Ice Age in 1850, when glaciers had reached their last maximal extension (Figure 11). In fact, during the last two decades an accelerated retreat of glaciers has been reported [34, 35]. Furthermore, recent model calculations predict, that European Alpine glaciers could almost completely disappear by the year 2100, assuming a temperature increase scenario of 5°C [36].

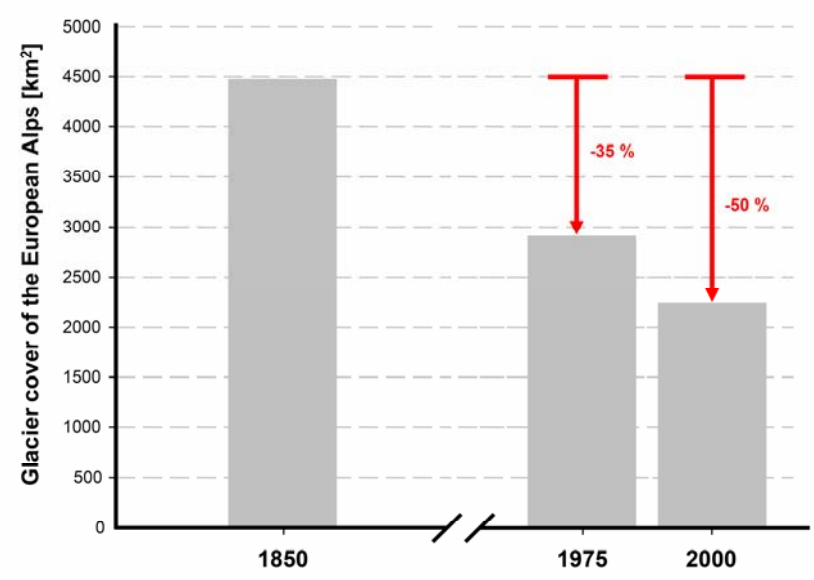

Figure 11: Spatial extension of Alpine glaciers between 1850, 1975 and 2000. The data illustrates a retreat rate of about 3 % per decade between the years 1850 and 1975 and a retreat rate of 9 % for the decades between 1975 and 2000 respectively (After Zemp et. al 2006).

3.3.3 Implications of glacier retreat for Europe and Switzerland

On the global scale European glaciers are comparably small and their melt-off will not significantly contribute to global sea level rise [37]. European glaciers, however, are located in areas that are far more densely populated and closer to settlement and infrastructure than most of the other glaciated areas of the world. This is especially true for Switzerland. Freshly exposed non-glaciated sediments on slopes can become instable, especially after intense precipitation events. From a hydrological point of view, glaciers represent important natural reservoirs for water. They play an important role in the regulation and storage of water on many time scales [38] such as melt-induced diurnal cycling and concentration of annual flow during the melt-off. Increased melt-water runoff from glaciers can induce flooding in associated river systems. Furthermore, glaciers play an important role for the generation of hydroelectric power. In Switzerland 95 % of the running power potential is already used up for hydroelectric power production [39]. Overall, Switzerland obtains about 65 % of its electricity form hydroelectrical power [40]. Besides, glaciers are also attractive for tourism and winter sport activities and they are after all a part of the cultural heritage of the country.

3.4 From rocks to soils and nutrients

3.4.1 Weathering

The alpine landscape is often shaped by rocks and scattered boulders. The local profile of the landscape therefore frequently appears cleft; elevations are shortly followed by depressions and within a short distance the altitude may vary considerably [21]. On slopes or in steep valleys the surface is often highly mobile due to rock and landslides. In front of a receding glacier, dynamical processes within the ice body, often result in the deposition of rocks as well as fine sediments.

Weathering and erosion lead to a fragmentation of compact rocks and the dissolution of minerals captured in the rocks. These processes ultimately lead to the development of soil [41]. The chemical composition of the parent rock material also affects vegetation, by influencing the properties of the developing soil [42]. For instance the Dammaglacier forefield (the field site of this thesis) is dominated by granite rock material. The weathering of this rock material is associated with low pH. Only specially adapted plants can prosper under the acidic and nutrient poor soil properties, which are typically found along this glacier forefield.

Biological processes can make important contributions to the weathering of parent rock material. Generally, activities, such as root-growth, root-exudates nutrient cycling and water uptake ultimately lead to the modification of parent rock material and increase weathering.

Lichens are frequently found on bare rock surfaces, those symbiotic associations (a symbiosis between fungi and algae) are well known for their ability to cause weathering of the rock on which they grow [43-46]. In general these symbiotic assemblages of microorganisms lead to the degradation of rock material by lowering the pH and by the penetration of hyphae into the

rock material. Recent studies have documented the weathering of granite rock material by bacterial processes, reporting the formation of pits and micropores on the granite substrate surface by the adhesion of bacteria to preferential sites on the granite, from which the bacteria elute essential minerals [47]. Similarly fungal activity on dolomite substrate material resulted in the formation of calcium-oxalates and glushinskite ($MgC_2O_4 \cdot 2H_2O$), involving processes of metal mobilization and recycling from the substrate material into newly formed minerals [48]. Other examples which illustrate the role of microorganisms to access nutrients from rock surfaces describe microorganisms in the rhizosphere of cactus plants that grow on rocks. Here microbial activity is involved in providing the plant with inorganic nutrients such as iron, calcium or nitrogen [49]. Also in this example the release of organic acids leads to modification of the parent rock material and the dissolution of nutrients that can be incorporated by the plant.

3.4.2 Establishment of a chronosequence

3.4.2.1 Soil development

Due to retreating ice masses, in front of glaciers new land, which is soon colonized by bacteria, is being uncovered. Plants and animals gradually lead to the formation of new soil [26]. This process is facilitated by weathering processes that lead to the release of nutrients from the parent rock material and which contribute to the morphology of the landscape. The land which is further away from the glacier terminus has been uncovered form the glacier ice for longer periods. Consequently it has been subject to longer periods of abiotic and biotic processes of weathering and soil formation [26]. This establishing structure is usually referred to as chronosequence. The chronosequence has important implications to predict and define the establishing ecosystem [7].

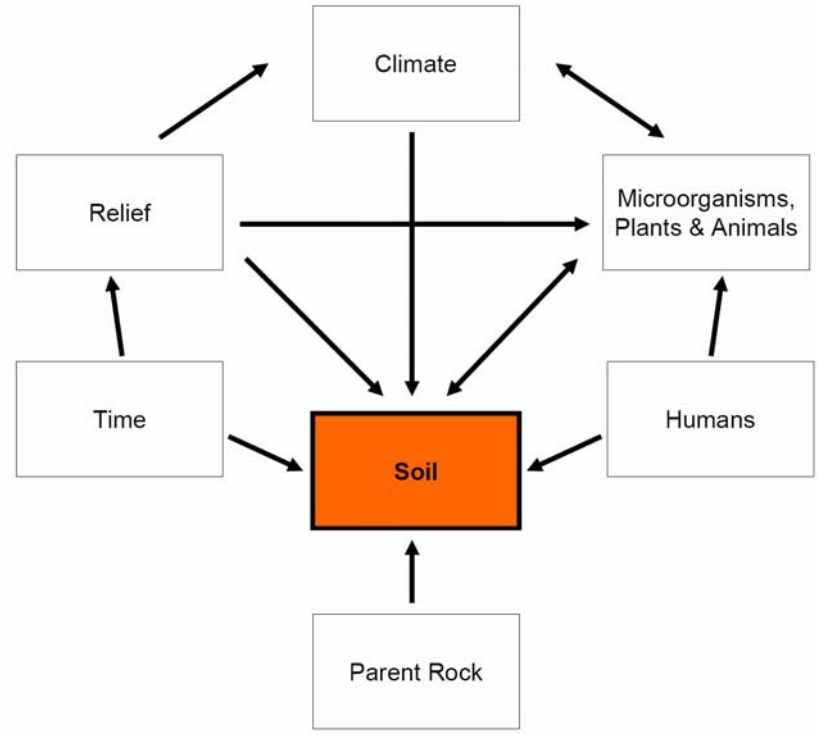

Figure 12: Major interactions (arrows) leading to the development of soil (adapted from AFU Luzern, 2000). Further details see text.

Soil is a complex term referring to the entirety of mineral and organic matter, as well as living organisms [41]. It is the layer between bedrock and vegetation and it represents the interface between rocks, the biosphere, atmosphere, and hydrosphere. Soil development in the glacier

forefield involves the interaction of processes such as the transport of sediments, weathering and interactions between organisms and substrate over time (Figure 12) [50]. Macro- and micro-climatic variables influence the rate of soil development, with faster rates under wet and warm conditions [51].

Distance from glacier [m]	Time since deglaciation [y]	Elevation [m a.s.l.]	Soil type	Total C [%]	Total N [%]	Total P [%]	pH	Plant cover
0	0-1	2053	Coarse	0.02	0	0.05	6.1	-
60	10	2052	Sand	0.06	0	0.05	5.2	+/-
100	46	2058	Fine sand	0.38	0.02	0.05	5.1	+
350	70	1985	Loamy sand	0.63	0.05	0.06	4.5	+++
500	100	1979	Loamy sand	0.97	0.07	0.07	4.5	+++

Table 2: Changing chemical, physical and biological properties along the chronosequence of the Dammaglacier. Adapted from: Sigler and Zeyer (2002).

The organic content as well as nitrogen are accumulating with time, along the chornosequence (Table 2). Usually, acidity increases and phosphorus and cation concentrations decrease along the chronosequence. Many of these changes are connected through positive feedback loops (e.g. higher nutrient availability, higher litter quality and higher decomposition rates) [51]. These soil development processes are linked with a typical transformation of the land. The immediately uncovered land in front of the glacier is vegetation-free, but within few meters from the glacier front, first pioneering plants are patchily appearing. And with increasing distance from the glacier the vegetation cover becomes more dense and abundant [26, 52]. Likewise, the combination of biological and chemical-physical processes slowly induces the development of characteristic soil profiles [42].

3.4.2.2 Nutrients

During the various stages of soil development, characteristic nutrient accumulation patterns can be observed (Figure 13). These patterns are the result of interactions between parent material and biological and chemical processes. In the case of phosphate and organic matter, a

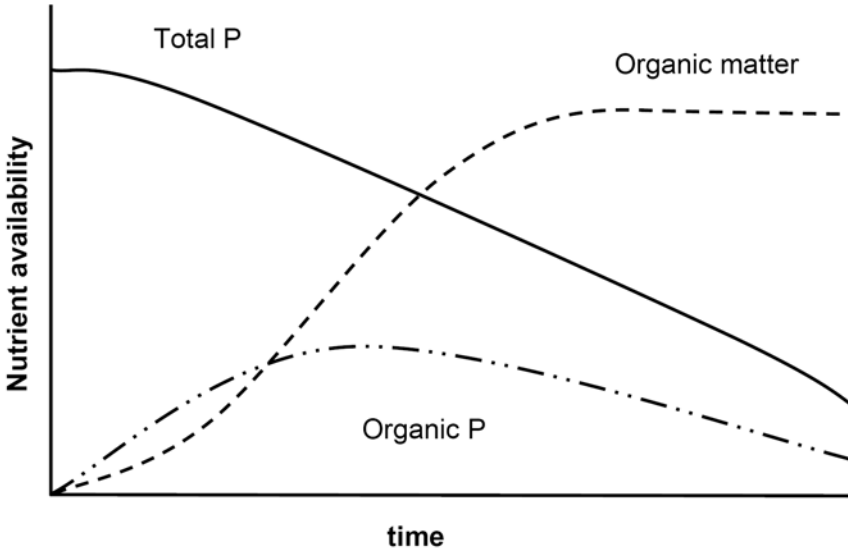

Figure 13: Various stages of soil development in relation to nutrient accumulation patterns in soil over time. Details see text (modified after Bardgett 2005).

build-up of organic matter is commonly observed in the initial stages of succession [16, 17, 50, 53]. Organic matter build-up is correlated to the gradual accumulation of microorganisms, plants and animals over time along a chronosequence. While the initial colonizing plants are usually non-mycorhrhizal ruderal plants, with ongoing succession these plants are gradually replaced by plants with symbiotic associations to arbuscular mycorrhizal fungi [50]. Arbuscular mycorrhizal fungi facilitate the uptake of phosphate from the parent material and make it available for plants. This results in the accumulation of organic phosphate in the soil. However, over time mineral phosphate resources in the soil are gradually depleted, which then in turn also leads to an exhaustion of organic phosphate in the soil.

These descriptions illustrate that the composition of vegetation and microorganisms of a soil determines soil development as well as the nutrient status of a developing soil.

Plants can induce further weathering of the soil by the excretion of organic acids, or alter its properties by introducing organic material in the form of plant litter [54]. At our field site, the Dammaglacier forefield, the acidic, young, nutrient deficient soils support only a limited number of pioneering plants, some nutritionally modest grasses and low growing shrubs such as *Salix helvetica*.

Climatic factors directly act on the availability of nutrients introducing nutrients by precipitation in the form of rain or snow, or in the form of aeolic sedimentation of organic or inorganic matter such as pollen or dust [26, 54]. Simultaneously, climate also affects plant-growth which in turn also influences the soil development process and biological processes of nutrient immobilization. Biological activities along the chronosequence of the forefield lead to the gradual accumulation of organic nutrients in the developing soil. In contrast to lowland soils, soil organic matter is more persistent in alpine soils, due to the reduced turnover of soil organic matter at higher altitudes [21]. Nevertheless soil organic matter accumulates only very slowly in alpine environments, due to the reduced primary production of alpine plants and to the predominant acid conditions in many alpine soils, which often reduce further plant primary production and the turnover process of dead organic matter. Therefore soil-layers in alpine environments are often only a few centimetres thick and the formation of thicker soil-layers of 20 or 30 cm as cited by Körner [21] may take thousands of years. To summarize: alpine life is frequently subjected to the limitation of nutrients. Alpine vegetation is often carbon [55] and nitrogen limited [56-58]. Nutrient availabilities may fluctuate considerably throughout the seasons [59, 60] and may also be spatially very heterogeneously distributed.

3.4.2.3 Colonization

Walker and del Moral [7] have summarized important factors concerning the establishment of species in newly colonized environments. They state, that the dispersal and arrival of organisms on a new site may initially be influenced by abiotic forces such as wind, water and gravity but also by animal vectors [13, 26, 54, 61]. The properties of the new barren site will then define whether seeds or spores that arrive at a new site can further germinate and allow organisms to grow. However, the degree of stress, such as drought, low nutrient availability or temperature at the site will condition the initial biomass accumulation and species turnover [54]. New arriving species will have to be able to reproduce at the site otherwise they will soon face extinction. Even if their presence will only be temporarily, the early arriving species might still contribute to the amelioration of local conditions and facilitate the arrival and establishment of other organisms. The moulding of communities at early successional stages is often greatly influenced by stochastic factors. Therefore, communities of young successional systems may sometimes be very dynamic and unstable. As a result early successional communities may be less predictable [54].

While in the earlier stages of succession bacteria dominate the microbial communities, in the later stages fungi take over a more dominant role [53]. While bacteria are probably more successfully degrading simple organic components in the early successional soil stages, fungi in the later stages of succession are increasingly involved in the degradation of accumulating recalcitrant organic matter such as lignin and complex organic acids and they are then also frequently encountered as mycorrhizal symbionts of plants [50]. Soil microorganisms, together with the rest of the soil fauna, largely control the decomposition of organic matter and the recycling of nutrients.

3.4.2.4 Adaptation

A fundamental challenge for alpine organisms is defined by the particularity of the alpine climate. Life in the alpine world has developed particular strategies to cope with these challenges. Even though alpine environments often receive higher amounts of precipitation than lowland environments [22], it may be difficult to generalize with regard to this parameter; water availability is often subjected to strong seasonal fluctuations and the retention ability of water in this environment is often reduced. Therefore, the scarcity of water remains a central problem for alpine vegetation.

A challenge for many alpine plants results from reduced humidity of the atmosphere which has led to the evolution of a thickened cuticula to reduce the loss of humidity from their cellular tissues.

A further challenge for alpine life, are extreme temperature fluctuations. Since alpine vegetation is highly dependent on the emitted heat energy from the surface, many alpine plants have evolved special adaptations such as dwarf growth forms to exploit the rapidly reflected surface heat energy during the night [20, 21]. *Silene acaulis spp. exscapa* has a dense flat growth form that covers the surface and maintains a special microclimate.

Other challenges arise from the short vegetation periods in the Alps or the low nutrient content of alpine soils. Plants have adapted to these challenges by evolving a number of strategies such as rapid growth, extended root-systems, clonal-growth, high photosynthesis rates, and efficient nutrient uptake [1, 23].

Challenges for bacterial organisms are most likely similar to those of alpine plants. Although, there appears to be no systematic study to characterize challenges for alpine bacteria, low temperatures throughout a majority of the year are most likely also a critical parameter that requires special strategies of adaptation in order to successfully survive and propagate in this habitat. Any decrease of temperature exponentially affects the rate of biochemical reactions.

Bacterial cells surviving at temperatures near or below the freezing point of water (psychrophiles) are consequently faced with problems related to a decreased membrane fluidity, which challenges the transport of nutrients and waste products. But also the inappropriate folding of proteins and intracellular formation of ice represent critical challenges. The subject of cold-adapted organisms has recently been extensively reviewed [62-64] and several mechanisms, that show how bacterial microorganisms overcome these complications, have been described. Membrane fluidity problems are apparently frequently compensated by cold bacterial organisms with special membrane properties [63]. In general, the cell membranes of many cold-adapted organisms are provided with higher content of unsaturated, polyunsaturated and methyl-branched fatty acids [64]. This altered composition is thought to play a key role in increasing membrane fluidity. Other mechanisms are related to the synthesis of antifreeze proteins which have the ability to bind to ice crystals through a large complementary surface and thereby create thermal hysteresis and lower the temperature at which an organism can grow [63]. But also special sugars and exopolysaccharides have been recognized in relation to cryoprotection of bacterial cells [63].

3.4.2.5 Interactions

As early colonizing species help to ameliorate the environmental condition of the site, they increase the probability for the invasion of less stress-tolerant species [54]. After the initial establishment in the new pristine habitat, which at the beginning is strongly dominated by abiotic conditions, biotic interactions start to increasingly gain importance and condition the further development of the ecosystem [26, 54]. The exploitation of resources in nutrient limited environments is often achieved by interactions between different specialized organisms [65-67]. While one partner is mobilizing nutrients, for instance by dissolving mineral nutrients from parent rock material, the other partner has developed special mechanisms to immobilize and incorporate these nutrients. In turn, the partner may be compensated by organic substrates or by a more stable and protected microenvironment.

Generally, it can be assumed that under favorable physiological circumstances (considering the physiological cost versus the benefit of an symbiotic interaction) interactions (e.g. a symbiotic relationship) may provide a competitive advantage in a nutrient limited environment [66, 68].

Important interactions in glacier forefields occur between bacteria and plants. Such interactions involve nutrient translocation (or exchange). Microorganisms and plants possess the ability to exude organic acids or phelnolics in order to solubilize sparingly soluble inorganic compounds [69]. Some important examples that illustrate how inorganic nutrients such as iron or manganese are being made available for plant-uptake are given in Figure 14. Plants have specific mechanisms to regulate the colonization of the rhizosphere by soil bacteria. One such mechanism is related to the translocation of photosynthetically fixed carbon compounds as root-exudates to the rhizosphere [52, 70]. Plant-root exudation activities usually lead to an increase in the number of bacterial organisms in the plant rhizosphere [69] .

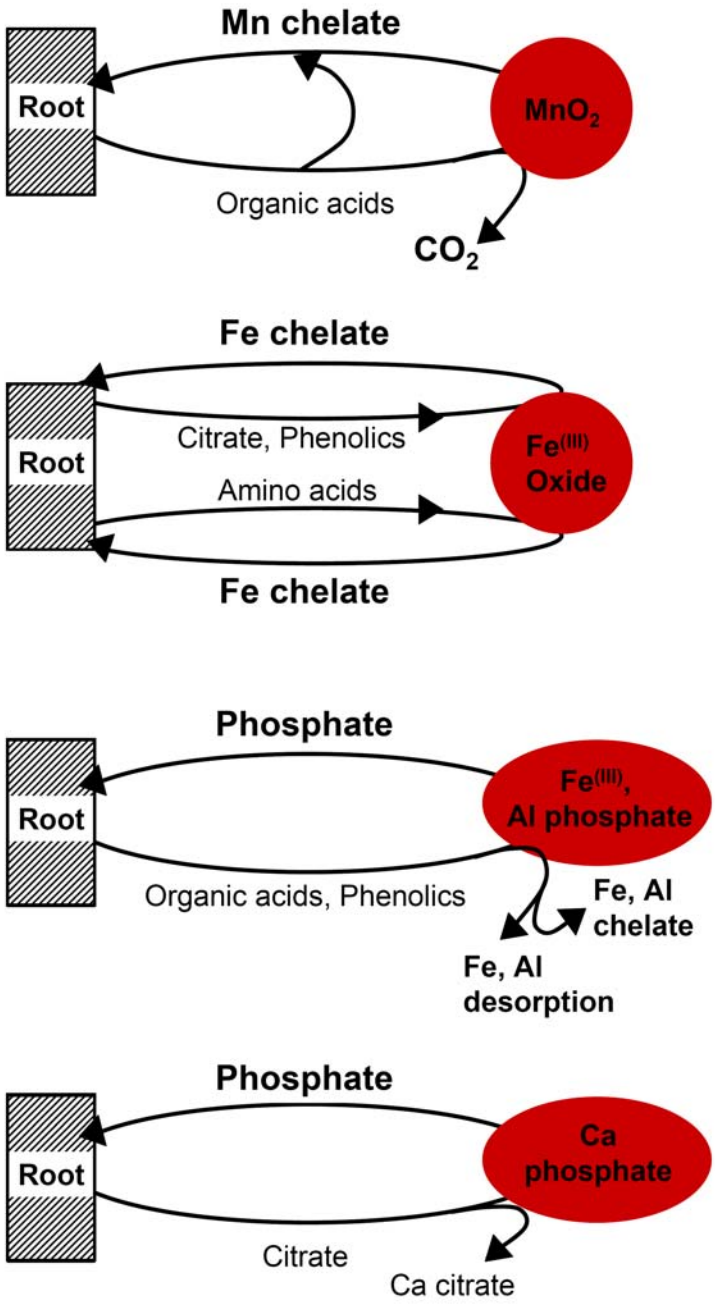

Figure 14: Principal reactions for the solubilization of inorganic nutrients by root exudates in response to nutrient deficiency of plants (modified after Marschner, 1995).

A major portion of root-exudates consists of organic acids, as well as carbohydrates and vitamins. Furthermore, roots also secrete mucilage, sloughed-off cells and tissues [69, 71, 72]. Figure 15 illustrates how some factors can affect root exudation and how this might be related to microbial activity. Mucilage might mediate close contact to soil-particles and create a soil-root interface where special processes take place that can be of considerable importance for

the uptake of mineral nutrients via phytosiderophores. Similarly, also low molecular weight compounds (LMW) play an important role for the mobilization of mineral nutrients that can then be taken up by the plant.

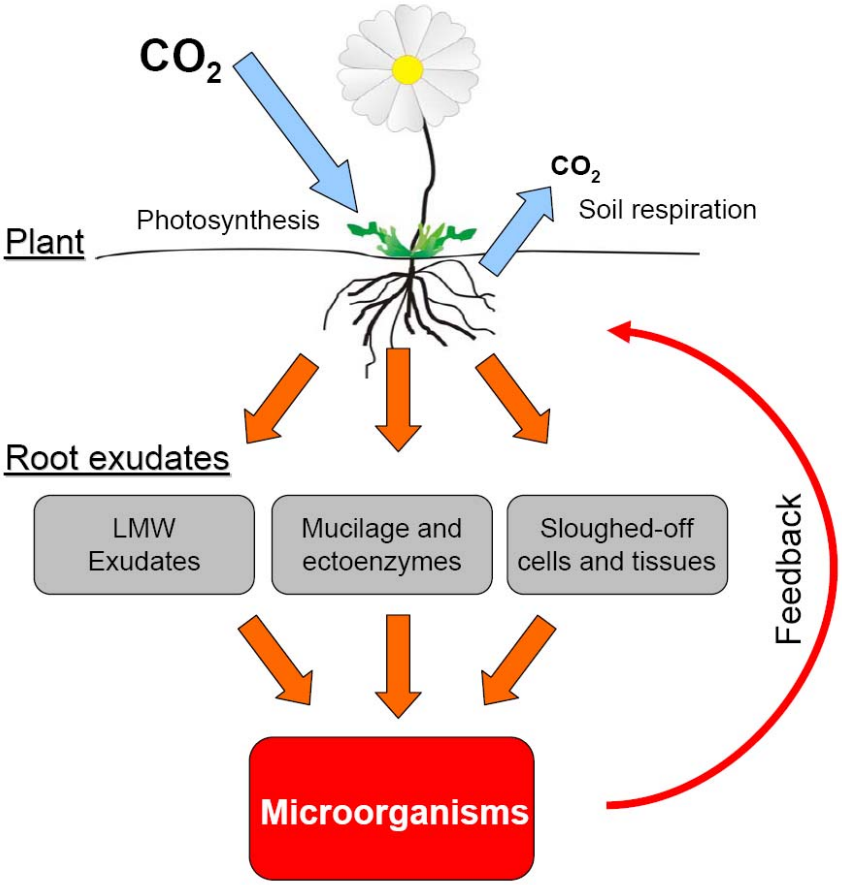

Figure 15: Factors affecting root-exudation. (LMW = low molecular weight). LMW-exudates include organic acids, sugars, phenolics, amino acids (modified after Marschner, 1995).

3.5 Thesis project

3.5.1 Field site

The field site of this project has been the forefield of the Dammaglacier, which is located in central Switzerland in the canton of Uri (Figure 16). The study site is located at approximately 2000 m above sea level and receives an annual precipitation of about 2400 mm [22].

Figure 16: The Dammaglacier forefield is located in central Switzerland in the canton of Uri. The research site is situated about 2000 m above sea level.

The Dammaglacier has been experiencing a cumulative length change of approximately 400 m since the last 85 years of observation (Figure 17). Since the 1990s the Dammaglacier has been continuously retreating [73].

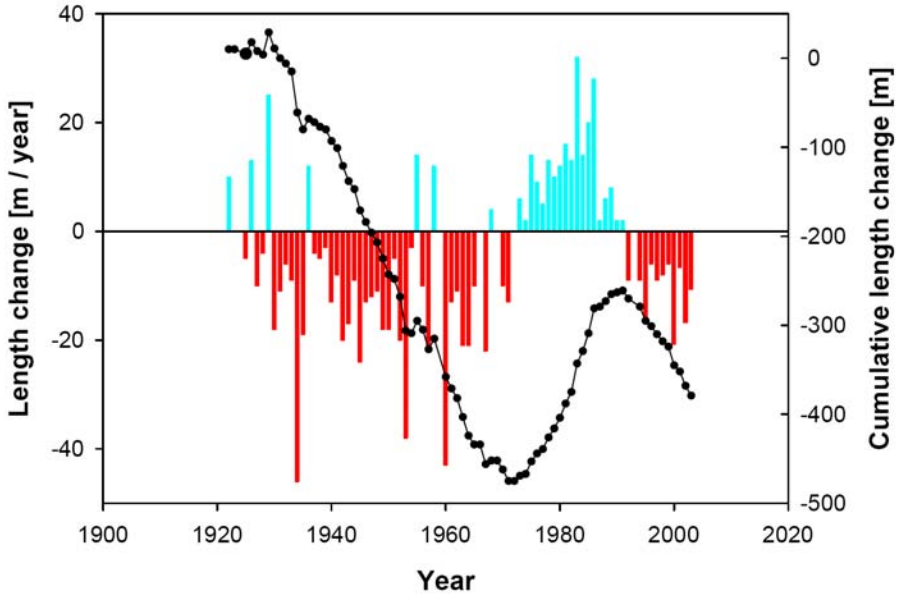

Figure 17: Length variation measurements of the Dammaglacier between the year 1921 and 2003 (from Swiss Glacier Monitoring Service, 2006).

3.5.2 Research Outline

The research described in this thesis is organized in four chapters, each addressing different aspects of microbial colonization in the forefield of a receding glacier. This outline gives a general overview on the different research topics and the underlying research questions of this thesis.

In chapter 4.1 the focus was on the investigation of interactions between the pioneering plant *Leucanthemopsis alpina* (L.) Heywood (*L. alpina*) and microorganisms at an early successional time-point of the glacier forefield chronosequence. The main questions were:

(i) How are microbial biomass, activity and diversity, affected by the presence of the pioneering plant *L. alpina*?

(ii) How far do plant-microbial interactions extend into the space around the rhizosphere of this plant?

Chapter 4.2 is extending the first study by introducing the aspect of seasonal variability. The main questions in this chapter were:

(i) How is the interplay between the pioneering plant *Leucanthemopsis alpina* (L.) Heywood and microorganisms affected by seasonal change.

(ii) How does the seasonal variability of environmental parameters such as temperature, precipitation, vegetation-cover and nutrient-availability correlate with microbiological parameters such as bacterial abundance and diversity?

Chapter 4.3 focusses on plant-microbial interactions along the chronosequence of the Dammaglacier forefield. The main questions in this chapter were:

(i) How does the microbial community respond to the successional age of the landscape?

(ii) How do environmental properties in the rhizsophere and bulk soil of *Leucanthemopsis alpina* (L.) Heywood change along the chronosequence?

Chapter 4.4 discusses the Denaturing Gradient Gel Electrophoresis (DGGE) method, which has been extensively used throughout this thesis to study genetic patterns of microbial diversity. The main questions in this chapter were:

(i) What is the importance of key parameters that effect DGGE-fingerprinting results?

(ii) How can the DGGE method be optimized?

(iii) Which factors have to be considered for the proper implementation of this method in studies addressing microbial community composition?

4. Results

4.1 Effects of pioneering plants on microbial structures and functions in a glacier forefield

Miniaci, C., M. Bunge, L. Duc, I. Edwards, H. Bürgmann and J. Zeyer (2007) **Biology and Fertility of Soils (DOI 10.1007/s00374-007-0203-0)**

4.1.1 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 Dammaglacier (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, as well as 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, since the bacterial communities were similar regardless of distance to the plant.

4.1.2 Introduction

The majority of Swiss glaciers are currently receding as a consequence of global warming [1]. Since 1850, around the end of the little Ice Age, European glaciers lost about 50 % of their original glaciated area [2]. The forefield of receding glaciers is initially vegetation free, with low nutrient content [3, 4]. In a continuous glacier retreat, greater distance from the termini corresponds to longer periods of ice free exposure, resulting in a successional chronosequence [5].

Whereas the succession of flora and fauna has been extensively studied in this system [5-9], only few studies addressed bacterial processes in glacier forefields [10-13]. Sigler and Zeyer [12], using molecular methods, reported that community structures and metabolic functions change along the chronosequence of a glacier forefield. Tscherko et al. [14] 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 % to 60 % of their photosynthetically fixed carbon into the rhizosphere, which may stimulate microbial communities and their associated enzymatic activities [15, 16]. However, along a glacier forefield chronosequence the interaction between microorganisms and vegetation might differ at the various successional stages. Tscherko et al. [13] 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. [17] 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.[18]. A major focus of this study were 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 Dammaglacier forefield soils, and because there are previous studies on the plant-microbial interactions for this plant [18]. 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 rDNA genes using RFLP and 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.

4.1.3 Materials and methods

4.1.3.1 Field site and sample collection

The research site at the terminus of the Dammaglacier 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 [12]. Between July and October 2003 we noted very large day-night temperature fluctuations, the observed maximum and minimum soil surface temperatures ranged between 38°C 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 ha 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. Sub-samples were dried at 105°C for 24 h and subsequently weighed for dry mass determination of root material and soil.

4.1.3.2 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 94086, USA) was used to measure nitrate, phosphate, lactate, acetate, propionate, formate, butyrate, pyruvate, oxalate, and citrate in their anionic forms [18]. Total soluble sugars were determined by acid hydrolysis as glucose equivalents [19]. 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 [20]. 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 was 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 (PBS; pH 7). Total bacterial cell numbers were determined after DAPI (4'6-diamidino-2-phenylindole) staining using an epifluorescence microscope [21]. Active bacterial cells were counted after FDA (fluorescein diacetate) hydrolysis by bacterial cells based on the method cited by Alef [22] 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; 10,000 x 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 were 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. [4], and pooling triplicate extracts for subsequent PCR-

amplification. For analysis of restriction fragment length polymorphisms (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 [23]. PCR products were purified with equal volumes of chloroform and re-precipitated with isopropanol. Subsequently, the PCR products (2 µl) were digested with *Hae* III (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 Laboratories, Hercules, California) was used, the running buffer was 1 x 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 minutes with GelStar® (Cambrex Bioscience, Baltimore, MA), 1:10,000 in 1 x TAE buffer. The restriction patterns were photographed under UV light using the GelDoc 2000 system and Quantity One® software (Bio-Rad Laboratories, Hercules, CA). After using a rolling disk algorithm for background correction, band intensities and relative band position were determined. Digital image data was 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 denaturing gradient gel electrophoresis (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. [24]. Each PCR reaction contained 1 x PCR buffer, 2 mM MgCl, 0.2 mM of each dNTP, 0.5 µM of each primer, 0.2 mg ml^{-1} BSA, 1U of *Taq* polymerase (Invitrogen, Carlsbad, CA 92008, 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. [25]. Gels were run in 1 x 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

$$H = -\sum_{j=1}^{X} p_i \ln p_i$$

with p_i 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 [26], 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 [27]. 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 [28] by colorimetrical determination of the ammonia released after incubation of field moist soil samples (5 g) with 2.5 ml urea solution [28]. Estimation of alkaline and acid phosphatase activity were performed according to the method described by Tabatabai [27]. 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 1h 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.

4.1.3.3 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 pairwise 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.

4.1.4 Results

Table 3: Chemical and physical properties across the investigated distance classes of central *L. alpina* plants in the Dammaglacier forefield. Values are given ± 1 standard error of the mean. The distance effect was tested with ANOVA.

	Distance from plant			ANOVA	
	0 cm (n = 3)	20 cm (n = 11)	40 cm (n = 10)	$F_{1,24}$-ratio	p-value[4]
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)$^{-1}$][3]	13.08 ± 3.58	10.94 ± 1.37	10.42 ± 1.32	0.32	0.73
Total organic acids [µg (g dry soil)$^{-1}$] [3)]	344.62 ± 23.11	283.91 ± 17.81	313.56 ± 29.35	0.86	0.44
Lactate [µg (g dry soil)$^{-1}$] [3)]	268.03 ± 9.62	240.43 ± 10.81	248.00 ± 14.82	0.53	0.60
Formate [µg (g dry soil)$^{-1}$] [3)]	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[1)]	< 100[1)]	< 100[1)]	-	-
Nitrate [µg N (g dry soil)$^{-1}$] [3)]	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)$^{-1}$] [3)]	108.32 ± 62.54	< 24 [2)]	< 24 [2)]	-	-

[1)] below detection limit (100 µg N (g dry soil)$^{-1}$)
[2)] below smallest standard concentration (24 µg [g dry soil]$^{-1}$)
[3)] measurements were done in 0.01M $CaCl_2$ extracts
[4)] significance-threshold 0.05

The measured chemical and physical soil properties showed no significant effect with distance from the plant (Table 3), 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*. Total organic C (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 4: Biological properties across three investigated distance classes of central *L. alpina* plants. Values are given ± 1 standard error of the mean. The effect of distance was tested by ANOVA.

	Distance from plant			ANOVA	
	0 cm (n = 3)	20 cm (n = 11)	40 cm (n = 10)	$F_{1,24}$-ratio	p-value[1)]
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

[1)] significance-threshold 0.05

In contrast to chemical data, cell count data showed significant trends with distance from the plant (Table 4). 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 4). DAPI counts were highest in the plant center soils (7.88 x 10^8 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.

Restriction fragment length polymorphism (RFLP) analysis revealed complex restriction patterns (Figure 18). 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 (Figure 18).

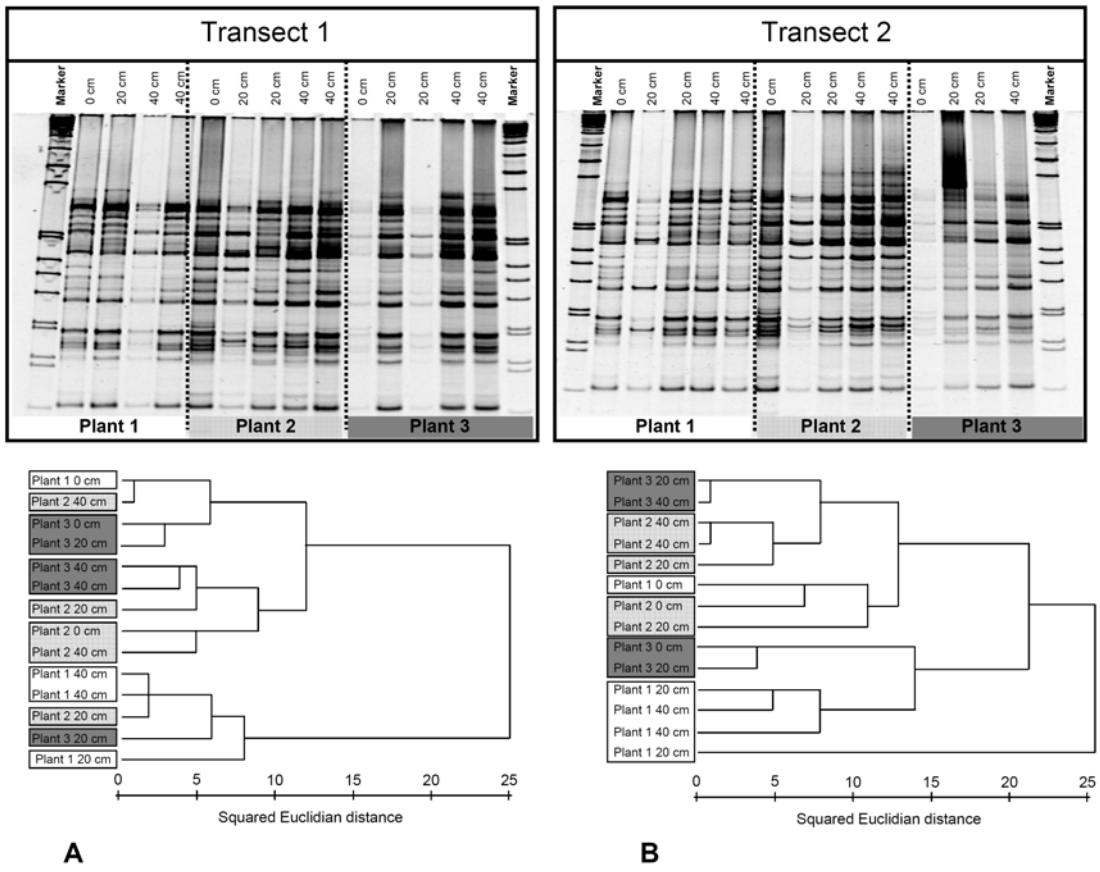

Figure 18: RFLP fingerprinting patterns of 16S rDNA fragments, originating from different distance classes (top panel) obtained from two transects (A and B) across *L. alpina* plants together with corresponding results obtained by hierarchical clustering using Ward's method (lower panel).

Fingerprinting by denaturing gradient gel electrophoresis (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 phylotypes [25]. We therefore used DGGE banding information to investigate two different diversity parameters: richness (number of different phylotypes), and Shannon-Index (Table 5), 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 5). 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 5 : Summary of diversity parameters calculated from DGGE banding patterns for samples from three distance classes. Values are given ± 1 standard error of the mean (n = 27).

	Distance from plant					
	0 cm	**20 cm**	**40 cm**	**PC[1)]**	**sig[2)]**	**p-value[3)]**
Shannon-Index	2.66 ± 0.07	2.51 ± 0.08	2.39 ± 0.11	0.352	0.07	p = 0.06
Richness	18.67 ± 1.20	17.55 ± 1.55	16.20 ± 1.69	-0.202	0.31	p = 0.25

[1)] Pearson Correlation
[2)] two-tailed significance
[3)] 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 4). 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 4). The ratio of saccharase activity divided by the number of FDA stained bacterial cells also decreased with distance (Table 4), 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 4). 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 4). 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 4).

4.1.5 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. [18], 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 centre soils, as would be expected based on numerous studies on the rhizosphere effect (e.g. [29-32]). 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 rhizophere in young soils [13, 18]. 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. [18] 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 [33, 34]. Acid

phosphatase, which also showed a significant trend with distance, may originate from the plant as well as microbes [35]. The soil we studied has been shown to become both P and N limited in the presence of sufficient carbon (B. Bleikolm, H. 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 [18]. Unlike our results, Tscherko et al. [13] did not observe enhanced enzymatic activities in the rhizosphere of the alpine grass *Poa 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 [36]. 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 [37]. 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 4). E.g the saccharase and glucosidase activity per FDA stained cell in 40 cm soil was 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 with 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 [38, 39]. 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. [5]. 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 [40]. Specific compounds in the root exudates might even selectively stimulate certain beneficial bacterial groups, which have mechanisms to potentially improve plant growth [41-43]. Edwards et al. [18] observed different microbial communities in rhizosphere of *Leucanthemopsis alpina* and interspace soil also sampled in this study using DGGE. The different findings may be explained by the different sampling procedures, since Edwards et al. [18] sampled rhizosphere soil directly within the root zone. However, Tscherko et al. [17] studied the microbial community directly in *Poa alpina* rhizosphere (root adhering soil) using PLFA analysis and found similar microbial community structure in bulk soil and rhizosphere in young soils. Our results support the conclusion of Tscherko et al. [17] 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 to 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 [44]. In conclusion, the currently available data remains inconclusive 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. [18] 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") [45, 46]. 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.

4.1.6 References

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4.2 Seasonal changes of microbial communities at two successional time-points in a glacier forefield

4.2.1 Abstract

In this study microbial communities at two different successional time-points in the glacier forefield have been investigated. The aim of this study was to investigate whether bacterial abundance and diversity of the microbial communities as well as the interactions between the pioneering plant *Leucanthemopsis alpina* (L.) Heywood (*L. alpina*) and root-associated microorganisms is affected by seasonal variability. The main objective of this study was to compare if microbial communities from different successional stages of *L. alpina* rhizosphere and bulk soil respond differently to changing environmental conditions throughout the snow-free season. Our study revealed the highest precipitation and strong temperature fluctuations at the beginning of the snow-free season (shortly after snowmelt). While foliage-cover was constantly increasing during the snow-free season at the young successional site, foliage-cover at the older successional sites remained more constant throughout the season. Our results indicated that microbial communities from young and old successional soils are differently affected by seasonal change. While the microbial communities in young successional soils show a strong seasonal effect, microbial communities from older successional soils did not reveal a clear seasonal effect. It is suggested that seasonal fluctuations of environmental parameters more strongly affect microbial communities in young successional soils where they trigger a seasonal community shift. However, a generally higher nutrient content and a more developed vegetation-cover may support a more constant microbial community profile in the older successional soils of the glacier forefield.

4.2.2 Introduction

Within the last 150 years European Alpine glaciers lost about half of their original area and they may completely disappear by the year 2100 [1]. One of the outstanding attributes of the accelerated glacier retreat are the uncovered forefields in front of the glaciers. The distance from the glacier front correlates with time since deglaciation, and processes of weathering and soil formation become more apparent with increasing distance from the glacier front. This makes the chronosequence of glacier forefields a unique and exceptional study site to ascertain natural processes of ecosystem development.

The harsh environment of recently exposed terrains consisting of rock material, boulders, and fluvial sands permits growth of only a limited number of organisms adapted to these hostile conditions, but the more distant regions in the forefield are gradually colonized by microorganisms, plants and animals. As plant growth is one of the most striking changes along the chronosequences, glacier forefields have been widely studied with respect to the primary succession of plants (*e.g.* [2-5]).

Although microorganisms are key players in the accumulation of organic matter in the developing soil and are among the first biota present at the newly exposed rocks and sediments, the role of microorganisms with regard to nutrient cycling and soil development at glacier forefields has been less well documented. Recent reports, however, demonstrate that microbial community structure is changing with distance from the glacier terminus, representing microbial succession in a similar manner as shown for plants [6-9].

Due to the reduced primary production by alpine plants and the low temperatures for decomposition of plant litter, the turnover of organic matter at higher altitude is generally

decreased. On the other hand, the rhizosphere of pioneering plants may provide a preferential microenvironment for an increased turnover of nutrients leading to a more rapid build-up of biomass around the plant, and thus may assist their pioneering lifestyle. Such plant-bacteria interactions have been reported for *Leucanthemopsis alpina* (L.) Heywood (*L. alpina*) and their root-associated microbes at the Dammaglacier forefield, Switzerland [10]. We recently showed that interactions between pioneering *L. alpina* plants are not restricted to the zone immediately beneath the plant, but extend comparably far into the space around the plants and thus affect microbial activity and community structure [11]. Hence, such areas may represent hot spots for microbial abundance and activity, and may significantly contribute to soil formation and ecosystem development.

Recent results by Edwards *et al*. suggest that the degree of interaction between *L. alpina* and microorganisms change along the chronosequence of a glacier forefield, exhibiting strongest plant-bacteria interactions in the *L. alpina* rhizosphere from young successional stages [10]. Presumably the dynamics of interactions are regulated by the plants through root exudates, suggesting one strategy of plants and their associated soil microbes to cope with the most unfavorable growth conditions. However, alpine environmental conditions are extremely variable throughout the seasons. During winter time constant soil surface temperatures near the freezing point are guaranteed by a thick cover of ice and snow. In the Central Alps the vegetation period is usually short, determined by a snow-free season of about 5 months at 1800 m altitude, and only 2 months at 2500 m. That also means that with increasing altitude the relative temperature changes are temporarily more pronounced, *i.e.*, they occur over a shorter time resulting in steep seasonal temperature gradients. Furthermore, during the summer period surface temperatures exhibit strong day-night fluctuations due to increased thermal radiation at higher altitudes [12]. Between night and day, temperature fluctuations of 50°C can easily be measured on soil surfaces [12]. Also,

precipitation is subject to extreme seasonal variation [12]. It remains uncertain to what extent microbial communities themselves, or the plant-bacteria interactions are susceptible to the changes in temperature, precipitation, foliage-cover, or nutrient availability within the short time of an alpine summer.

The scope of our study was to investigate whether bacterial abundance and diversity of the microbial communities as well as the interactions between the pioneering plant *L. alpina* and root-associated microorganisms are affected by seasonal variability. An important aspect of our research was to compare if microbial communities from different successional stages of *L. alpina* rhizosphere and bulk soil respond differently to changing environmental conditions throughout the snow-free season.

4.2.3 Materials and methods

4.2.3.1 Field site

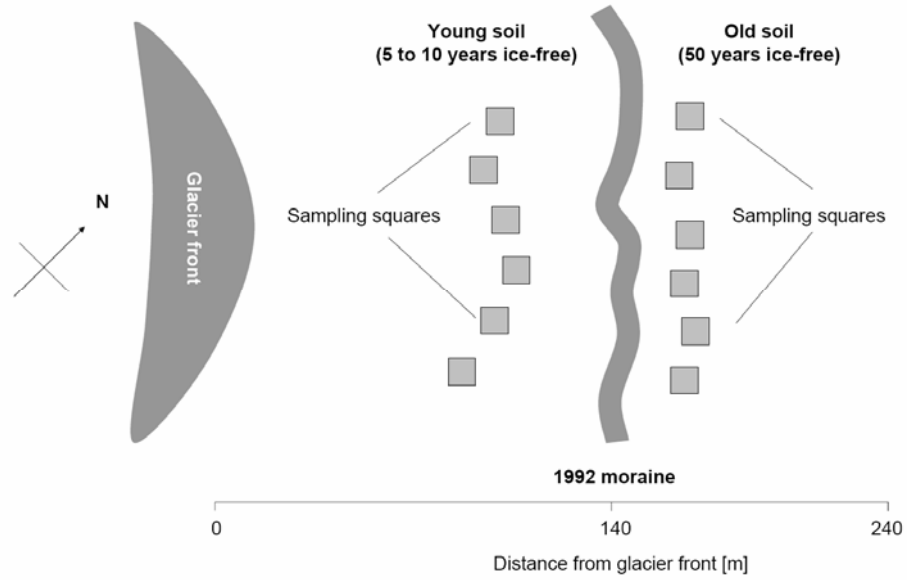

Figure 19: Schematic representation of the sampling area at the Dammaglacier forefield.

We investigated sampling squares at two successional time-points along the chronosequence of the Dammaglacier forefield in the Central Alps (Canton Uri, Switzerland), located about 2100 m above sea level. The two sampling areas corresponded to about 5 to 10 years (referred to as "young" soil) and 50 years (referred to as "old" soil) of ice-free development, respectively (Figure 19). Further details about the Dammaglacier forefield can be found in the recent literature [7, 8, 10, 11].

4.2.3.2 Sampling of glacier forefield soils

Soil material was collected for determination of soil-chemical and biological parameters during one vegetation period between June and September 2004. At both sampling areas and for each sampling date we randomly selected six sampling squares which were at least partially covered with *L. alpina*. Each sampling square had the dimensions of 1 x 1 m, and was photographically recorded prior to sample collection. Bulk and rhizosphere soil from the top 5 cm, as well as plant-material of *L. alpina*, was collected and brought to the lab for further analysis.

4.2.3.3 Estimation of foliage-cover

We used digital image analysis to estimate foliage-cover of the investigated sampling area.

Digital images were taken with a Nikon Coolpix 990 camera. One-mega pixel images were further analyzed using the open source image software ImageJ 1.34s (Wayne Rasband, National Institute of Health USA, http://rsb.info.nih.gov/ij/). Prior to analysis, digital images were manually adjusted for brightness and contrast to compensate for the varying light conditions during the sampling period. The raw image data was digitally split into its red, green and blue components. The green image channel was subtracted from the blue channel. The resulting image displayed green, vegetated areas (from which the percentage cover of the total area was calculated using ImageJ) as black areas which could then be detected and analyzed with the software program.

4.2.3.4 Estimation of nitrogen leaching with ion-exchange resin bags

For the estimation of nitrogen leaching in the glacier forefield soils ion-exchange resin bags made of nylon-stocking material that contained 7 g of ion-exchange resins (BioRad 501-X8(D), BioRad Laboratories, Hercules, CA) were horizontally placed at the edges of each sampling square, avoiding disturbance of the upper soil layers inside the square. Within each sampling square three ion-exchange resin bags were placed at 5 cm and 15 cm soil depth, respectively. For each time-point a total of six sampling squares were selected for sampling of the ion-exchange resin bags. Ion-exchange resin bags which had been in the field for one month were excavated, collected, and brought to the laboratory for further analysis.

4.2.3.5 Precipitation measurements

The precipitation at the sampling site was monitored from July to October 2004. Three rain collectors were placed in the glacier forefield which were distributed between the two sampling locations (Figure 21). The rain collectors consisted of 20 l plastic water barrels that were partially buried and covered by aluminum foil on the top of the barrel to protect them from sunlight. To the opening of each barrel we attached a plastic funnel with a diameter of 15 cm. The precipitation volume was measured every second week.

4.2.3.6 Soil temperature measurements

Between June 2004 and April 2005 temperature profiles were obtained at three sampling squares that were selected at the young and old succession time-points. Temperature was recorded every four hours at soil depths of 0, 5, and 15 cm, using temperature data-loggers (iButton DS1921G-F5, Maxim/Dallas, Sunnyvale, CA).

4.2.3.7 Analysis of soil-chemical properties

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. Ammonium was determined colorimetrically by extracting soil and resin samples with 2 M KCl (ratio 1:5 of soil to extractant). Samples were shaken for 1 h at room temperature. Nitrate was measured using ion chromatography with suspended conductivity detection (DX-100, Dionex, Sunnyvale, CA) from the same extracts after 1:20 dilution with deionized water. Total organic carbon (TOC) and total nitrogen was determined by combustion of ground air-dried soil samples using a LECO 932 CHNS device (Leco Instruments, Krefeld, Germany).

4.2.3.8 Determination of bacterial cell numbers

We adapted a method from Zarda *et al*. for the determination of bacterial cell counts [13]. Briefly, one g of field-moist soil from each sample was fixed with 4 % paraformaldehyde in phosphate-buffered saline (pH 7). Total bacterial cells were counted after DAPI (4'-6-diamidino-2-phenylindole) staining using an epifluorescence microscope (Axioplan, Carl Zeiss, Oberkochen, Germany).

4.2.3.9 DNA fingerprinting of soil bacteria communities

To monitor the pattern of the bacterial community development throughout the vegetation period DNA was extracted from triplicate soil samples of both the young and old successional soils (0.7 g each) using the bead-beating method previously described by Sigler and Zeyer [8]. PCR reactions were performed in 50 μl samples containing 1 x PCR buffer, 2.0 mM $MgCl_2$, 0.2 mg ml^{-1} bovine serum albumin, 200 nmol of each dNTP, 0.5 μmol of each primer, 1 U of *Taq* DNA polymerase (Invitrogen, Basel, Switzerland), and approximately 1 ng of template DNA. 16S rRNA gene

fragments were amplified using the universal bacterial primers U968f and L1401r and the PCR protocol previously described [14]. The amplicons were separated by DGGE (denaturing gradient gel electrophoresis) [15, 16]. Equal amounts of DNA were loaded on a 35 – 60% gradient 8 % polyacryalamide gel, and the DGGE gel was run in 1 x TAE buffer at 60°C for 5 h and 200V. Subsequently, the gel was stained with GelStar® (Cambrex, East Rutherford, NJ) before capturing and processing the image with the GelDoc 2000 system and the Quantity One® software (Bio-Rad Laboratories, Hercules, CA).

4.2.3.10 Statistical analyses

Statistical analyses were carried out using the *SPSS 11.0* software package and the open source language and environment for statistical computing and graphics R (http://www.r-project.org/). The calculation of uncertainty in hierarchical cluster analysis was performed using the R package pvclust by Suzuki and Shimodaira (http://stat.ethz.ch/CRAN/index.html). Redundancy Analysis (RDA) was performed with the *CANOCO 4.5* Software.

Mean values are given with their standard errors (± 1 standard error of the mean). The significance-threshold was set to 0.05 for the p-value. Analysis of variance (ANOVA) was performed to test for the seasonal effect on chemical, physical and biological parameters. We applied one-way ANOVA on each parameter set (n = 22) and used four classes for the levels of the fixed factor (June [n = 6], July [n = 6], August [n = 6], September [n = 4]).

Cluster analysis was performed to investigate microbial community fingerprinting patterns, using the Ward's method [17]. Bootstrap values were calculated to test the probability of the resulting clusters under random expectations.

RDA analysis was carried out to indicate the relationships of environmental parameters with microbial diversity structure.

4.2.4 Results

4.2.4.1 Seasonal changes in soil temperature

Soil temperature measurements showed that the top layers of glacier forefield soils were subject to strong fluctuations. We observed the greatest differences between average maximum and minimum temperatures at the beginning of the snow-free season in June (Figure 20). During that month temperature differences of up to 33.5 °C were measured at the soil surface. These high temperature

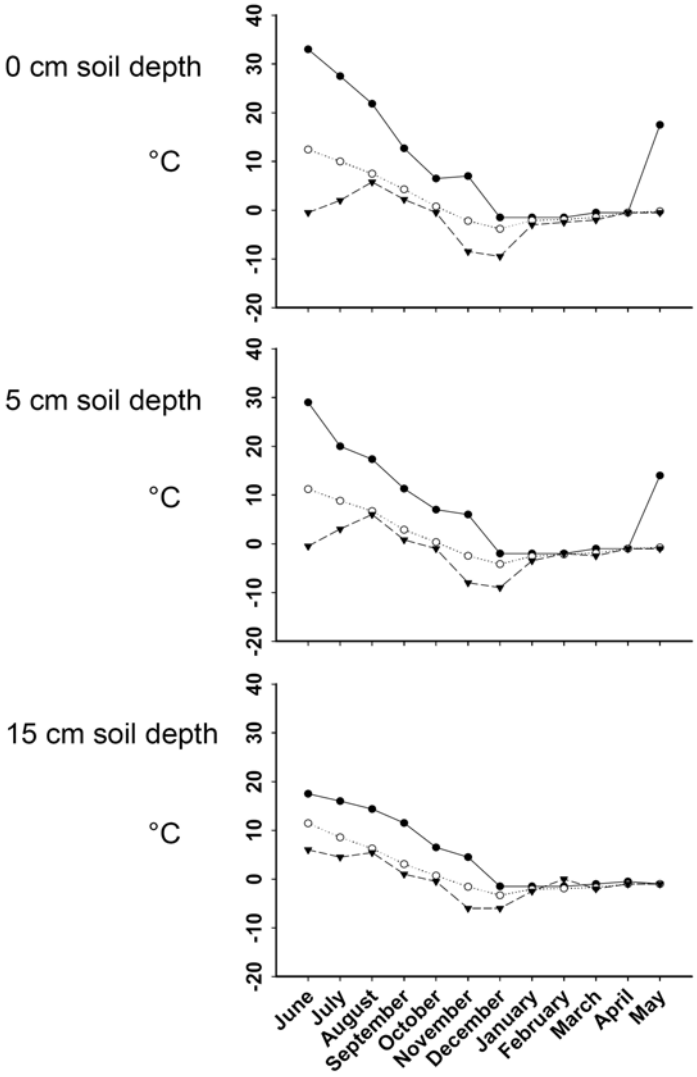

Figure 20: Soil temperature profiles recorded at the Dammaglacier forefield between the years 2004 and 2005 (● maximum temperatures, ○ average temperatures, ▲ minimum temperatures).

fluctuations were less prominent in the deeper soil layers and decreased towards wintertime for all the soil layers (Figure 20). The highest soil surface temperature (33° C) was registered on June, 18, 2004, at daytime. The lowest soil surface temperature (-9.5° C) was recorded on December 19, 2004, at night.

4.2.4.2 Precipitation data

During our 106 days of observation 403 mm of precipitation was measured (Figure 21). The highest precipitation was measured at the beginning of the observation period in July 2004, yielding an average precipitation of more than 6 mm of rain per day (Figure 21). In August the average daily precipitation was less than 3.5 mm per day, but between the end of that month and the beginning of September it reached a second peak yielding mean daily precipitation values of 5.5 mm of rain per day. Overall, the average daily precipitation values were higher than at the lower situated meteorological station at Göscheneralp (Swiss Meteo, 1745 m above sea level, approximately 3.5 km horizontal distance from our sampling site), where a total of 307 mm of rain was recorded during the observation period.

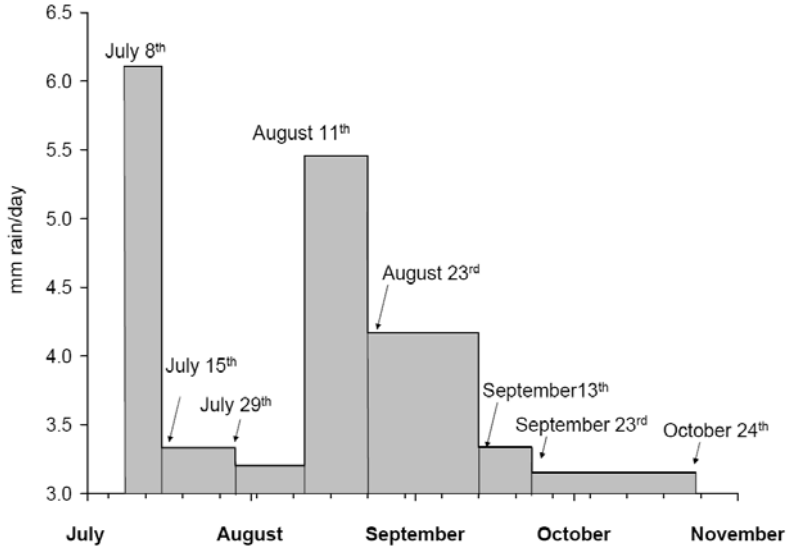

Figure 21: Pre-cipitation monitored at 2100 m above sea level at the Dammaglacier fore-field site. During the sampling period between July and October 2004 a total of 403 mm rain was recorded.

4.2.4.3 Foliage-covered area

The digital evaluation of the sampling squares revealed different seasonal trends for the foliage-covered area of the two successional time-points. While for the young successional soils a constant increase of the foliage-covered area between the beginning and end of our observation period was observed (Figure 22) we noted a rather constant foliage-covered area for the old successional soils. The investigated vegetated sampling squares of the young successional site revealed an average foliage-covered area of about 3 % at the beginning of the snow-free season in June 2004 (Figure 22). With proceeding season, the foliage-covered area strongly increased, reaching an average foliage-cover of about 44 % in September 2004. By contrast, at the older successional site the sampling squares were already covered with more than 50 % of foliage and revealed a less prominent increase of foliage-cover throughout the sampling period, reaching their maximum area in July 2004 (71.5 %), and then declining again to 63.6 % in September 2004 (Figure 22).

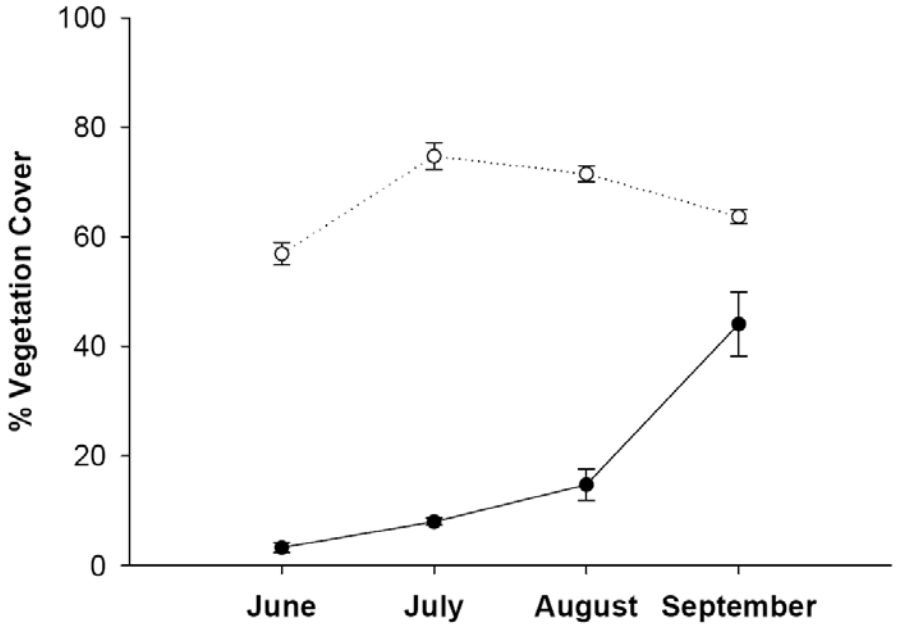

Figure 22: Foliage-cover dynamics. Digital images of the investigated vegetated sampling plots were analyzed for the development of foliage-cover. Foliage-cover development at the 5- to 10-year (●) old glacier forefield site and foliage-cover development at the 50-year old sampling site (○). For each time-point and soil type six sampling plots covering an area of 1 m^2 were measured. For the last sampling time-point (September) only four sampling plots were available for foliage-cover analysis.

4.2.4.4 Soil chemical properties

Our measurements revealed an acidic character of the Dammaglacier forefield soils: pH values ranged between 4.16 and 5.68 (Table 6). Overall, pH at the 50-year old sampling site was lower than at the 5- to 10 year old successional site. Average values at the 5- to 10-year old sampling site were between 4.73 and 5.68, whereas for the 50-year old sampling site average pH values ranged between 4.16 and 4.98. During the course of the summer we observed significant shifts in the pH values of bulk and rhizosphere soils for both successional stages. The average pH values in young bulk soils revealed an overall increasing trend from 4.73 to 5.23 (ANOVA F = 9.581; p = 0.001), and showed a seasonal peak in August (pH 5.34). In the rhizosphere of L. alpina at the young successional soils we measured a similar trend (ANOVA F = 4.425; p = 0.017) of pH 4.93 in June to pH 5.39 in September, and also a high-season peak of pH 5.68 in August. The pH in old successional bulk soils increased from 4.16 in June to 4.49 in September (ANOVA; F = 5.495 and p = 0.009), but revealed even higher values in July and August. In old rhizosphere soils average pH values increased from 4.68 in June to 4.98 until August (ANOVA; F = 2.631 and p = 0.081), and then decreased again to pH 4.49 in September.

	5 to 10 year old successional soil				50 year old successional soil			
pH	**June**	**July**	**August**	**September**	**June**	**July**	**August**	**September**
in bulk soil	4.73 ± 0.07	4.93 ± 0.02	5.34 ± 0.14	5.23 ± 0.08	4.16 ± 0.04	4.80 ± 0.11	4.76 ± 0.12	4.49 ± 0.09
in rhizosphere soil	4.93 ± 0.20	5.57 ± 0.17	5.68 ± 0.12	5.39 ± 0.10	4.68 ± 0.16	4.70 ± 0.09	4.98 ± 0.10	4.49 ± 0.09

Table 6: Soil-pH values during the snow-free season.

4.2.4.5 Soil nitrogen

Soil ammonium measurements in the rhizosphere of *L. alpina* showed a characteristic trend for the summer period (Figure 23). In rhizosphere soils at the young successional time-points mean values of 5.4 μg N (g dry soil)$^{-1}$ were determined at the beginning of the snow-free season. These values decreased until August (2.6 μg N (g dry soil)$^{-1}$) and tended to increase again towards the end of the snow-free season, reaching an amount of 4.3 μg N (g dry soil)$^{-1}$ in September. This trend was even more pronounced in old successional rhizosphere soil where the mean values of 13.0 μg N (g dry soil)$^{-1}$ in June decreased to 3.4 μg N (g dry soil)$^{-1}$ in August 2004. In September, an ammonium concentration of 6.1 μg N (g dry soil)$^{-1}$ was determined. Despite an initial increase in ammonium concentrations, we observed a similar development also for bulk soil at the old successional site (Figure 23). No such trend was visible for the amount of ammonium in young bulk soil. Here, the mean values fluctuated between 1.4 μg N (g dry soil)$^{-1}$ and 3.4 μg N (g dry soil)$^{-1}$ In comparison to the data from ammonium analyses, nitrate measurements revealed low concentrations ranging between 0.06 and 0.40 μg N (g dry soil)$^{-1}$ (Table 7).

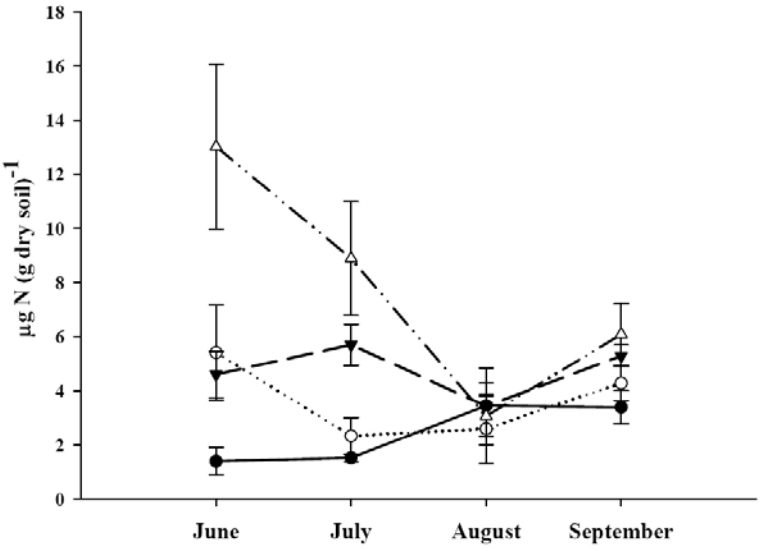

Figure 23: Soil ammonium concentrations. ● Bulk soils from the 5- to 10- year old sampling site; ○ rhizosphere from 5- to 10-year old sampling site; ▼ bulk soils from 50-year old sampling site; △ rhizosphere soils from 50-year old sampling site.

	5 to 10 year old successional soil				50 year old successional soil			
Nitrate (NO_3^-)	**June**	**July**	**August**	**September**	**June**	**July**	**August**	**September**
in bulk soil [µ g N (g dry soil)$^{-1}$]	0.29 ± 0.29	0.06 ± 0.60	nd	nd	0.40 ± 0.13	0.20 ± 0.26	nd	nd
in rhizosphere soil [µ g N (g dry soil)$^{-1}$]	0.07 ± 0.07	nd	nd	nd	0.07 ± 0.07	nd	nd	nd
Ammonium (NH_4^+)								
in bulk soil [µ g N (g dry soil)$^{-1}$]	1.40 ± 0.51	1.52 ± 0.13	3.45 ± 0.86	3.39 ± 0.62	13.02 ± 3.04	8.89 ± 2.11	3.06 ± 0.76	6.08 ± 1.15
in rhizosphere soil [µ g N (g dry soil)$^{-1}$]	5.41±1.77	2.33 ± 0.67	2.59 ± 1.28	4.28 ± 0.65	4.60 ± 0.84	5.70 ± 0.77	3.43 ± 1.42	5.28 ± 0.41

	5 to 10 year old successional soil			50 year old successional soil		
Resin Measurements	**July**	**August**	**September**	**July**	**August**	**September**
Retrieved Nitrate (NO_3^-)						
from Resins buried at 5 cm soil depth [µ g N day^{-1} (g resin)$^{-1}$]	59.56 ± 4.53	25.95 ± 5.86	52.98 ± 16.05	111.75 ± 12.49	29.16 ± 5.48	68.27 ± 46.08
from Resins buried at 15cm soil depth [µ g N day^{-1} (g resin)$^{-1}$]	91.56 ± 29.98	8.23 ± 14.08	48.85 ± 34.22	71.49 ± 6.98	23.12 ± 6.64	77.12 ± 48.91
Retrieved Ammonium (NH_4^+)						
from Resins buried at 5 cm soil depth [µ g N day^{-1} (g resin)$^{-1}$]	1.96 ± 0.38	0.44 ± 0.21	0.13 ± 0.05	5.65 ± 1.21	4.12 ± 0.84	0.22 ± 0.14
from Resins buried at 15 cm soil depth [µ g N day^{-1} (g resin)$^{-1}$]	3.13 ± 1.65	0.17 ± 0.1	0.19 ± 0.12	6.95 ± 2.00	3.27 ± 0.69	3.84 ± 0.94

nd: < 0.05 µ g N (g dry soil)$^{-1}$

Table 7: Soil-nitrogen values obtained from two different successional forefield soils at the Dammaglacier forefield

4.2.4.6 Estimation of nitrogen availability and nitrogen leaching properties in the glacier forefield soils

The examination of resin bag data suggested a significant seasonal trend for the availability of nitrogen. At the beginning of the season, a higher amount of nitrogen was available than towards the end of the season (Figure 24).

This finding is supported by nitrate availability data (ANOVA F = 4.6116 p = 0.033), as well as by ammonium availability data (ANOVA F = 11.761, p = 0.001) obtained from resin bags buried at 5 cm soil depth in the 5 to 10 year old soil. Additionally, a seasonal trend is also supported by data obtained from resin bags buried in 50 year old successional soil buried at 5 cm and 15 cm soil depth (ANOVA F = 8.125, p = 0.006, F =. 5.357, p = 0.022). In July, at the beginning of the sampling season, a significant nitrate leaching effect could be observed in the 50 year old successional soils (t-test, p = 0.42).

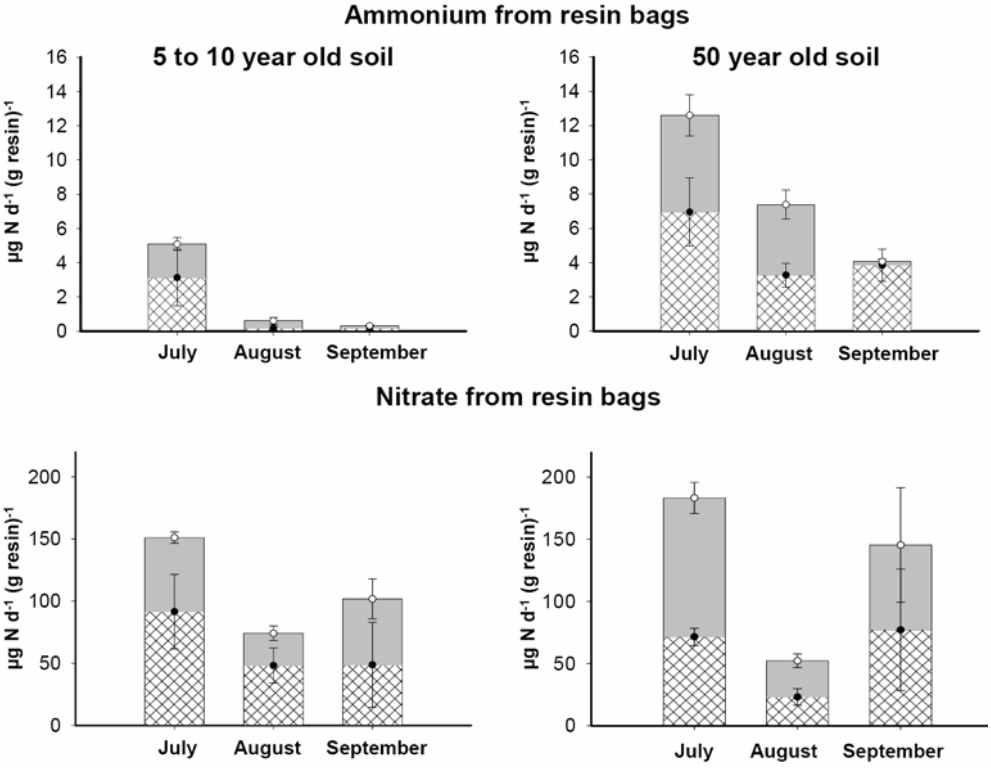

Figure 24: Ammonium and nitrate concentrations retrieved from resin bags that were incubated at two different soil depths for a period of one month ((▒) 5 cm and (▒) 15 cm).

4.2.4.7 Microbial abundance

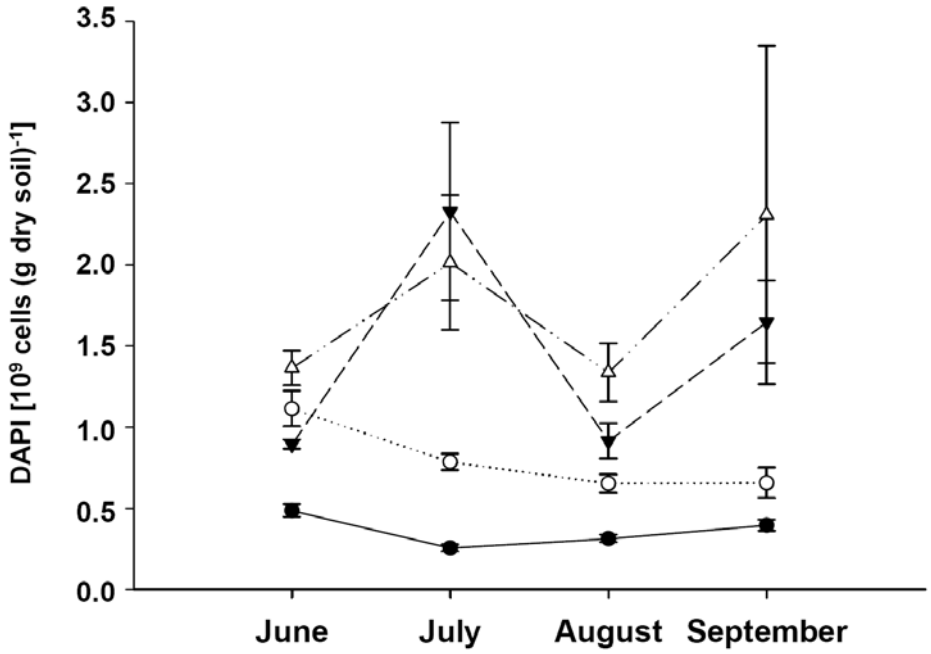

Figure 25: Bacterial cell counts in soils originated from 5- to 10-year old bulk soil (●) and rhizosphere (○), and from 50-year bulk soil (▼) and rhizosphere soil (△).

We investigated the seasonal trends for microbial abundance (Figure 25). Throughout the sampling period, bacterial cell numbers significantly decreased in the rhizosphere (ANOVA $F = 7.513$ $p = 0.002$) and bulk soil (ANOVA $F = 12.121$ $p < 0.001$) of the young successional stage. Throughout the entire sampling period cell numbers in the rhizosphere were higher than in the bulk soils. Whereas in the young bulk soil cell numbers were between 2.6×10^8 cells (g dry soil)$^{-1}$ and 5×10^8 cells (g dry soil)$^{-1}$, in the rhizosphere cell numbers ranged between 6.5×10^8 (g dry soil)$^{-1}$ to 1.1×10^9 (g dry soil)$^{-1}$. Cell numbers from the 50-years old sampling site were generally higher than those from the 5 to 10 year old sampling site. Although considerable differences between the time-points (ranging between 8.9×10^8 cells (g dry soil)$^{-1}$ and 2.3×10^9 cells (g dry soil)$^{-1}$) and a drop of total cell numbers were observed in August, we determined an overall increase in bacterial cell numbers for rhizosphere as well as bulk soil throughout the observation period (ANOVA $F = 4.463$ $p = 0.009$).

4.2.4.8 Molecular characterization of soil microbial communities

Molecular characterization of the soil microbial communities by DGGE fingerprinting and subsequent hierarchical cluster analysis revealed that fingerprinting patterns cluster in four different branches (Figure 26). Bacterial DNA fingerprinting patterns obtained from 5 to 10 year old soil samples branched into the clusters young 1 and young 2. Whereas cluster young 1 grouped samples collected in June and September, cluster young 2 grouped samples collected in July and August together. Within the clusters young 1 and young 2 bulk and rhizosphere soil samples could clearly be differentiated indicating that samples could be separated on the basis of their origin from bulk or rhizosphere soil, although the clustering effect was more dependent on the season.

DGGE patterns of the old successional soils branched in the clusters old 1 and old 2. Cluster old 1 contained patterns from bulk and rhizosphere samples collected in June, July and September. Cluster old 2 grouped bulk and rhizosphere patterns from August as well as June and July together. The reported clustering data are supported by high bootstrapping values which indicate a high probability under random expectations.

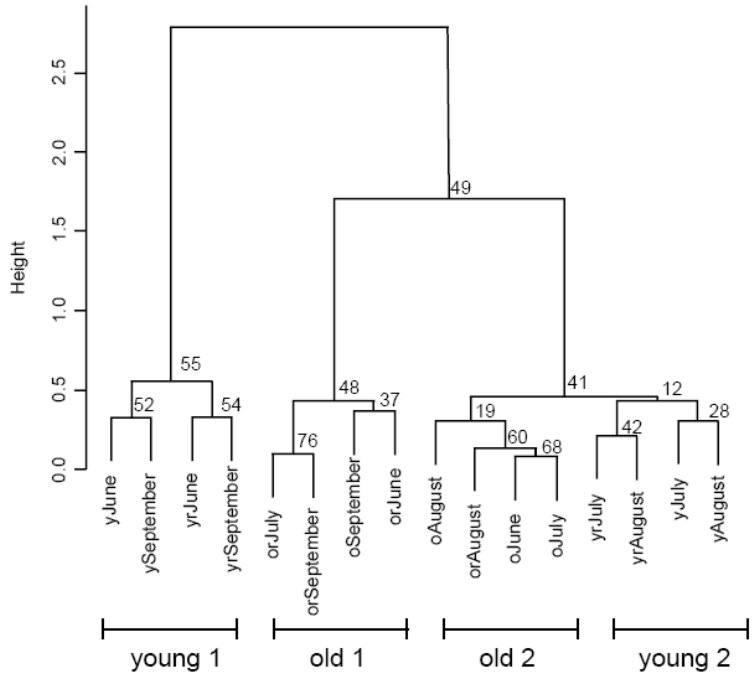

Figure 26: Hierarchical cluster analysis of DGGE fingerprinting data using the Ward-Method. Bootstrap values were calculated to test the probability of the resulting clusters under random expectations; bootstrap values are indicated at each ramification. (The first two letters of the sample-name designate the origin of the investigated samples: y: 5 - 10 year old bulk soil, yr: 5 – 10 year old rhizosphere soil. o: 50 year old bulk soil, or: 50 year old rhizosphere soil)

4.2.5 Discussion

In this study, we investigated the effect of seasonal changes at two successional time points in a glacier forefield by monitoring a set of biotic and abiotic environmental factors. Our objective was to identify key parameters that explain seasonal patterns of microbial abundance and differences in soil microbial community structure at two stages along the Dammaglacier forefield chronosequence.

Temperature is a critical parameter for life, since the speed of biochemical reactions is exponentially related to temperature. Hot as well as cold temperatures generally require specific strategies for survival and growth. While constant soil temperatures near the freezing point are maintained during winter when the soil is covered by snow and ice, our data suggests that especially at the beginning of the vegetation period (June) the surface soils are exposed to strong temperature fluctuations posing a particular challenge for the Alpine plants and microorganisms. These strong temperature differences are likely to be related to the fact that solar radiation, although already strong at the beginning of the snow-free season, is not able to compensate for the cold air masses, occurring at this period of the year. Furthermore, a sparser foliage-cover at the beginning of season may result in a weaker thermal insulation of the soil surface. We did not discriminate between temperatures at the young and old successional site. However, in agreement with the lesser foliage-cover as a potential reason for the different amplitudes at the beginning of the vegetation period, we expect similar variable conditions in young successional soil compared to old soil.

The precipitation recorded at the site indicated the strongest precipitation events at the beginning of the observation period. During the entire observation period, a total of about 400 mm rain was recorded. The total annual precipitation for our research site is reported with 2400 mm [18]. This

illustrates that less than 20 % of the annual precipitation was received during the summer period at our research site. A strong geographic variability of the annual precipitation regime has been reported for the Alps [19]. The regions of the Alps facing north, like our research site, receive their major precipitation during the winter period. The winter precipitation is received as snow and it accumulates until the beginning of the melting period in spring or early summer, when the melting ice and snow masses cause a pronounced discharge of water from the mountain regions [20]. Together with the melting water in spring and early summer at the Dammaglacier, this creates a completely different water regime compared to all other periods of the year.

This study indicates enhanced soil ammonium concentrations at the Dammaglacier forefield early in the season that were presumably released from melting snow and ice masses. The winter snow cover is not only a water storage compartment, it also acts as compartment for the accumulation of nutrients which are delivered by either dry or wet precipitation throughout the winter season. Within the snowpack, freeze-thaw cycles induce the fractionation of nutrients and lead to their concentration in the deeper snow layers [21]. As a result, at the beginning of the melting period, a concentrated discharge of nutrients may occur [21]. With regard to nitrogen, it has been reported that during this period the released nutrients may cover a considerable part of the nitrogen demand of Alpine plants [22]. In this context, the bulk and rhizosphere soils from the 50-year old sampling sites as well as the rhizosphere soils from the young sampling site revealed a decreasing trend in ammonium concentrations until August. The most probable explanations for this trend may be related to microbial ammonium assimilation, ammonium uptake by plants, or nitrification. Ammonia-oxidizing Archaea may dominate nitrogen cycling processes in soil [23, 24]. This is apparently also relevant to glacier forefields; recent studies have found that the microbial communities along a glacier forefield chronosequence (Rotmoosferner, Austria) were dominated by members of a non-thermophilic group of Crenarchaeota [25]. The data in this study indicates

that old successional soils reveal higher ammonium concentrations than young successional soils. This is supported by the resin bag data obtained from resin bags buried at 5 cm soil depth. This may be related to the circumstance that soils from the old sampling site have a generally higher content of soil organic matter, and contain more rhizosphere material [7, 8, 10], which both facilitate a rapid absorption of ammonium [26, 27]. On the other hand, a more porous structure, a lower content of soil organic matter, as well as lower vegetation density, limit the retention ability of ammonium in the young successional forefield soils. In the rhizosphere consistently higher ammonium concentrations could be observed, regardless of soil age. In addition to a more effective retention, a further increase in ammonium concentrations in the rhizosphere can be achieved by decomposition of dead plant material or nitrogen fixation by free-living diazotrophs, most likely supporting the observed increasing trends in September.

The seasonal relative changes in nitrate and ammonium recovered from the resin bags that were buried at 5 and 15 cm soil depth may help to understand the fate of nitrogen throughout the season. We found a significantly decreasing trend for ammonium concentrations in young and old soils with ongoing season. While assimilation by living organisms is one reason, the differences can also be explained by nitrification. The observed differences could possibly be explained by distinct groups of ammonium-oxidizing microorganisms that occur at the various successional stages and have different activity patterns throughout the season and at the two soil depths.

In accordance with our ammonium data, our resin bag data confirmed a seasonal trend for nitrate, revealing decreasing concentrations of nitrate in the top soil throughout the season.

Although resin bags buried in the young soil at 15 cm soil depth generally revealed higher nitrate concentrations than the resin bags buried at 5 cm, a significant leaching effect could not be determined.

Despite the data at the beginning of the season when bacteria may have to boost their metabolism, microbial total cell numbers resemble the seasonal trends observed for soil ammonium concentrations. Although the drop in bacterial cell number might also be related to the availability of other nutrients or other seasonally changing environmental factors, we noted the lowest values of total bacterial cells in old soil in August, similar to the ammonium concentrations. Most likely, because of the higher microbial biomass and vegetation cover, the ammonium demand in old successional soils is much larger than in young soils. Especially at the beginning of the sampling season, ammonium concentrations in older successional soils are markedly higher than in the younger successional soils. Elevated bacterial cell numbers in the younger successional soils at the beginning of the season may be related to nitrogen stress for bacterial organisms. Throughout the season, nitrogen deficiency in younger successional soils may become more acute, due to the fact that vegetation is steadily growing, thereby contributing to an increased nitrogen demand. In contrast, at the old successional site, the initial ammonium burst from melt water at the beginning of the season is presumably increased by the degradation of dead plant material. Bacterial cell numbers in the older successional soils, although characterized by a certain degree of variability, do not reveal a clear seasonal trend. However, they remain on a relatively high abundance level, suggesting that throughout the season they experience a more constant nutritional situation.

Microbial diversity data, obtained from molecular fingerprinting experiments, revealed seasonal patterns in the microbial community structure. For the young successional soils, cluster analysis and the calculation of bootstrap values showed that the microbial communities are deeply branching into two main microbial community clusters, thereby clearly grouping the microbial communities from early and late season in one cluster (June and September) and separating them from the microbial communities of high season (July and August). A rhizosphere effect was less prominent compared to the clustering for the old forefield soils.

Furthermore, the microbial fingerprinting data support the view that the impact of different abiotic and biotic factors on the microbial communities is different at the two successional time-points. Fingerprinting data from young soils suggests that seasonality may have a strong effect on the microbial community profiles in young successional soils. Our data indicates that throughout the investigated time period parameters such as precipitation and temperature are subject to strong seasonal changes. Such parameters may have a more accentuated effect on microbial communities in young successional soils, since the environmental parameters appear to be less buffered at this site. Clustering data for the old successional soils do not reveal a seasonal effect. However, fingerprints suggest that the origin from rhizosphere or bulk soil affect microbial community composition.

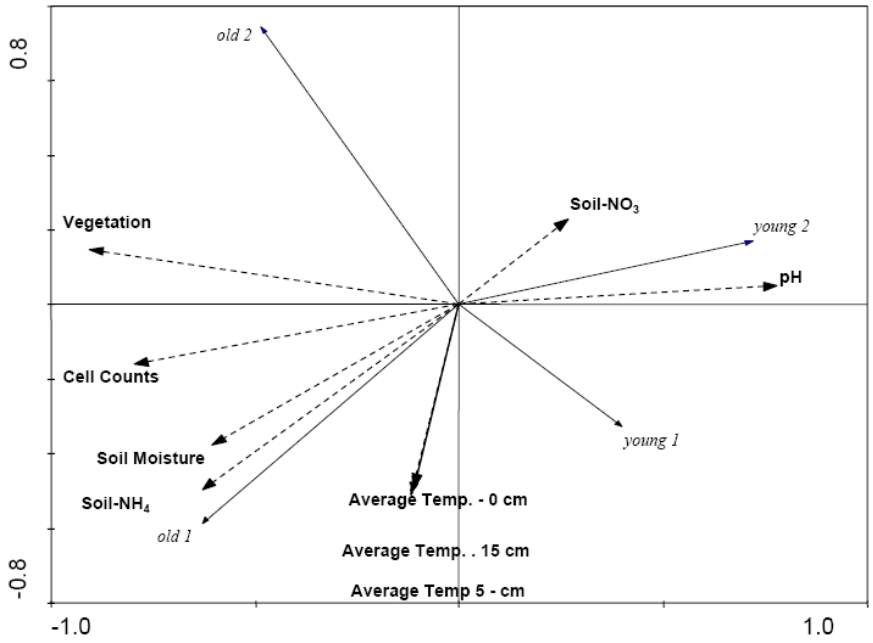

Figure 27: Redundancy analysis indicating the relationships of molecular fingerprinting data and environmental parameters.

These findings suggest that ecosystems in early successional stages are less robust with regard to microbial species composition and species abundance. However, the gradual accumulation of biomass and nutrients in the forefield results in a more stable microbial community structure with ongoing succession. The data presented in this study indicates that young and old successional

soils are differently affected by seasonal change. Microbial diversity may be differently affected by seasonal change at different successional stages of the forefield. It could be observed that, "season" had a lower impact on the community composition in old soil where clustering was mainly determined by the origin of the samples from rhizosphere or bulk soil. As an exception, the fingerprinting profiles from rhizosphere in August were more similar to bulk soil than to the other rhizosphere samples. Since this finding coincided with a tremendous drop in bacterial abundance and the most dramatic decrease of ammonium concentrations, these findings suggest that N source availability is an important triggering factor for the observed changes in microbial diversity patterns and abundance.

Seasonally changing environmental factors such as precipitation and temperature may have a larger impact on the microbial communities in young successional soils, due to the lower buffering capacity of this site. This buffering capacity may be determined by the lack of thermal insulation due to sparse foliage-cover or due to the generally lower amount of available nutrients. This argumentation is supported by redundancy analysis which indicated that the distinct clusters of the microbial communities from young and old glacier forefield soils are correlating with different environmental parameters (Figure 27).

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4.3 Variation in microbial community composition and culturability in the rhizosphere of *Leucanthemopsis alpina* (L.) Heywood and bare soil along an alpine chronosequence

Edwards, I. P., H. Bürgmann, C. Miniaci and J. Zeyer (2006) *Microbial Ecology* **52(4), 679–692**

4.3.1 Abstract

We compared the size, culturability, diversity, and dominant species similarity of the bacterial communities of *Leucanthemopsis alpina* (L.) Heywood rhizosphere and adjacent bare soil (interspace) along a chronosequence of soil development time (5, 50, and 70 years) in the forefield of the Dammaglacier (Switzerland). We found no evidence that the size of the bacterial community was significantly affected by either soil age or the presence of *L. alpina*. In contrast, the proportion of the bacterial community that could be cultured on nonselective agars, and which was taken as an indication of the proportion of r-selected populations, was significantly higher in the 50-and 70-year-old soils than in the 5-year-old soil, and was also significantly higher in the rhizosphere of *L. alpina* at all time points. RDA indicated significant correlations between the increased culturability of the bacterial community over time and increasing concentrations of labile N, and between the increased culturability in the rhizosphere and increased concentrations of labile C and N. *Hae*III-amplified ribosomal DNA (rDNA) restriction analysis of a library of 120 clones of 16S rDNA revealed 85 distinct phylotypes. Hurlbert's probability of interspecific encounter (PIE) values derived from this library ranged from 0.95 to 1.0, indicating a very high genetic diversity. There was no significant difference in the PIE values of rhizosphere and interspace communities. Detrended correspondence analysis (DCA) of 16S ribosomal RNA (rRNA) denaturing gradient gel electrophoresis (DGGE) community profiles clearly distinguished the rhizosphere from the interspace community in the 5-year-old soils and also clearly distinguished between these communities and the rhizosphere and interspace communities of the 50-and 70-year-old soils. However, 16S rRNA DGGE revealed little difference between rhizosphere and interspace communities in the 50- and 70-year-old soils. The relative similarity of the 16S rRNA profiles strongly reflected labile carbon and nitrogen availability. Overall, our

results suggest that improved C and N availability in the rhizosphere of *L. alpina* increases the size of r-selected bacterial species populations, but that the influence of *L. alpina* depends on soil age, being maximal in the youngest soils and minimal in the oldest. The reduced influence of *L. alpina* in the older soils may reflect a feedback between improved nutrient availability and reduced rhizodeposition.

4.3.2 Introduction

Increasing global atmospheric temperatures over the last decades have resulted in the recession of alpine glaciers, exposing fresh glacial till to colonization by plants and microorganisms and creating ecosystems in which plant and microbial community development can be studied in the context of primary succession [1-4]. The colonization of recently deglaciated sediments by pioneering plants initiates a process of soil development, which, in turn, creates environmental conditions suitable for the establishment of a more diverse and productive plant community [1]. Key factors of this process appear to be the long-term accumulation of soil organic matter and associated changes in nutrient availability and soil pH [1, 5]. These same factors are likely to influence the development of the soil microbial community, as microorganisms in soils are typically carbon and nutrient limited for growth [6-8]. Carbon and nitrogen limitations may be exacerbated in young alpine soils which typically have a high fraction of coarse-grained mineral skeleton and low total carbon and nitrogen contents [5, 9]. Nitrogen limitation has been recorded in the microbial communities of both arctic [10] and alpine soils [11], and a positive relationship between microbial biomass and soil organic carbon content has been observed in the forefields of the Lyman Glacier (Washington, USA) [3], and the Rotmoosferner and Ödenwinkelkess glaciers (Austria) [12, 13]. Our previous work in the forefields of the Dammaglacier and Rotfirnglacier in

the Swiss Alps also revealed considerable differences between the dominant bacterial populations along chronosequences of soil age [14], and similar differences have also been recorded in other alpine glacier forefields [12]. Such differences may be an example of long-term succession analogous to that seen in the plant community and may reflect gradients of substrate and nutrient availability [14, 15].

In addition to long-term changes, the exudation of photosynthetically fixed carbon in the rhizosphere of plants [16] creates local patches of enhanced nutrient availability in which microbial growth is stimulated [17]. Quantity and quality of rhizodeposits is often plant specific and may favor the development of characteristic rhizosphere microbial communities [18-21]. For example, distinct bacterial communities have been observed in the rhizospheres of *Bromus tectorum*, *Stipa hymenoides*, and *Hilaria jamesii* growing together in the Colorado plateau [11], and between the bacterial communities of adjacent *Nardo-Galion*-and *Lolio-Plantaginion*-type grasslands in Ireland [22]. Alpine plant species exhibit adaptations to their environment, such as perennial growth, extensive fine root development, and increased root longevity [23], which may enhance the selective effect of rhizodeposition [24, 25]. The colonization of recently deglaciated soil by pioneer plants could therefore create nutrient enriched hotspots in which microbial growth and activity exceed that of unvegetated patches of similar age and characterized by distinct bacterial communities [4]. We hypothesized that in the forefields of receding alpine glaciers, the development of the microbial communities is therefore driven by exposure to two gradients of carbon and nitrogen availability. These two gradients are a long-term gradient resulting from soil development and a local gradient created by current plant growth and rhizodeposition. We predicted that (1) the long-term gradient would lead to qualitative and quantitative differences between the microbial communities of recently deglaciated and older soils, but also that (2) as a result of rhizodeposition, alpine plants would develop characteristic rhizosphere microbial

communities independent of the age of the soil in which the plants were located. To test our hypotheses, we chose to target the bacterial communities developing in both the root zone of *L. alpina* and in adjacent patches of unvegetated soil along a chronosequence of soil age. *L. alpina* was chosen because in the forefield of the Dammaglacier, it colonizes both recently exposed glacial tills and older soils. We used a combination of traditional methods and culture-independent techniques to examine the relative size and compositional similarity of the bacterial communities, and ordination techniques to relate these observations to the C and N gradients.

4.3.3 Methods

Field Site and Collection of Samples. The forefield of the Dammaglacier (8° E 27' 30'' 46° N 38' 00'') in Göscheneralp, Canton Uri, Switzerland, is ~2000 m above mean sea level at the glacier toe. The site includes over 150 ha and slopes downward (25 % slope) over 1500 m toward the northeast. The glacier has probably been receding since ca. 1850, and a net recession of 400 m has been recorded since 1922 [9], despite advancing between ca. 1972 and 1992 (Figure 28).

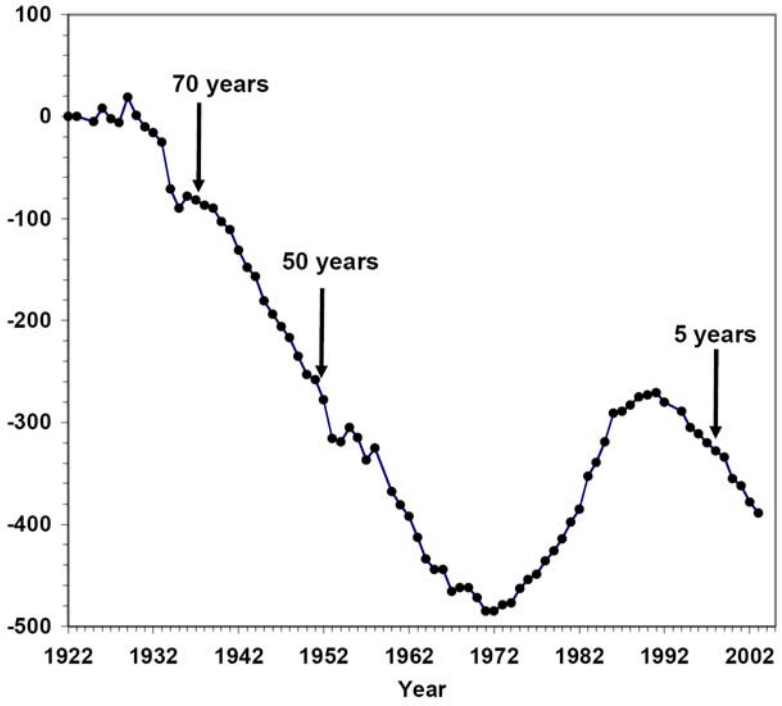

Figure 28: Cumulative recession of the Dammaglacier since 1922, indicating the three stages of soil age sampled in this study.

During the last decade, the glacier retreated with a mean rate of 11 m year^{-1}. The site has a mean annual temperature of between 0 and 5°C, and a mean precipitation of 2400 mm year^{-1} [26]. Samples were collected shortly after snowmelt in May 2003 at three distances from the glacier toe representing different soil ages (Figure 28). At each distance, three collections of *L. alpina* soil (rhizosphere, sample designation: Rh) and three collections of nonvegetated soil (interspace, sample designation: In) were made. *L. alpina* plants (2 – 5 cm diameter) were chosen such that an

adjacent interspace patch was located within 15 cm of the plant. For interspace samples, a small soil core ~2 cm in diameter and 2 – 3 cm deep was taken, bagged, and transported on ice back to the laboratory. For *L. alpina* rhizosphere, the plant was excavated and the soil within the root zone was retained. The youngest soil was sampled 40 – 60 m from the current toe of the glacier and has been ice-free for approximately 5 years (samples Rh5, In5). Soils here have a coarse sandy-gravel texture, and plant community development is restricted to scattered pioneer plants. The second youngest soil was sampled at 80 – 100 m from the glacier terminus, corresponding to a soil age of 50 years (samples Rh50, In50). Here, plant community development is more advanced, although vegetation cover is still patchy. The oldest soil was sampled 320 – 350 m from the glacier terminus, corresponding to a soil age of 70 years (samples Rh70, In70). Vegetation cover here is essentially contiguous. Within the 5-year-old soil, where *L. alpina* was sparsely distributed, we were only able to locate and sample two plants; in total, 16 samples were taken. All soils were sieved to remove the >4 mm coarse fraction and stored at -20°C until analysis.

4.3.3.1 Chemical properties of rhizosphere and interspace soils

Field moist subsamples of each soil (1.0 – 2.0 g) were extracted with cold deionized water for 1 h (1 : 5 soil / extractant ratio). Nitrate and the concentrations of lactic, acetic, butyric, propionic, malic, oxalic, and citric acids (in their anionic forms) were determined by ion chromatography with suppressed conductivity detection (Dionex® Corporation Document 031373). Soil pH was determined in the same solution [27]. Dissolved organic carbon (DOC) was determined by combustion of an aliquot of the cold-water extract (TOC-5000, Shimadzu Europa, Duesberg, Germany). Dissolved organic nitrogen (DON) was estimated as the increase in nitrate after

oxidation of an aliquot of the cold-water extract with alkaline $K_2S_2O_8$ [28]. Total sugars in solution were determined as glucose equivalents after acid hydrolysis [29]. Potentially available ammonium was extracted in 2 N KCl (1 : 5 soil / extractant) for 1 h at room temperature and determined colorimetrically [30]. Total organic carbon (TOC) was determined by combustion of ground air-dried soil samples (LECO 932 CHNS, Leco, Krefeld, Germany).

4.3.3.2 Total cell counts and proportion of culturable bacteria

Field moist subsamples of each soil were treated with 4 % paraformaldehyde in phosphate-buffered saline (pH 7) to fix bacterial cells, and the total number of bacteria in each sample was estimated by direct microscopic counting following 4'6-diamidino-2-phenylindole (DAPI) staining [31]. Both samples and slides were prepared in duplicate, and 10 fields per slide were counted. Culturable bacteria were enumerated on tryptone soy agar (TSA), R_2A, and cold soil extract agar (CSEA) media. TSA was prepared as per instructions of the manufacturer (Fluka Production, Buchs, Switzerland). R_2A medium was prepared from (per liter) 0.50 g yeast extract, 0.50 g proteose peptone, 0.50 g casein hydrolysate, 0.50 g glucose, 0.50 g soluble starch, 0.30 g sodium pyruvate, 0.30 g dipotassium hydrogen phosphate, 0.05 g magnesium sulfate, and 12.0 g agar. CSEA was prepared as described previously [32, 33]. Briefly, 1 kg of a composite forefield soil (equal parts 5-, 50-, and 70-year-old bulk samples collected for a separate study) was extracted with shaking in 1 l of sterile deionized water overnight. After centrifugation (20 minutes, 4500 rpm), the supernatant was filtered (0.2 mm pore size). CSEA contained (per liter) 12.0 g agar, 400 ml cold soil extract, and 1.0 ml of a nutrient stock solution (10.0 g l^{-1} each of sodium citrate, sodium succinate, glucose, fructose, xylose, peptone, and yeast extract). Cycloheximide was added to all media at 50 mg ml^{-1} to inhibit fungal growth. Bacterial inoculum was obtained by serial dilution of a 1.0 g field moist soil in 10 ml sterile water suspension. Plates were incubated in the dark at 20° C for 3 days.

4.3.3.3 Extraction of DNA

Total DNA was extracted from 1.0-g aliquots of field moist soil using a bead-beater technique as previously described [14], except that the final precipitation was performed with a 20 % polyethylene glycol 6000 in 2.5 M NaCl solution at 37°C to remove traces of organic material [34]. The concentration of DNA was determined by UV absorbance at 260 nm [35]. Triplicate DNA extracts from each sampling point were subsequently pooled to facilitate PCR amplification from these low DNA soils (<1 – 25 $\mu g\ g^{-1}$ DNA).

4.3.3.4 Denaturing gradient gel electrophoresis

The similarity of bacterial communities was investigated after amplification of partial bacterial 16S rRNA gene fragments with the universal bacterial primers U968f (5'-GC clamp-AAC GCG AAG AAC CTT AC-3') and L1401r (5'-CGG TGT GTA CAA GAC CC-3') [21, 36-39]. Reactions were performed in a 50 µl solution containing 1 x PCR buffer, 2.0 mM MgCl2, 0.2 mg ml^{-1} bovine serum albumin (BSA), 200 nmol of each dNTP, 0.5 mmol of each primer, approximately 50 ng soil extract DNA, and 1 U *Taq* DNA polymerase Invitrogen, Carlsbad, CA 92008, USA. PCR amplicons were subsequently extracted once with an equal measure of chloroform, concentration was estimated by comparison with a mass standard after agarose gel electrophoresis, and approximately 500 ng was loaded in each lane of a 35 – 60 % denaturing gradient 8 % polyacrylamide gel. Electrophoresis was performed at 200 V and 60°C for 5 h and gels were stained with GelStar (Cambrex Bioscience, Baltimore, MA) before image capture. Quantity One software (Bio-Rad Laboratories, Hercules, CA) was used to determine the intensity and relative position of each band after applying a rolling disk background subtraction algorithm. These data were then used to construct a phylotype relative abundance matrix. We assumed that

each distinct denaturing gradient gel electrophoresis (DGGE) band represented a distinct phylotype and that relative intensity is a reasonable measure of phylotype relative abundance [14, 36-38, 40].

4.3.3.5 16S random clone libraries

Nearly full-length bacterial 16S ribosomal DNA (rDNA) genes were selectively PCR amplified with the primer set 8-27F (5'-AGA GTT TGA TCC TGG CTC AG-3') [41] and L1401r. Amplification was performed in 50-ml reactions containing 1 x PCR buffer, 2.0 mM $MgCl_2$, 0.2 mg ml^{-1} BSA, 200 nmol of each dNTP, 0.5 mmol of each primer, approximately 50 ng soil extract DNA, and 1 U *Taq* DNA polymerase. Purified PCR products were cloned into vector pGEM® -T using a 3 : 1 insertion/vector ratio according to instructions of the manufacturer (Promega, Madison, WI). Ligations were transformed into competent *Escherichia coli* DH5a (Invitrogen, Karlsruhe, Germany) and 30 white colonies per microhabitat were picked and grown overnight in Luria broth. Plasmids were recovered with Wizard® SV R Plus minipreps (Promega), and the insert was reamplified with 8-27F-L1401r as described above except that BSA was omitted from the reaction mixture. To select the dominant bacterial species for sequencing, amplified rDNA restriction analysis (ARDRA) was performed. Plasmid PCR products were digested overnight in appropriate buffer with 1 U of *Hae*III (Invitrogen). Restriction fragments were separated by electrophoresis on 2 % Agarose-1000 (Invitrogen) gels at 80 V for 90 minutes in 1 x TAE buffer, stained with ethidium bromide, and photographed under UV light. The U968f-L1401r partial 16S rDNA gene fragment of plasmids with a common *Hae*III ARDRA profile were subsequently screened by DGGE under the same conditions as used for soil community analysis. Where differences in DGGE migration behavior were observed, the *Hae*III ARDRA-type was considered

to be a composite of several distinct phylotypes. Sequences representing the most common phylotypes have been submitted to GenBank (AY672655– AY672670).

Table 8: Soil chemical and microbiological data for *L. alpina* rhizosphere and interspace soils at three time points along a chronosequence of ice-free soil development time as determined in May 2003.

							p	
	Interspace			Rhizosphere			Soil age[a]	Rhizo-sphere[b]
Age (years)	5	50	70	5	50	70		
pH	6.54 ± 0.06a	6.24 ± 0.06b	6.09 ± 0.10b	5.37 ± 0.15	5.67 ± 0.06	5.78 ± 0.08	0.004	0.03
TOC[c]	0.08 ± 0.04	0.29 ± 0.17	0.25 ± 0.09	0.60 ± 0.12	1.07 ± 1.32	0.53 ± 0.42	0.23	0.013
TDOC[d]	79.2 ± 20.9	158 ± 118	172 ± 49.0	1688 ± 962	321 ± 180	241 ± 117	0.48	0.10
Sugars[d]	137 ± 49.3	71 ± 35.8	209 ± 16.4	1175 ± 460	703 ± 358	612 ± 417	0.15	0.011
TDOA[d]	22.8 ± 2.90	16.1 ± 1.78	18.2 ± 6.05	427 ± 115	75.4 ± 64.6	20.6 ± 7.78	0.30	0.11
Oxalate[d]	0.87 ± 0.65	0.20 ± 0.09	1.15 ± 1.01	182 ± 93.6	20.6 ± 26.9	1.16 ± 0.70	0.08	0.004
Citrate[d]	12.6 ± 3.85	8.60 ± 0.77	8.76 ± 2.95	215 ± 25.5	41.0 ± 33.1	8.56 ± 2.78	0.27	0.24
Malate[d]	0.34 ± 0.31	0.51 ± 0.04	1.19 ± 0.61	12.2 ± 3.06	2.58 ± 1.99	1.07 ± 0.82	0.13	0.17
Acetate[d]	4.02 ± 0.28	3.85 ± 0.52	3.83 ± 0.82	11.5 ± 4.49	6.52 ± 2.32	4.61 ± 2.72	0.94	0.21
Formate[d]	2.64 ± 0.85	2.23 ± 0.40	2.21 ± 0.60	5.29 ± 2.54	3.83 ± 1.25	3.07 ± 0.94	0.70	0.01
Lactate[d]	2.39 ± 0.78	0.72 ± 0.64	1.06 ± 0.90	0.10 ± 0.02	0.85 ± 0.79	2.15 ± 0.82	0.14	0.22
TDN[e]	1.26 ± 0.57a	1.92 ± 0.28b	3.76 ± 0.34c	9.15 ± 1.35	5.03 ± 1.53	5.07 ± 2.17	0.002	0.04
NH_4^+-N[e]	1.05 ± 0.48a	1.63 ± 0.13a	2.63 ± 0.47b	4.54 ± 0.85	4.17 ± 1.33	3.43 ± 2.10	0.015	0.05
NO_3^- - N[e]	0.25 ± 0.15	0.10 ± 0.06	0.23 ± 0.37	4.61 ± 2.67	0.33 ± 0.40	0.83 ± 0.84	0.61	0.10
DON -N[e]	<0.01a	0.19 ± 0.15b	0.91 ± 0.17c	<0.01	0.53 ± 0.52	0.81 ± 0.78	0.002	0.65
DAPI[f]	2.05 ± 0.07	2.12 ± 0.60	3.84 ± 1.32	3.17 ± 1.02	3.90 ± 1.80	4.07 ± 1.89	0.10	0.19
TSA[g]	0.78 ± 0.01a	9.60 ± 3.48b	26.4 ± 1.22c	21.2 ± 12.7	70.0 ± 74.7	73.3 ± 15.6	0.001	0.08
pTSA[h]	0.04 ± 0.01a	0.45 ± 0.03b	0.67 ± 0.18b	0.77 ± 0.65	1.54 ± 1.07	1.98 ± 0.70	0.005	0.003
R2A[g]	1.32 ± 0.77a	10.5 ± 2.17b	24.2 ± 14.9b	33.8 ± 5.80	27.1 ± 7.62	72.9 ± 24.8	0.003	0.14
pR_2A[h]	0.06 ± 0.04a	0.50 ± 0.04b	0.61 ± 0.21b	1.16 ± 0.55	0.78 ± 0.41	1.89 ± 0.53	0.01	0.03
CSEA[g]	1.02 ± 0.20a	14.6 ± 2.53b	39.8 ± 22.9c	35.0 ± 23.6	86.9 ± 86.2	113 ± 44.4	<0.001	0.11
pCSEA[h]	0.05 ± 0.01a	0.71 ± 0.12b	1.01 ± 0.29b	1.29 ± 1.16	1.94 ± 1.11	2.92 ± 0.95	0.01	0.002

Data are mean values ± S.D. Interspace values in the same row and denoted by different letters are significantly different at $\alpha = 0.05$.
[a]One-way ANOVA interspace samples only, $F_{2,7}$.
[b]Two-way PROC MIXED ANOVA $F_{1,12}$.
[c]% Dry soil (w/w).
[d]$\mu g\ g^{-1}$ dry soil. Note that TDOC, sugars, and TDOA were extracted with different methods and measured independently; thus, TDOC is not equal to the sum of sugars and TDOA.
[e]$\mu g\ N\ g^{-1}$ dry soil. TDN is the sum of NH_4^+,NO_3^-, and DON.
[f] $x\ 10^8\ g^{-1}$ dry soil.
[g] $x\ 10^5$ CFU g^{-1} dry soil.
[h] Proportion of total DAPI-stained cells (%).

4.3.3.6 Phylogenetic analysis

Chimeric sequences were identified with the Chimera Check tool [42] and three clones were excluded from further analysis. Remaining clone sequences and best matching sequences obtained from BLAST searches [43] for each clone sequence were subjected to phylogenetic analysis using the ARB program package [44]. Sequences were aligned using the ARB fast aligner to an existing database of 2849 16S rRNA sequences based on the Ribosomal Database Project alignment. The alignment was manually checked and corrected. The phylogenetic tree was constructed based on maximum parsimony insertion of the new sequences. The tree was tested using maximum parsimony analysis with 100 bootstrap samples on the reduced species set selected for presentation.

4.3.3.7 Statistical analysis

The effect of soil age on soil properties was examined through one-way analysis of variance (ANOVA) of the interspace samples, with Tukey's honestly significant difference (HSD) used as the means separation test. The overall effect of *L. alpina* was examined with the model x = *L. alpina* + soil age + *L. alpina* x soil age using the PROC MIXED procedure in SAS 9.1. *Post hoc* t-tests with Bonferroni corrections [45] were used to assess the difference between *L. alpina* rhizosphere and interspace samples at each time point along the chronosequence individually. We took this two-step approach to the analysis because initial analyses using the factorial model in PROC GLM (SAS 9.1) revealed significant and confounding interactions between soil age and *L. alpina* effects for 16 of the 22 variables examined. For both ANOVA procedures, normality and equivalence of variance assumptions were checked for all soil chemical and microbial parameters, and log transformations were performed where necessary.

The relationship between the number and proportion of culturable organisms and the soil

chemical data were assessed by a species-centered and standardized redundancy analysis (RDA), and the significance of the correlation was determined by Monte Carlo permutation tests [46]. DCA was used to examine the similarity of the bacterial community DGGE profiles, and mean values of the quantitative environmental parameters were added to this ordination as supplementary passive variables. RDA and DCA were performed in CANOCO vs 4.5 (Biometris, Wageningen, The Netherlands). A collector's curve for the 16S rDNA clone library and values of Hurlbert's probability of interspecific encounter (PIE) [47] for the entire library and for each of the microhabitats represented within the library were calculated after rarefaction to a standard sample size using the Species Diversity package within ECOSIM [48].

4.3.4 Results

4.3.4.1 Soil chemistry

The mean values for all soil chemical parameters in interspace and rhizosphere soils along the chronosequence are reported in Table 8. In the interspace soils, only pH and the concentrations of DON, NH_4^+, and total dissolved nitrogen (TDN) showed significant variation with soil age (Table 8). Generally, Tukey's HSD tests indicated no significant difference between the mean values of these parameters in the 50- and 70-year old soils, but indicated a clear difference between these and the youngest 5-year-old soil (Table 8).

In comparison with the interspace soils, the rhizosphere of *L. alpina* was characterized by significantly lower pH and significantly higher concentrations of TOC, soluble sugars, ammonium, and TDN (Table 8). Interestingly, for pH, soluble sugars, ammonium, and TDN concentrations, the magnitude of the difference between rhizosphere and interspace soils decreased steadily with increasing soil age (Figure 29), whereas for concentrations of total DOC

(TDOC), total dissolved organic acid (TDOA), and nitrate, the magnitude of the difference between rhizosphere and interspace soils decreased dramatically between 5- and 50-year-old soils (Figure 30). For TDOC, TDOA, and nitrate, there was therefore no significant overall rhizosphere effect (Table 8), although *post hoc* t-tests revealed that the concentrations of all of these parameters were significantly higher in the rhizosphere of *L. alpina* on the 5-year-old soils and that TDOA concentrations were also significantly higher in the rhizosphere of *L. alpina* on the 50-year-old soils (Figure 30). Of the six organic acids consistently recovered from all soil samples, citrate was the most abundant in both interspace and rhizosphere soils (Table 8) and represented ~50% of TDOA. As for TDOA, higher concentrations of citrate tended to be recovered for the rhizosphere of *L. alpina* (Table 8), although the difference between rhizosphere and interspace concentrations was only significant in the 5- and 50-year-old soils (Figure 30). In the *L. alpina* rhizosphere, oxalate was typically the second most abundant organic acid (Table 8), representing, on average, 20 % of TDOA. In contrast to this, oxalate represented on average only 3.6 % of TDOA in interspace soils, and these proportions were significantly different (one-sided t-test, $p = 0.015$). Nevertheless, as for TDOA and citrate, concentrations of oxalate were only significantly higher in the rhizosphere of *L. alpina* than in the interspace in the 5- and 50-year-old soils (Figure 30). Although concentrations of acetate, formate, malate, and lactate were often higher in *L. alpina* rhizosphere than interspace, these differences were not significant (Table 8). Acetate, however, did represent a significantly greater proportion of the interspace TDOA (mean interspace, 21 %; mean rhizosphere, 10 %; onesided t-test, $p = 0.012$).

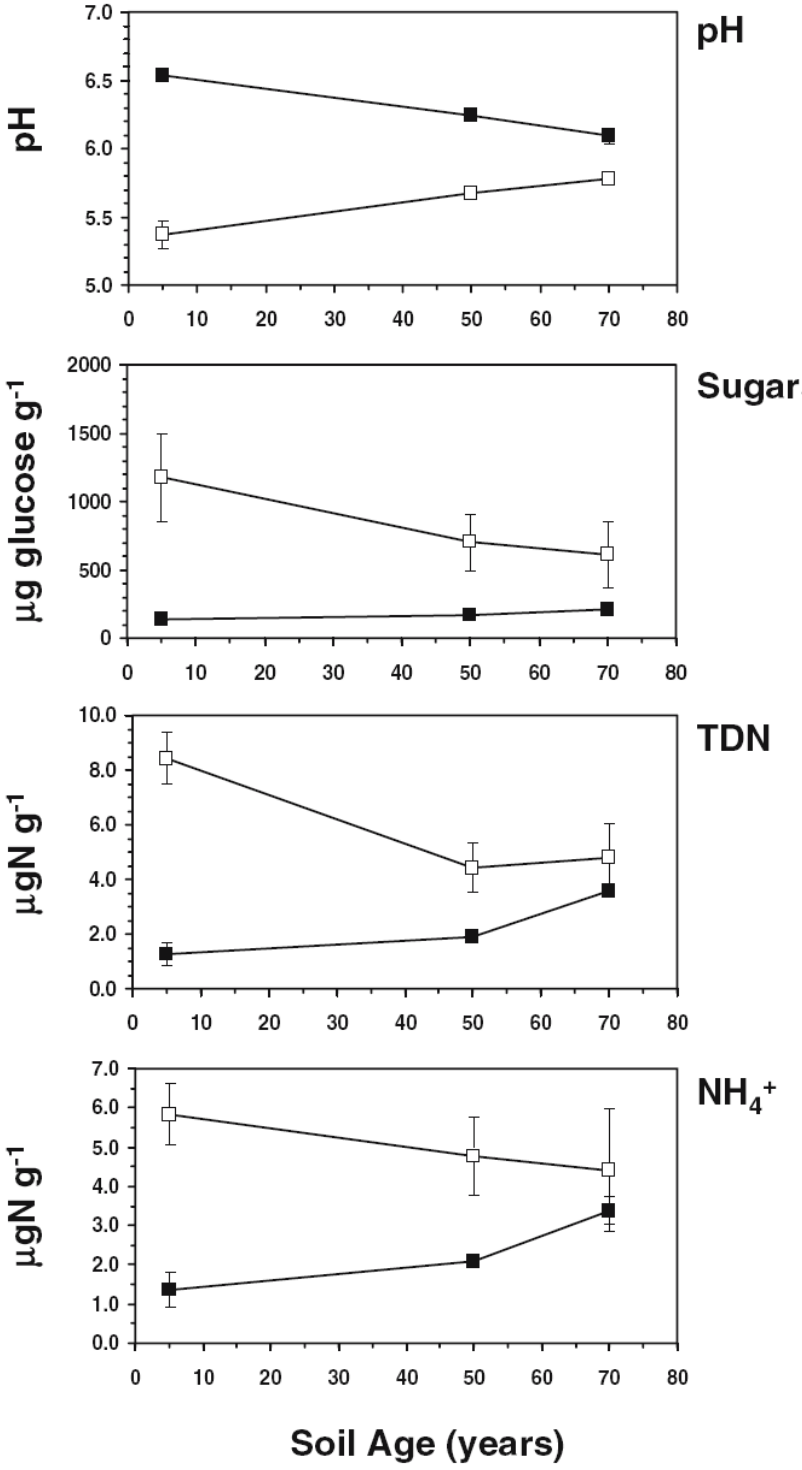

Figure 29: Chronosequence trends in pH, soluble sugars, TDN, and ammonium.□ Rhizosphere, ■ interspace. Error bars are ± 1 SE.

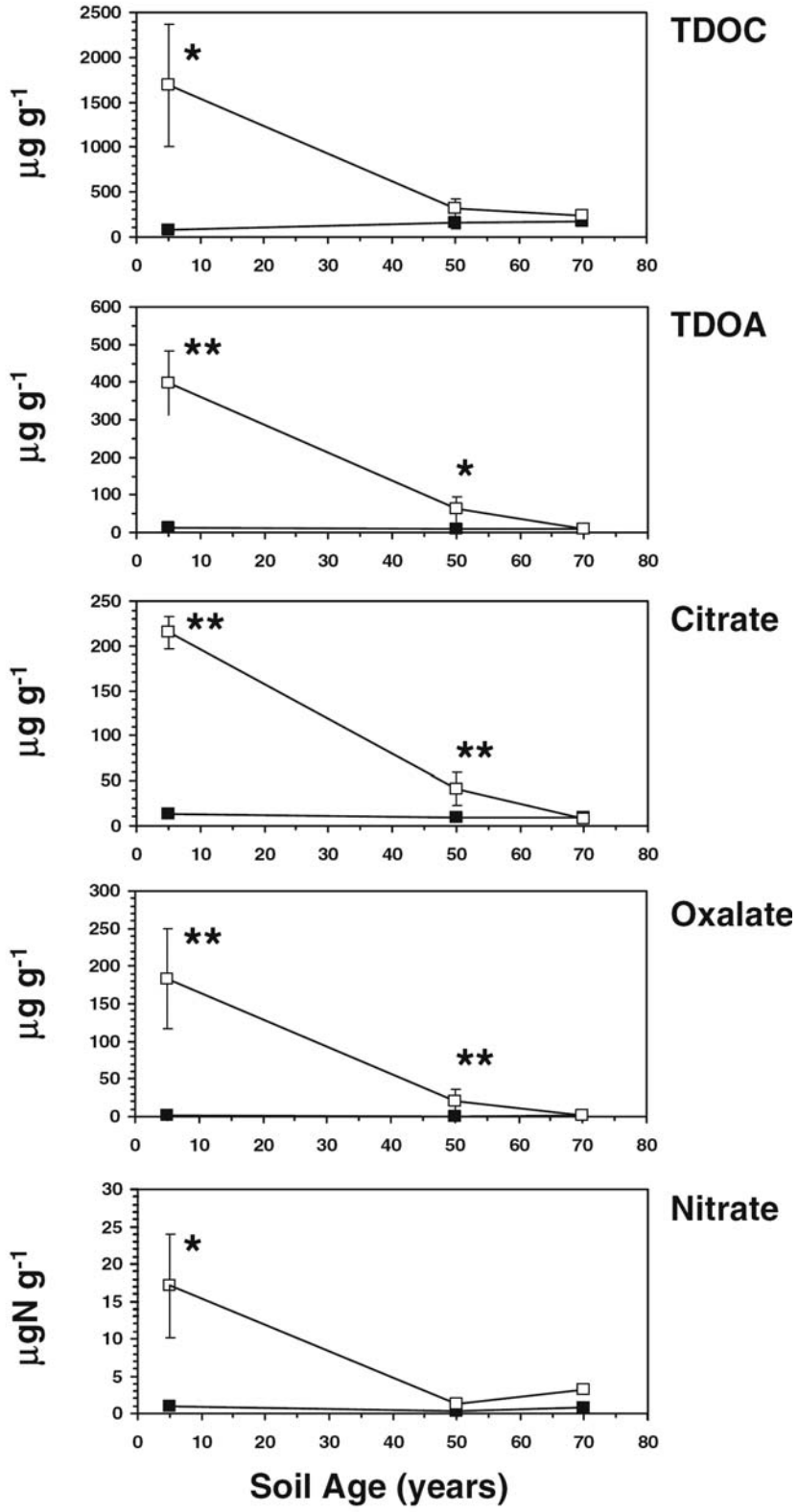

Figure 30: Chronosequence trends in TDOC, TDOA, citrate, oxalate, and nitrate concentrations showing the strong conditionality of the "rhizosphere effect" for these parameters. □ Rhizosphere, ■ interspace. Error bars are T1 SE, and the difference between rhizosphere and interspace is significant at *p < 0.05 or **p < 0.01 at the time points indicated.

4.3.4.2 Total and culturable cell numbers

The mean values for all microbiological parameters in interspace and rhizosphere soils along the chronosequence are reported in Table 8. In the interspace soils, total cell numbers estimated by DAPI generally increased with increasing soil age (Table 8), but this was not statistically significant. In contrast to this, soil age had a significant effect on both the number and the proportion of colony-forming units (CFU) recovered from interspace soils on TSA, R_2A, and CSEA agars (Table 8). Generally, the number and proportion of cells recovered in the 50-and 70-year-old soils were similar, and significantly higher than observed in the youngest, 5-year-old soils (Table 8).

The presence of *L. alpina* had no significant effect on the total cell numbers estimated by DAPI overall (Table 8) or at any of the three individual time points (Figure 31). ANOVA also revealed that the presence of *L. alpina* had no overall effect on the number of CFU recovered on TSA, R_2A, or CSEA (Table 8), although for each of these media, numbers of CFU were significantly higher in the *L. alpina* rhizospheres of 5-and 50-year-old soils (Figure 31). The culturable proportion of the bacterial community (i.e., pTSA, pR_2A, and pCSEA) was significantly higher in *L. alpina* rhizosphere at all time points (Table 8).

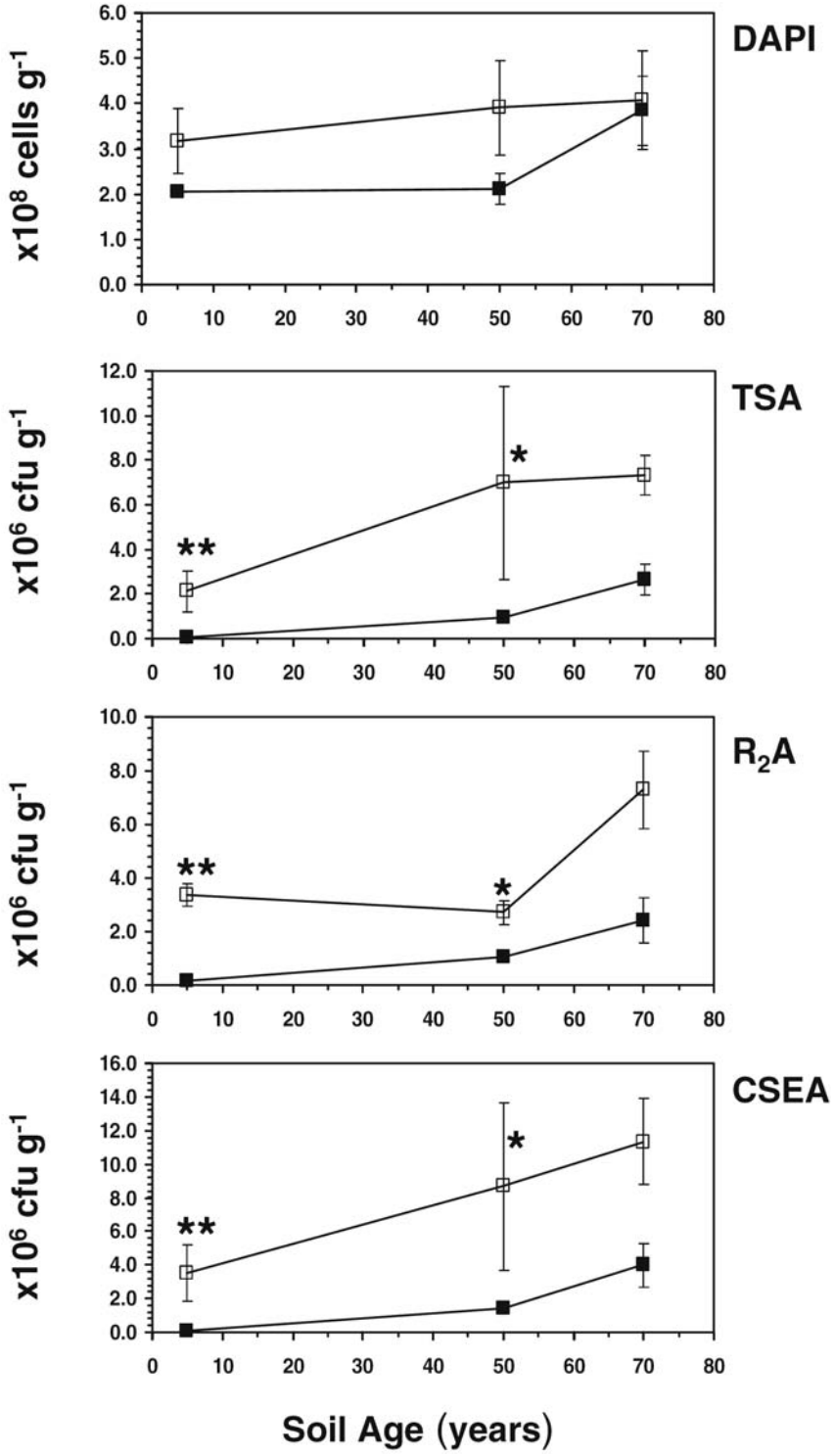

Figure 31: Chronosequence trends in total cell counts (DAPI) and in the numbers of culturable bacteria recovered on TSA, R_2A, and CSEA nonselective plates. □ Rhizosphere, ■ interspace. Error bars are ± 1 SE, and the difference between rhizosphere and interspace is significant at *p < 0.05 or **p < 0.01 at the time points indicated.

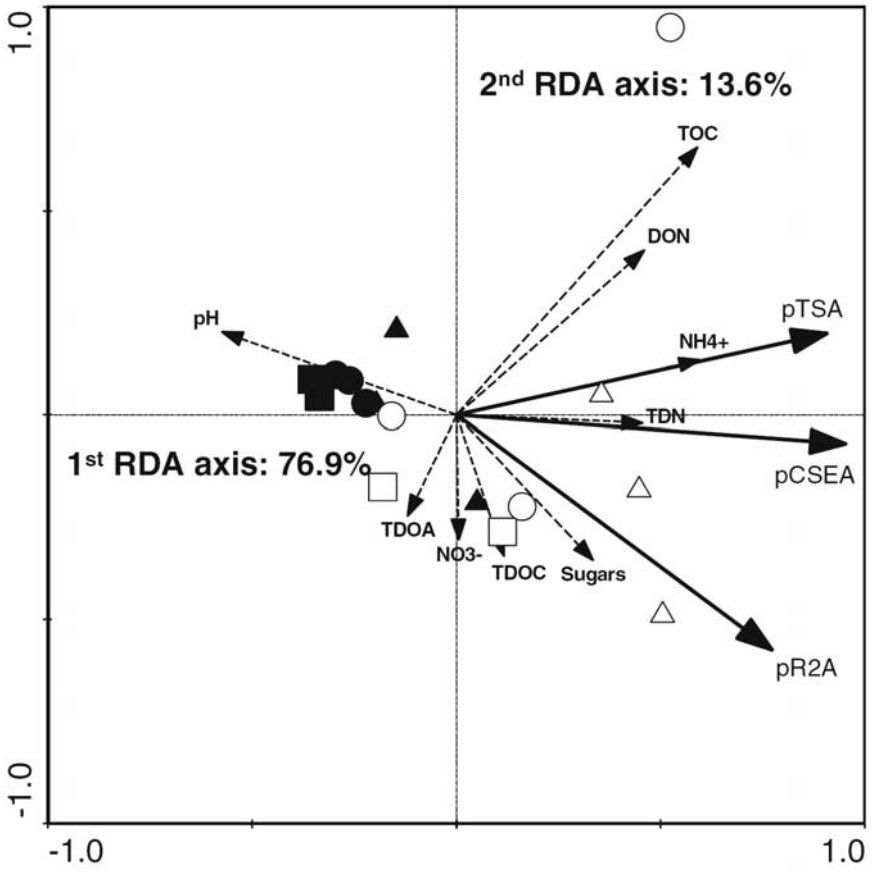

Figure 32: Species-centered and normalized biplot based on RDA phylogeand showing the relationship between the proportion of the bacterial community culturable on three nonselective agars and the soil chemistry of the samples from which the bacteria were extracted. Trends in culturability are shown as solid line vectors, and trends in soil variables by dashed line vectors. ■ In5, ● In50, ▲ In70, □ Rh5, ○ Rh50, △ Rh70.

4.3.4.3 Redundancy analysis

The primary two axes of the RDA (Figure 32) accounted for over 90% of the variation in culturable cells numbers and community culturability, and indicated a statistically significant correlation between bacterial colony counts and the chemistry of the environment from which the cells were recovered (Monte Carlo permutation tests p = 0.02, 199 permutations).

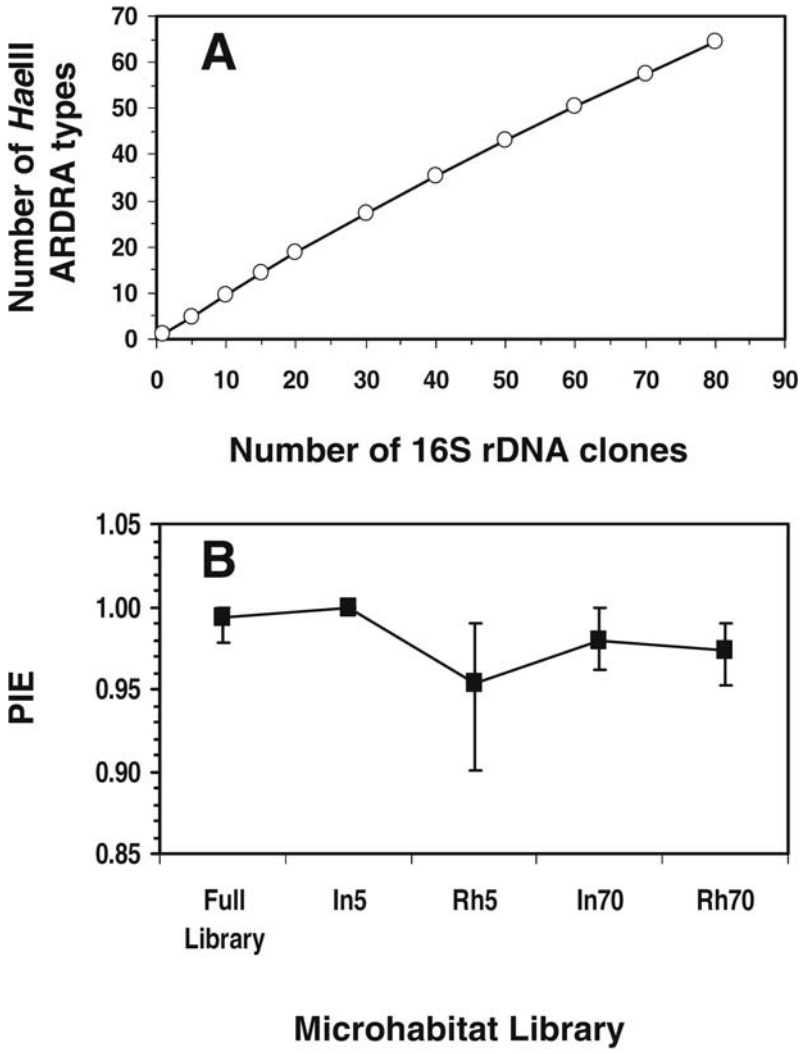

Figure 33: (A) Collector's curve for the library of 120 randomly cloned 16S rDNA sequences recovered from the Damma forefield soils. (B) Variation in PIE values obtained for each of four Damma forefield micro-habitats and for the full library of 120 clones. Error bars indicate 95 % confidence intervals for the estimates of PIE.

4.3.4.4 Molecular analysis

A total of 85 *Hae*III ARDRA types were recovered from the 120 clones that we examined. Sixty-seven of the ARDRA types were recovered only once, and the remaining 18 ARDRA types were recovered two to five times each. A collector's curve for the full library of 120 clones showed no evidence of reaching an asymptote (Figure 33), indicating that bacterial 16S rDNA diversity in the Dammaglacier forefield soils greatly exceeded the number of sequences recovered. To compare the relative diversities of the four microhabitats represented in the clone library (In5, Rh5, In70, and Rh70), we therefore calculated Hurlbert's PIE for each one separately. Mean PIE values for each microhabitat ranged from a low of 0.95 in Rh5 to a high of 1.0 in In5; however, examination of the 95% confidence intervals for these estimates provided no evidence of a significant decrease in bacterial diversity either in the rhizosphere of *L. alpina* or along the chronsequence (Figure 33). DGGE was used to assess the similarity of the six microenvironments in terms of their dominant bacterial populations. The DGGE patterns (Figure 34) revealed clear differences between Rh5 and In5, and between both of these and all of the older soils, but no characteristic community associated with *L. alpina* rhizophere. DCA based on band-normalized relative intensity (Figure 34) clustered the 50- and 70-year-old rhizosphere and interspace samples together, and indicated less of a difference between these communities and the 5-year rhizosphere sample than between 5-year rhizosphere and 5-year interspace.

High-quality nonchimeric sequences of the fragment of the 16S rDNA gene amplified with the DGGE primer set were obtained from 12 of the 18 *Hae*III ARDRA types that occurred more than once in the library, and subsequent Basic Local Alignment Search Tool (BLAST) searches returned strong matches to *Pseudomonas*, *Pedobacter*, *Acidobacterium*, and *Aquaspirillum* / *Janthinobacterium* 16S rDNA sequences for 5 of these 12 (Table 9). The remaining seven 16S rDNA partial sequences could not be identified through BLAST (Table 9). A phylogenetic

analysis (Figure 35) provided strong support for the BLAST matches and, in addition, revealed that six of the seven unidentified sequences clustered together in a clade possibly related to the Cytophaga–Flexibacter–Bacteroides (CFB) group, whereas the seventh clustered with Pedobacter. Although, because of the short length of the analyzed 16S rRNA gene fragments (358 – 392 bp), the phylogenetic placement of the sequences is putative, the good agreement between BLAST results and phylogenetic analysis and high bootstrap values supporting most of the branches containing clone sequences indicate that the current analysis is valid. Table 9 also shows that three of the *Hae*III ARDRA types included sequence variation not sampled by restriction analysis, resulting in 15 common phylotypes. The DGGE migration distances for each of these 15 unique phylotypes under denaturing conditions are shown in Figure 34.

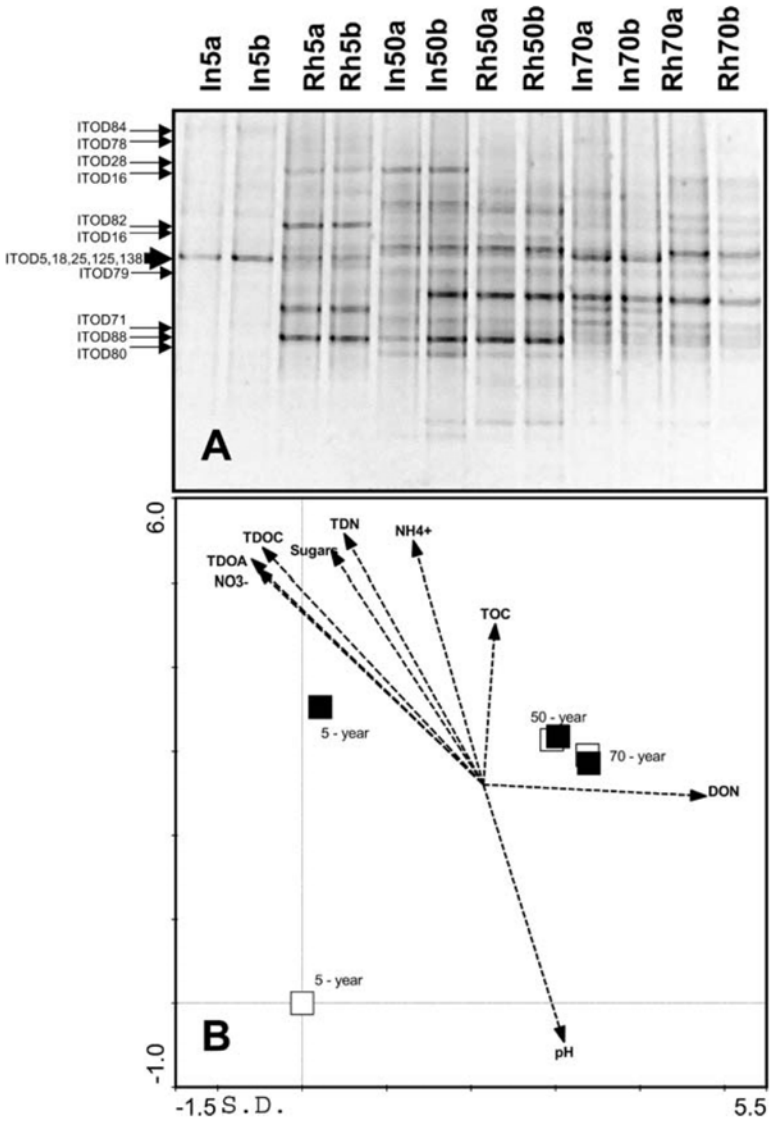

Figure 34: (A) DGGE patterns produced from 16S rDNA templates isolates from interspace (In) and rhizosphere (Rh) samples across the Dammaglacier forefield chronosequence. Suffix a and b indicate separate PCR amplifications. Band migration distance of selected ITOD sequences is indicated. (B) DCA of 16S rDNA DGGE phylotype distributions. The first axis explains 42 % of the variance in phylotype relative abundance, and the second axis a further 22 %. Gradients in the underlying soil chemistry are plotted as passive vectors. □ Rhizosphere, ■ interspace.

Table 9: BLAST search results for Dammaglacier forefield sequences.

Soil	Phylotype[a]	Abundance (%)[b]	Sequenced clones	Best-match database sequence	Percent homology (%)	bp[c]
Rh5	A1	16.7	ITOD 80 (AY672664)	*Antarctic bacterium* (UBA 441004)	99	388
Rh5	A2	–	ITOD 88 (AY672667)	*Pseudomonas veronii* (AY144583)	98	383
Rh5/In5	B1	10	ITOD 84 (AY672666)	*Pedobacter cryonitis* (PCR 438170)	98	381
In5	B2	3.3	ITOD 78 (AY672663)	*Pedobacter cryonitis* (PCR 438170)	99	377
Rh5	C	10	ITOD 71 (AY672661)	*Pseudomonas syringae* (AF511511)	99	394
Rh5	D1	10	ITOD 82 (AY672665)	*Aquaspirillum autotrophicum* (AB074524)	99	370
Rh5	D2	–	ITOD 79 (AY672662)	*Janthinobacterium lividum* (AF174648)	99	370
Rh70/In70	E	6.7/6.7	ITOD 28 (AY672669)	Uncultured *Acidobacterium* (AJ619064)	98	352
Rh70	F	6.7	ITOD 5 (AY672657)	Uncultured bacterium (AJ318124)	91	344
Rh70	G	6.7	ITOD 1 (AY672655)	Uncultured bacterium (AJ318124)	91	344
Rh70	G	6.7	ITOD 16 (AY672658)	Endosymbiont of *Acanthamoeba* (AY549545)	97	379
Rh70	H	6.7	ITOD 8 (AY672656)	Uncultured bacterium (AJ318124)	90	350
Rh70	I	6.7	ITOD 25 (AY672659)	Uncultured bacterium (AJ318124)	90	340
In70	J	6.7	ITOD 138 (AY672670)	Uncultured bacterium (AJ318124)	89	340
In70	K	6.7	ITOD 125 (AY672660)	Uncultured bacterium (AJ576410)	94	364

[a]Each letter indicates a different *Hae*III ARDRA pattern. Letters followed by a number are ARDRA types that were subsequently revealed to contain more than one DGGE phylotype.
[b]Abundance as percentage of 30 random clones.
[c]Sequence length in base pairs.

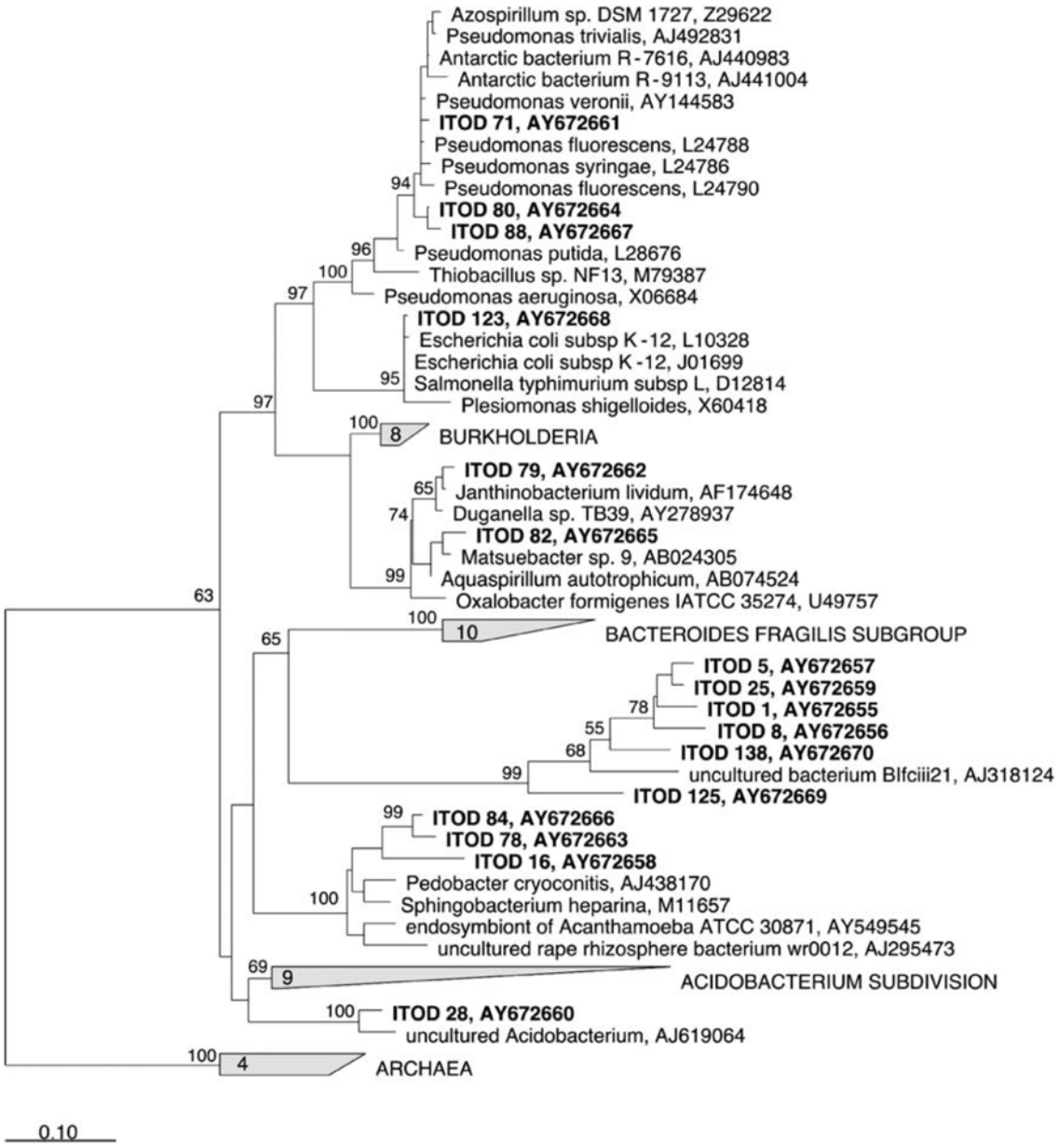

Figure 35: Maximum parsimony analysis of partial 16S rRNA genes from 5- and 70-year-old rhizosphere and interspace microhabitats of the Dammaglacier forefield and published sequences. Percentage of 100 bootstrap samples supporting main branches is indicated if > 50%. The number of species in collapsed groups is indicated in the shaded area.

4.3.5 Discussion

4.3.5.1 Glacier forefield C and N gradients

Previous studies in the forefields of the Damma [14] and other alpine glaciers [3, 12, 49] have suggested that the long-term development of the soil microbial community in such environments is at least partly driven by the accumulation of soil TOC and total nitrogen and by changes in soil pH over time. Although, in this study, we also examined longterm trends in total C and soil pH, we primarily focused on labile, water-soluble forms of C and N, because these are the most likely forms to be readily bioavailable to bacteria [17]. In addition, by concentrating on forms of labile C and N, we were able to provide a more detailed comparison of rhizosphere and interspace soils along the chronosequence.

As with previous work [14], we found that soil TOC content increases rapidly in the Damma soils in the first 50 years following deglaciation and then levels off (Table 8), and we show also that TDOC follows the same pattern of accumulation (Figure 30). Despite this, because of a high degree of spatial heterogeneity, we found no significant difference between the TOC or TDOC contents of the interspace soils of increasing age (Table 8). In contrast, our study revealed that the ammonium, DON, and TDN contents of interspace soils increase steadily along the chronosequence, whereas concentrations of nitrate are unaffected (Table 8). The principal geochemical gradient that we observe in the 70-year chronosequence is therefore one of increasing N availability. A similar pattern of labile N accumulation during the first decades of primary succession has been reported on the floodplain sediments of the Tanana River, Alaska [15], and in both cases a small but steady supply of atmospheric nitrogen deposition may account for much of this increase. Interestingly, it can also be seen from our results (Table 8) that the proportion of DON in the labile N pool also increases with time, from < 1 % in the youngest soils

to ~ 24% in the oldest, whereas that of nitrate decreases. Increased DON suggests biological transformation, and the consequence is a qualitative as well as a quantitative change to the labile N pool over the 65-year period that we examined.

The colonization of deglaciated soils by vascular plants and bryophytes begins early in the Damma forefield, and isolated individuals of *L. alpina* can be found just a few meters from the glacier toe (I. Edwards, personal observation). In comparison with the interspace soils, our results clearly show that the rhizosphere created by *L. alpina* has a significantly higher TOC content (Table 8) and contains significantly higher concentrations of soluble sugars, ammonium, and TDN at all time points along the chronosequence (Figure 29). Unexpectedly, however, inspection of Table 8 and Figure 29 shows that the magnitude of the difference between rhizosphere and interspace was much less in the oldest soils than in the youngest, and that this could not be fully explained by increased concentrations of, e.g., ammonium or TDN in the interspace of the oldest soils. This apparent "conditionality" of the *L. alpina* rhizosphere effect was most clearly seen for TDOC and TDOA (Figure 30) where rhizosphere concentrations were up to 20-fold higher than interspace in the youngest soils, but were no longer significantly different in the oldest soils despite little increase in the TDOC or TDOA concentrations of the interspace. Although the precise source of the organic acids in soil solution cannot be known without ^{14}C tracer experiments, both citric and especially oxalic acids are commonly reported to be major components of plant rhizodeposits [17, 50] and were the major components of TDOA in the *L. alpina* rhizosphere soils. As the decline in rhizosphere TDOA was primarily because of a dramatic reduction in the quantity of citric and oxalic acids recovered from older soils, and as TDOA and TDOC were strongly correlated, we feel that our results may point to differences in *L. alpina* exudation patterns across this chronosequence. The reason for this is not clear, but may be

related to the nutritional status of *L. alpina* at the various time points. For example, increased carboxylic acid exudation has been linked to phosphorous deficiency in several plant species [16, 51]. Further studies should therefore seek to elucidate the nutritional stress experienced by pioneering plant species, such as *L. alpina*, and to examine their rhizodeposition response to such stress.

4.3.5.2 Microbial community size and culturability

Previous work in the Dammaglacier forefield has shown that the size of the bacterial community in 50-year-old soil can be 200 – 400 times larger than in the ice at the glacier toe, but that the size of the community is only slightly affected by soil age beyond this point [14]. Our results broadly agree with this in that we found no significant difference in the size of the bacterial communities recovered from the interspace along the5-to70-year chronosequence (Table 8). Surprisingly, given the tendency for increased C and N availability in the rhizosphere of *L. alpina* discussed above, we also found no evidence to suggest that rhizosphere bacterial communities were significantly larger than their interspace counterparts (Table 8; Figure 31). In contrast, recent work in the forefield of the Rotmoos glacier, Austria [12], has shown larger microbial biomass in the rhizosphere of *Poa alpina* than in interspace soils, although this difference was only apparent in older soils (75 and 9500 years). Direct comparison of our results and those of Tscherko *et al*. [12] is not possible, however, as we did not determine cell volume.

Although the size of the bacterial community in the Damma forefield was not strongly affected either by soil age or by the presence of *L. alpina*, the numbers of culturable cells increased with soil age and were also much higher in the rhizosphere (Table 8). However, the magnitude of the rhizosphere effect on culturability showed the same trend as the TDOA, citrate, and oxalate concentrations (Figure 30 and Figure 31), with maximal difference in the youngest soil and

minimal difference in the oldest soils. Bacterial species culturable on nonselective media are often considered as copiotrophs or r-selected generalists [25], and it has been proposed that the degree of culturability, defined as the ratio of culturable to total cells [52], should decrease over time as the number of r-selected species in the community declines [38]. The culturability of the forefield bacterial communities ranged from 0.04 to 2.92 % (Table 8) and, surprisingly, the highest degree of culturability was observed in the rhizosphere of *L. alpina* growing on 70-year-old soils. Perhaps even more surprisingly, the culturability of the microbial communities recovered from 70-year-old interspace was very similar to that seen in the *L. alpina* rhizosphere of the 5-year-old soils (Figure 31). A previously proposed relationship between "successional stage" and culturability [52] is therefore not supported by our results, and we further examined the relationship between culturability and C and N availability through RDA. In a species-centered and standardized RDA, the length and direction of the vectors representing species and environmental variables provides a visual indication of the magnitude of the correlation between these and the redundancy axes [46]. The RDA (Figure 32) indicated that the size and proportion of the culturable fraction in the Damma forefield microbial communities is most likely determined by the interactive effects of plant colonization and soil age on C and N availability; the primary RDA axis separated interspace from rhizosphere communities, but also separates the communities within each of these classes on the basis of soil age (Figure 32). This primary axis, which therefore incorporates both plant and age factors, is most strongly correlated to the TDN gradient. The secondary RDA axis further distinguishes interspace from rhizosphere communities, and this axis is most strongly correlated with labile carbon availability. Our results therefore suggest that the microbial community of the youngest soils in the Damma forefield is dominated by populations of nonculturable cells, which correspond to stress-tolerant or K-selected species, and that the size of r-selected species populations (culturable on nonselective

media) increases over the first 70 years of succession, as nitrogen availability increases, or in the rhizosphere of a colonizing plant, where C and N availability increases.

4.3.5.3 Glacier forefield geochemical gradients and microbial community composition

Several studies have shown that distinct microbial communities can develop in the rhizospheres of individual plant species growing in the same soil [11, 18, 20, 21, 53]. At the same time, differences have been observed between the rhizosphere microbial communities of several plant species when these species are grown in different soils [53]. Our results (Figure 34) showed several dominant bacterial populations in the DGGE fingerprint obtained from the rhizosphere of *L. alpina* in the 5-year-old soil that are not present in the community fingerprint obtained from the interspace soil. Based on the DGGE migration distance of the 16S rDNA clones recovered from these soils, many of these dominant rhizospheric populations could be putatively assigned to be members of the *Pseudomonaceae*, *Sphingomonaceae*, and *Oxalobacteriaceae* (Figure 34). The DGGE fingerprints (Figure 34) also show that the age of the soil in the Damma forefield affects the dominant bacterial populations in both the interspace and the rhizosphere, and that the difference between the composition of the bacterial communities in *L. alpina* rhizosphere and interspace soil decreases with increasing soil age. Our second hypothesis, that *L. alpina* would be associated with a distinct rhizosphere community, was therefore rejected. As such, our results tend to agree with those studies of agronomic plant species that have indicated a soil effect [53]. Plotting the soil chemical data as vectors onto the DCA enabled us to examine the patterns of microbial community similarity in terms of the environmental gradients, and the degree to which the bacterial communities of the Damma forefield differed in composition strongly mirrored the differences in underlying soil chemistry (Figure 34). Our results therefore suggest that shifts in the composition of the dominant microbial populations in the rhizosphere are primarily the result

of increased soluble carbon and mineral nitrogen availability, whereas the long-term shifts in the community seen with increasing soil age may be more related to an increased availability of labile nitrogen. Interestingly, our results with *L. alpina* differ from those presented by Tscherko *et al*. [12], which tended to show that the influence of the grass *P. alpina* on microbial community composition increased with increasing soil age. Both *L. alpina* and *P. alpina* are recognized as pioneer species in the glacier forefield environment [54]. The reason for their apparently quite different effects on the rhizosphere microbial community is not clear. The difference may in part be because of differing analytical methodology: our DNA-based analysis targeted only the bacterial community, whereas Tscherko *et al*. [12] showed a pronounced reduction in the fungal component of the *P. alpina* rhizosphere with increasing soil age. In addition, we sampled plants very soon after snowmelt in the spring, whereas Tscherko *et al*. [12] sampled close to the end of the alpine growing season in September. As noted above, the extent to which proximity to a glacier affects either the degree of nutritional stress experienced by pioneering plants or the dynamics of plant activity is not yet clear.

4.3.5.4 Conclusion

In conclusion, our results support the hypothesis that a long-term gradient in N availability can develop in recently deglaciated soils, and that this can influence both the culturability and the composition of the bacterial communities. However, we found no clear evidence for a C gradient within the 70-year time frame that we examined. Our results also show that pioneering vascular plants such as *L. alpina* can increase C and N availability in their root zone and create a clear "rhizosphere effect" characterized by both a sharp increase in the degree of culturability of the microbial community and a shift in the dominant bacterial populations. For *L. alpina*, our results further showed that the magnitude of the rhizosphere effect was maximal in the earliest and most nutrient-poor soils, and was much smaller in older soils.

The high concentrations of oxalic acid in the rhizosphere of 5-year-old soils suggests that these soils are a stressful environment for *L. alpina* growth, possibly because of nutrient limitations, and that the plant compensates for this through increased levels of rhizodeposition. The increased culturability of the microbial communities recovered from older soils, together with much smaller evidence of population shifts in the *L. alpina* rhizosphere, suggests that the proportion of r-selected species in the bacterial community increases during the first 70–100 years of primary succession on nutrient-poor alpine soils.

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4.4 Electrophoresis time impacts the denaturing gradient gel electrophoresis-based assessment of bacterial community structure

Sigler, W.V., C. Miniaci and J. Zeyer (2004) *Journal of Microbiological Methods* **57(1), 17–22**

4.4.1 Abstract

We investigated the impact of denaturing gradient gel electrophoresis (DGGE) run time on the assessment of bacterial community structure. Results indicated that increased electrophoresis run time (while maintaining 1000 volt-hours) resulted in dissimilar profiles, likely due to instability of the denaturing gradient. We recommend that DGGE run times be minimized to provide optimal band resolution, as extended electrophoresis times can greatly impact subsequent band-based analyses.

4.4.2 Introduction

Denaturing gradient gel electrophoresis (DGGE) is a popular method for assessing the structure of microbial communities in environmental samples [1]. The technique is based on the electrophoretic separation of PCR-generated double stranded DNA in an acrylamide gel containing a gradient of a denaturant. As the DNA encounters an appropriate denaturant concentration, a sequence-dependent partial separation of the double strands occurs. This conformational change in the DNA tertiary structure causes a reduced migration rate and results in a DNA band pattern representative of the sampled microbial community. Modern image analysis systems have proven to be of value for the analysis of DGGE bands and their associated patterns. For instance, pairwise matching of DGGE bands in separate gel lanes has facilitated the calculation of similarity coefficients to describe relationships between communities [2, 3]. Additionally, the use of common diversity indices that incorporate band number and intensity as surrogates for phylotype number [4] and abundance [5-9], respectively, is also popular. However, this application of band information is often limited to

less complex systems due to PCR amplification biases including preferential-and nonspecific amplification (reviewed by van Wintzingerode; Suzuki and Giovannoni [10, 11]) and heterogeneity in rrn copy number [12]. Regardless of the methods chosen to interpret banding patterns, key to the success of DGGE-based community structure analysis is the separation of PCR products that results in the optimum resolution of as many potential phylogenetic markers as possible.

As with many molecular methods, the steps involved in DGGE analysis are more or less consistent among differing laboratories, but not standardized. In general, the PCR product length analyzed is between 200 and 600 base pairs (bp). The acrylamide percentage of the gel is commonly either 6 % or 8 % and most runs are performed at a temperature of 60° C across denaturant concentrations from as low as 20 % to as high as 70 % or more (a 100 % denaturing solution is defined as 40 % [vol / vol] formamide and 7 M urea). However, much inconsistency exists in the choice of electrophoresis volt-hours (V·h), which is a function of applied voltage and running time. This inconsistency is reflected in the applied V·h described throughout the DGGE literature, which ranges from a minimum of 455 V·h (130 V for 3.5 h; [13]) to 2100V·h (100 V for 21 h; [14]). In preliminary experiments we observed that extended electrophoresis times resulted in sub-optimal band separation and resolution.

4.4.3 Materials and methods

To further explore the idea that extended electrophoresis times can impact community analyses performed by assessing DGGE band patterns we generated a model microbial community from soil (a 100-year-old soil from Transect 2 of a glacier forefield as described in Sigler and Zeyer [9]). Triplicate soil samples (5 g each) were shaken in 50 ml of minimal

media supplemented with glucose (1 g l^{-1}; [15]) at 23° C (100 rpm) until the OD_{600} was approximately 0.5. Preliminary studies indicated that the glucose amendment helped to promote an enriched microbial community that ultimately generated a banding pattern suitable for optimizing DGGE conditions. The soil was allowed to settle and the supernatant was transferred to a sterile Falcon tube and centrifuged at 5000 x g for 10 min to pellet the cells. The supernatant was removed, the cells were resuspended in DNA extraction buffer and DNA was extracted, pooled, and quantified as previously described [9]. PCR was performed in triplicate according to the protocol of Muyzer *et al.* [16] with three different sets of oligonucleotide primers in order to generate three PCR product lengths for DGGE analysis. We used primers 341f-gc and 534r (~ 200 bp; [16]), 101f-gc and 537r (~ 440 bp; [17]) and 101f-gc and 705r (~ 600 bp; [18]) to generate a range of product lengths commonly used in DGGE. The total volume of product necessary to satisfy the requirements of the entire experiment was produced during a single PCR run, then aliquoted (25 µl) and frozen at –20°C until DGGE analysis was performed. All reactions produced a single DNA band of the correct size as estimated by agarose gel electrophoresis and comparison with a DNA size standard (not shown).

For all DGGE experiments we poured (7.5 ml min^{-1}) 15 x 15 cm gels containing 8 % acrylamide : bis-acrylamide (37.5 : 1) and 2 % glycerol, with a denaturing gradient of formamide and urea of 25 – 65 %. Because 1000 V·h represented a popular value among many published DGGE protocols, we chose it as a constant parameter to address the likelihood that varying the combination of voltage and time impacts the assessment of microbial community structure. Triplicate samples of each PCR product were subjected to DGGE under one of four combinations of voltage and time; 25 V for 40 h, 60 V for 16.7 h,

100 V for 10 h, and 200 V for 5 h (Figure 36). For each combination, we monitored the resistance (mΩ) during the run, which did not change appreciably. For each triplicate DGGE lane, band number and position were assessed for pattern similarity using Quantity One image analysis software (Bio Rad Laboratories, Hercules CA) and three single-value indices of similarity were calculated; (1) phylotype richness (*S*, the number of bands); (2) Dice similarity (S_D),

$$S_D = \frac{2N_C}{N_Q + N_T},$$

where N_Q represented the number of bands detected in the query soil, N_T represented the number of bands detected in the test soil, and N_C represented the number of bands common to both soils; and (3) Jaccard similarity (S_J),

$$S_J = \frac{P_C}{P_T},$$

where P_T represented the total number of gel positions occupied by a band (i.e. the number of bands of different migration distance) and P_C represented the number of gel positions in which both lanes featured a band. Each lane's band richness was subjected to a pairwise Student's t-test across the range of tested voltages in order to determine the significance of the differing electrophoresis regimes in assessing microbial community structure. Similarity indices were expressed numerically within a range of 0 (completely dissimilar) to 1.0 (perfect similarity).

4.4.4 Results

Results indicated that although the same V·h was applied during DGGE analysis, varying the electrophoresis time significantly altered band richness and impacted profile similarity (Table **10**). Specifically, pairwise comparisons of band richness indicated significant differences in all evaluations with the exception of one pair (10 vs. 5 h, 400 bp). It is also interesting to note that for all fragment sizes, decreased electrophoresis time resulted in a higher number of bands.

It is unknown if a shorter run time would have resulted in a continued increase in band number, as 200 V represented the working limit of our electrophoresis setup, which limited our shortest run time to 5 h (while maintaining 1000 V·h). Profile similarity as assessed by both Dice and Jaccard similarities indicated that altering the electrophoresis time promoted dissimilarity among all of the profiles. All profiles were found to be completely dissimilar to those resulting from DGGE at 25 V for 40 h (no bands in a common position). The highest similarities were observed upon comparing the 10-and 5-h regimes, but different fragment sizes produced the highest similarities for the Dice (600 bp; 0.80) and Jaccard (400 bp; 0.42) evaluations.

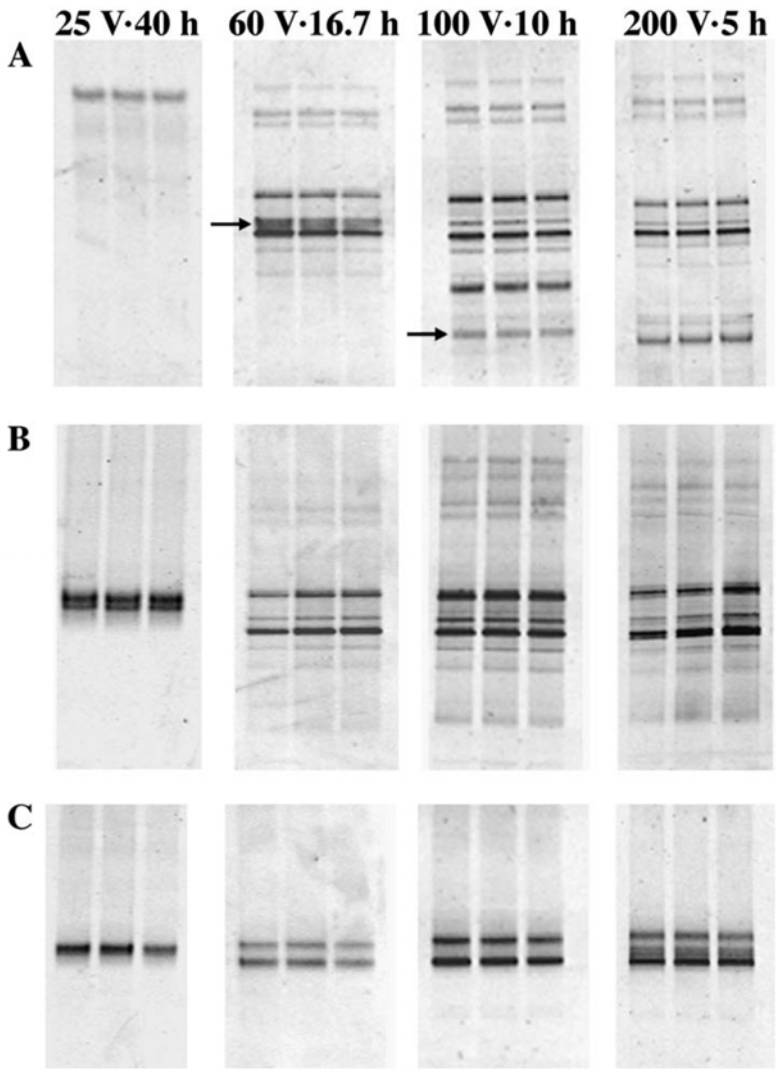

Figure 36: DGGE analysis of (A) 200 bp-, (B) 440 bp-, and (C) 600 bp-PCR products under differing V·h. Previous experiments indicated the position of single-stranded DNA (arrows); these bands were not considered for the analyses in this study.

Table 10: Summary of DGGE band-based comparisons of band richness(S), and Dice-(S_D) and Jaccard (S_J) similarity indices

Index	Time(h)	200 bp				440 bp				600 bp			
		Electrophoresis time to achieve 1000 V·h (h)											
		40	16.7	10	5	40	16.7	10	5	40	16.7	10	5
Band richness (S)		1a[a]	8b	10c	11d	2.3a	7b	11.4c	13c	1a	2b	2c	3.4d
Dice similarity (S_D)	40		0	0	0		0	0	0		0	0	0
	16.7			0.33	0.11			0.36	0.35			0.50	0.40
	10				0.19				0.60				0.80
Jaccard similarity (S_J)	40		0	0	0		0	0	0		0	0	0
	16.7			0.20	0.06			0.21	0.21			0.33	0.25
	10				0.11				0.42				0.25

[a]Richness values with different letters were significantly different as described in text.

4.4.5 Discussion

Based on our results, it is apparent that the selection of voltage–time ratio, not only the total V–H, has a significant impact on the ultimate band pattern generated during DGGE analysis as well as subsequent community structure assessments using all indices. By using the 200 bp product fingerprint as an example, we can conclude that in terms of estimated phylotype richness (*S*), which is a popular and easily quantified descriptor of DGGE results [19], altering the electrophoresis time as described above would theoretically result in either a minimum of 1 phylotype-(25 V for 40 h) or a maximum of 11 phylotypes observed (200 V for 5h) (Figure 36 A and 30 A and B). Such discrepancy will have a significant effect on band richness and thus the overall community structure assessment. Although it could be argued that differences in the applied voltage, which ranged from 25 to 200 V, caused the dissimilarity in band patterns, this is probably not the case. It is more likely that instability of the denaturing gradient was the cause of the pattern differences, especially with longer electrophoresis times. To show this, we extracted DNA from five different bacterial isolates, performed PCR to obtain the 200 bp fragment, combined the PCR products, and then ran DGGE of the mixture for 5 h at 200 V. Two gels were prepared identically to those described above, however DGGE was performed either (i) 2 h after pouring the gel, or (ii) after incubating the gel for 35 h at 60° C, which mimicked the 40 h electrophoresis time used in the first experiment. Although both samples were electrophoresed for 5 h at 200 V (1000 V·h), the preincubation at 60° C for 35 h greatly impacted the separation and resolution of the isolate DNA (Figure 37). This suggested that the prolonged incubation at an elevated temperature, and not the low voltage, played the major role in the differential band separation observed in Figure 36. Although the exact reason for this separation phenomenon is unknown, it is possible that

some internal diffusion of the denaturing gradient occurred following polymerization of the acrylamide. Furthermore, considering that the bottom of DGGE gels are open to the electrophoresis buffer, it is likely that diffusion of urea and formamide into the running buffer constantly occurs throughout the run, which promotes an enhanced breakdown of the gradient over longer runs.

Although DGGE fingerprinting has been recognized as an effective means for high throughput analysis of environmental samples [20], the current study confirms that results of DGGE-based community structure analyses should be interpreted cautiously. Our results indicate that under a constant volt-hour regime, shorter electrophoresis times might minimize instability of the denaturing gradient and result in a more complete band separation than can be achieved following longer runs. Furthermore, it is recommended that gel-to-gel comparisons only be attempted if all gels have been subjected to similar electrophoresis parameters, as our results have illustrated that although the total electrophoresis V·h were consistent for each run, changing the electrophoresis time can greatly impact subsequent band-based analyses.

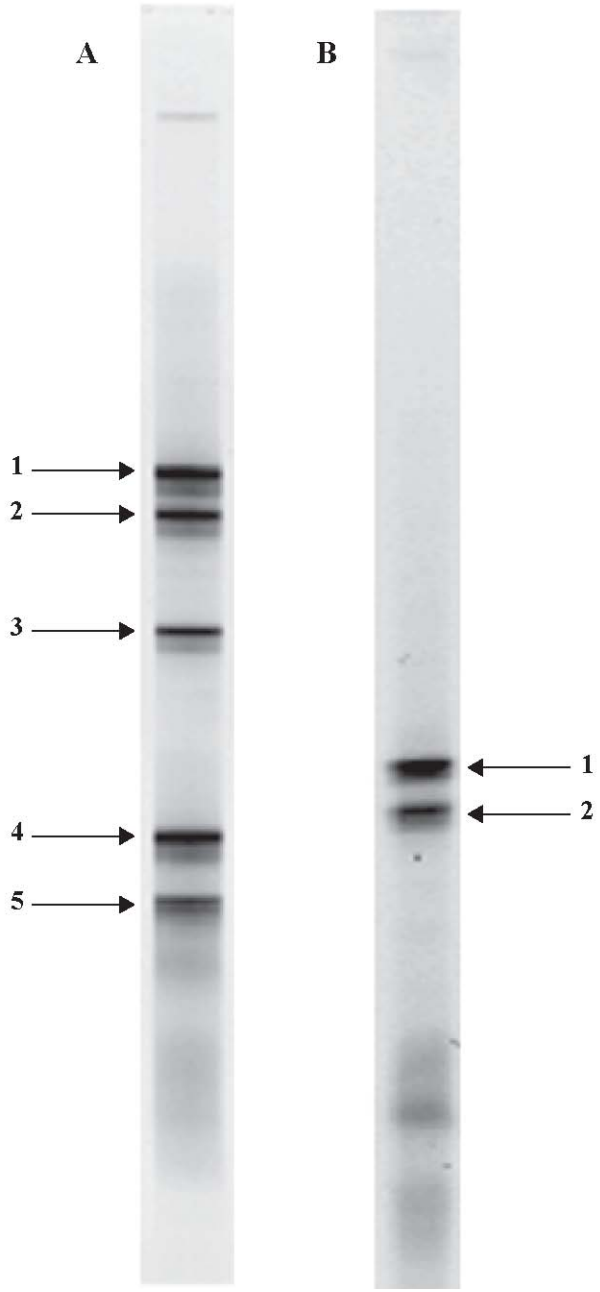

Figure 37: DGGE of a five bacterial isolates (200 bp PCR product). DGGE was performed at 200 V for 5 h (A) 2 h after pouring the gel, or (B) following 35 h of pre-incubation at 60°C. Isolates: (1) *Aeromonas baumannii*, (2) *Proteus mirabilis*, (3) *Ralstonia pickettii*, (4) *Escherichia coli*, (5) *Bacillis subtilis*.

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4.4.6 References

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5. Discussion

5.1 The interaction between plants and microorganisms

5.1.1 The rhizosphere of plants: a hotspot for microbial life

The number of bacterial cells in the rhizosphere is commonly 10 to 200 times higher than in the surrounding bulk soil [74-76], however, these figures vary greatly between plant species, soil type and developmental status of the plant. The data presented in chapter 4.1, 4.2 and 4.3 showed that bacterial biomass was concentrated in the rhizosphere of the pioneering plant *L. alpina*. This finding coincided with the observation of elevated amounts of nutrients and enhanced activities of enzymes related to carbon metabolization at the same location. These results confirm previous studies that have reported enhanced microbial activity and carbon turnover in the rhizosphere of plants [17, 77].

In addition, our results show that glacier forefield plants at different successional stages may reveal different patterns of interactions with soil bacteria. It was observed that *L. alpina* plants exhibited a more pronounced rhizosphere effect at early successional stages than plants from the later stages of succession (chapter 4.3). The stronger rhizosphere effect during early succession was associated with elevated concentrations of organic acids in the rhizospheric soils of *L. alpina*. The existence of elevated organic acid concentrations in the rhizosphere is consistent with the observation that the microbial community in the youngest rhizospheric soils differed from that in the surrounding bulk soil. The difference between rhizosphere and bulk-soil bacterial communities was less apparent at later successional stages. This is consistent with the similarity of the soil chemical-physical properties of bulk and rhizosphere soils. These results support the idea that plants adapt their exudation pattern in response to changes in soil nutrient availability [78-81] i.e. plants at early successional stages exhibit enhanced root-exudation activity due to higher nutrient stress. Studies have shown that the

regulation of root-exudation is a complex regulation process [69, 82]. Root-exudates contain a broad range of organic compounds that can be used as growth substrates by bacteria. This leads to increases in the number of bacteria in the rhizosphere [69, 83]. Therefore, the plant rhizosphere can be regarded as a hotspot for microbial life.

5.1.2 How far do plant microbial interactions extend?

This study has shown that enzymatic activities and sugar concentrations were highest in the rhizosphere of *L. alpina* (chapter 4.1 and chapter 4.3). Previous studies have shown that the activity of roots may influence soil compartments that extend over a scale of millimeters to centimeters from the root surface [84]. It is broadly recognized that the space around plant-roots is characterized by gradients of nutrients, pH, redox potential, root-exudates and microbial activity [69]. These gradients are affected by soil chemical and physical factors, as well as by the nutritional status of the plants and the microbial activity within the rhizosphere. The data presented in chapter 4.1 revealed that increases in the amount of bacterial biomass and nutrients and in enzyme activities were observed within 40 cm of the plants. The ability of the plant to extend its influence beyond the apparent root zone may be explained by fine roots, which could not be detected by the methods used in chapter 4.1. As cited by Hodge [85] roots with a diameter of less than 2 mm are generally referred to as fine roots. It is well documented that fine roots are quite remarkable in their ability to make physiological and morphological adjustments to resource heterogeneity in soil. Fine roots can be rapidly deployed into nutrient-rich patches to exploit available resources. [86-88]. Therefore, fine roots may play a major role in patch exploration and extend relatively far from the main root-cluster. Alternatively, young soils, which are characterized by low biomass and a

predominantly mineral character may allow diffusion or advection of dissolved organic carbon to a much greater extent than has previously been described for mature soils [89, 90].

5.1.3 Do plants select the microbial community in their rhizosphere?

Plants may select for specific microorganisms in their rhizosphere [91-93]. Growth promoting microorganisms have frequently been described as being among those whose growth is favored within the rhizosphere. Promotion of growth is achieved by protecting the host plant against phyto-pathogens, by solubilizing nutrients such as phosphate or by directly stimulating plant-growth [94-96]. In the discussion as to whether plants select for specific bacterial communities it is often argued that this effect is species specific (e.g. [97-100]). However, variations in soil type may interfere strongly with such plant-specific effects [82]. Indeed, this study showed that the development of a plant-associated community distinct from that observed in the bulk soil occurred only at the earlier stages of succession. Furthermore, a single plant species may exhibit differing patterns of exudation throughout its life cycle resulting in changes in the microbial community structure of the rhizosphere [101]. Root-exudates are assumed to play an important role in the selection of specific microbial communities in the rhizosphere of plants. However, root-exudates, such as sugars and organic acids, are also readily metabolized by a broad range of microorganisms [69]. It is therefore difficult to understand how the exudation of such readily degradable compounds leads to the selection of specific microbial communities within the rhizosphere of plants.

Plants are able to attract vital symbiotic organisms such as arbuscular mycorrhizal fungi or N_2-fixing bacteria. But, it is poorly understood to what extent plants can select for microorganisms with beneficial but non-symbiotic functions.

In chapter 4.1 the small-scale spatial impact of *L. alpina* plants in young glacial forefield soils was investigated, by analyzing soil samples directly underneath the plants as well as at distances of 20 cm and 40 cm from the plants. The results revealed no clear distance effect on the microbial community structure between 0 cm and 40 cm from the plant. However, these findings indicated, that increases in bacterial biomass and enzymatic activity were detectable beyond the root zone. These observations suggest that the observed enhancement of root-exudation in young successional soils may lead to non-specific stimulation of the locally present microbial community. Similar findings have been reported by Tscherko et al [102].

5.1.4 How susceptible is the microbial community to seasonal variability?

Chapter 4.2 focused on seasonal variability and examined its consequences for the microbial community and nutrient dynamics in the forefield. The results revealed that in general, early and late successional stages displayed different responses to seasonal changes. The young successional stage displayed a dynamic response whereas the older successional stage appeared to be buffered against seasonal variability. The different response patterns of young and old successional soils were particularly apparent in soil nitrogen availability. Increased nitrogen availability was observed soon after the snow melt at the beginning of the sampling season. Nitrogen availability then decreased throughout the season before increasing again towards the end of the sampling season in September. Similar patterns of nitrogen availability have previously been observed for other alpine ecosystems [103]. This pattern is thought to be related to the seasonal separation of nitrogen use between plants and microorganisms. Accordingly, plants primarily absorb nitrogen during the summer months and microbes immobilize nitrogen during the cold season (autumn, winter and early spring). It is assumed that such a mechanism helps to avoid nitrogen competition between plants and

microorganisms. Previous studies have described a decrease in the microbial biomass in spring, which has been associated with peaks in soil protein concentration and proteolytic activity [104]. This was followed by an increase in amino acid and ammonium concentrations in the soil. In summary, these studies suggest that the main release of nitrogen to alpine plants occurs soon after snow melt, as a consequence of the degradation of proteins released following the death of the winter microbial community.

The microbial abundance measurements from young successional soils, presented in chapter 4.2 are consistent with previous findings [60, 104]. The measurements indicate that microbial cell numbers were highest soon after snow melt and decreased during the summer. However, microbial abundance data from older successional stages did not reveal a clear seasonal effect. This suggests that microbial communities from early and later successional stages may be differently affected by seasonal change. The fingerprinting data reported in chapter 4.2, is consistent with these findings. The different response patterns of the soil microbial communities from early and late successional stage may be related to the different environmental conditions at the two investigated sites. According to the data reported in chapter 4.2, foliage-cover increases along the forefield chronosequence. The increases of foliage-cover occurred in parallel with the accumulation of soil organic matter and litter in older successional soils. The higher content of soil organic matter in the older successional soils may provide a better buffering against seasonal fluctuations in C- and N availability and thereby provide relatively more constant environmental conditions for microbial communities throughout the season. In contrast, the low biomass content of young succeesional soils may be responsible for the greater seasonal fluctuations in nutrient availability resulting in increased temporal variability in soil microbial community structure.

5.2 The applicability of the r-K framework on microorganisms

5.2.1 The r-K concept

The r-K concept was developed in the 1970s and was originally used to describe the life history traits of birds and other macroorganisms [105-108]. In r-K models, r is the growth rate and K the carrying capacity for a given species. Soon after its development the r-K concept was also applied to describe the life-history traits of microorganisms, e.g. [109]. Ever since then, the r-K concept has been widely applied in the field of microbial ecology (e.g. [110-115]. Culturing experiments from chapter 4.3 suggest that microorganisms at the various successional stages of the forefield are characterized by different life-strategies. Previous studies describing the microbial community development along a glacier forefield chronosequence also employed the r-K concept to explain their data [16].

The Verhulst equation is a simple mathematical model to describe density dependent population growth illustrating the meaning of the parameters *r* and *K*:

$$\frac{dN}{dt} = N \cdot r \cdot (1 - \frac{N}{K})$$

The equation expresses that growth of an initially small population (*dN/dt*) is in the first instance proportional to the size of the population (since more individuals have more offspring), but eventually becomes limited by the carrying capacity of the environment (*K*) so that the growth rate is reduced to zero when the population reaches it's limit ($N = K$). Solutions to the Verhulst equation *N(t)* for different values of *r* and *K* are shown in Figure 38. The curves reveal a characteristic sigmoid shape, showing that initially the growth is nearly exponential; this "growth phase" is dominated by the parameter *r*. In the "intermediate phase" the growth rate of a population eventually decreases; and eventually reaches the "plateau or maturity phase", during which the population size is dependent on the parameter *K*.

Organisms which maximize their rate of reproduction (*r*) are generally referred to as r-strategists. They are generally small organisms, with a short life-cycle and a rapid rate of renewal [116]. They are rapidly expanding organisms, which achieve the ability to reproduce in the early stages of their life-cycle. The availability of resources (e.g. food or space) is a critical limiting factor for these organisms. Therefore, r-strategists fare poorly in highly competitive environments. Many members of this group of organisms are colonizing species; they are frequently encountered in disturbed and unpredictable habitats, such as "young" ecosystems, which would in our case refer to the recently deglaciated environment of the glacier forefield. Conversely, organisms which maximize for the parameter (*K*) are referred to as K-strategists. These species have long life-cycles and low reproduction rates. These organisms are considered to represent stationary populations which reproduce in the later stages of their life-cycle. K-strategists occupy specialized ecological niches. This greater niche differentiation with regard to environmental nutrient resources, avoids close competition with other organisms. Their life expectancy is generally high and juvenile mortality is low.

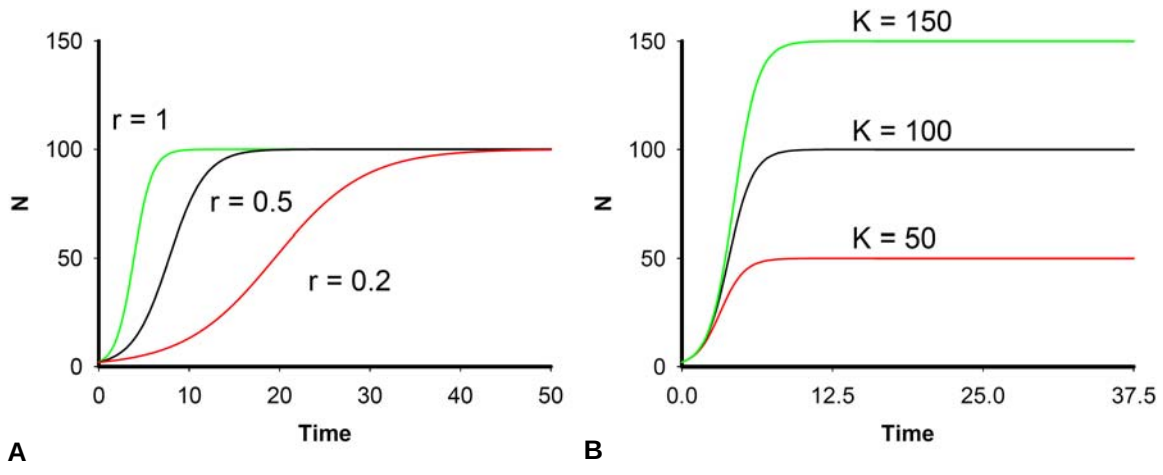

Figure 38: The effect of the parameters r and K on population growth for the solution of Verhulst equation considering. (A) same K's (K = 100) but different r's (B) same r's (r = 1) but different K's.

5.2.2 The R-C-S concept

The early ideas of r-K strategies [107, 108] were later expanded to plant ecology by Grime [68]. The resulting concept was the so-called 'Grime-triangle', in which three primary strategies, namely ruderals (R), competitors (C) and stress-tolerators (S) were postulated. The major difference between the r-K and the R-C-S concept lies in the recognition of a distinct stress-tolerant strategy, which enables the species to prosper despite conditions of limited productivity. Although this concept is predominantly applied in the field of plant ecology it has also found applications in the study of microbial ecology [117]. The R-C-S concept states, that species evolve three different strategies. Each species can be positioned in a triangular diagram (Grime-triangle) (Figure 39). The three life-strategies compete with each other. For example, during succession, species with a mainly ruderal strategy might become replaced by species with increasing stress tolerance [68].

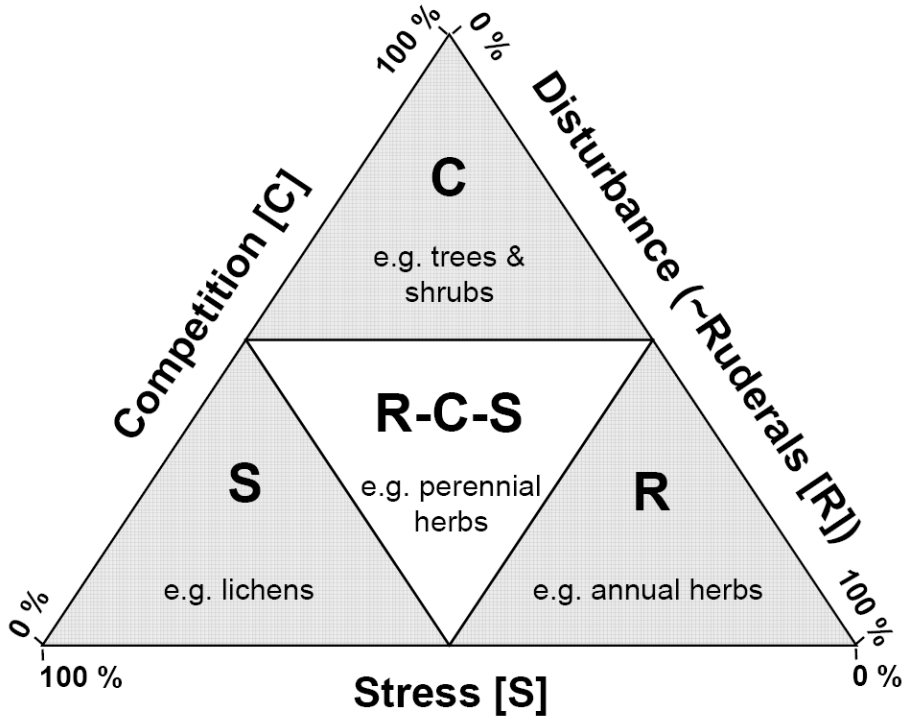

Figure 39: Example of a Grime-triangle. The triangle describes the various equilibria between competition, stress and disturbance in terms of three primary strategies for plants. The grey shaded areas indicate the domination of a particular life-strategy and to which organisms it may apply. In the central shaded area the relative importance of each life-strategy is balanced (Modified after Matthews, 1992).

The R-C-S and the r-K concept are widely used to describe the life-strategies of different groups of organisms [26, 118, 119]. Although these concepts provide a means to describe the general traits of organisms which are successful under a given set of environmental conditions, they do not provide a clear mechanistic explanation [120]. Although the life strategies of organisms are determined largely by their genetics, the number of genes involved is typically so large that it is impracticable at this point to classify organisms as r or K strategists based on the presence of a particular gene or gene network [121]. The r-K (or R-C-S) strategies are essentially community level descriptors, this overlooks the fact that a great deal of variation may exist within any given group of organisms [122]. They describe the role of a species in a community and within a particular environment. The environments usually referred to different successional stages in developing ecosystems. The utility of the r-K framework lies in its ability to describe observed patterns of growth vigor, mode of reproduction and competition between colonizing species. However the weakness of this concept is that it does not provide a clear mechanistic explanation of why a given strategy should dominate under a particular set of conditions. In applying these concepts to specific groups of organisms it is necessary to consider how specific observable traits (e.g. nutrient uptake affinities, enzyme half saturation constants, nitrogen fixation ability etc.) influence the competitiveness of the organisms in a particular environment. In addition the classifications "r-strategist" and "K-strategist" are relative terms and it is therefore only sensible to apply them to comparable organisms (e.g. between different species of bacteria or between plant species) [120]. Nevertheless such models certainly have a degree of power to predict which kinds of organisms will tend to prevail under a given set of environmental parameters.

5.3 Denaturing Gradient Gel Electrophoresis (DGGE) for the estimation of microbial diversity

5.3.1 Principles of DGGE fingerprinting

Denaturing Gradient Gel Electrophoresis (DGGE) is an established method in molecular microbial ecology [123, 124]. The DGGE method which is more explicitly described in chapter 4.4 separates DNA fragments that have the same length, but different sequences. The separation of these fragments occurs by electrophoresis on a polyacrylamide gel containing a linearly increasing denaturant gradient. The principle of this method is that DNA fragments of the same length, but with different sequences have different denaturing properties. Once a DNA fragment encounters the appropriate denaturant concentration in the gel it denatures and becomes essentially immobile. In this way DNA fragments are separated along the polyacrylamide gel according to their melting behavior. The outcome of a DGGE analysis is a characteristic banding pattern that can be used to describe the microbial community composition.

Although DGGE is a well established method in microbial diversity, it is not standardized. Examination of the literature reveals that experimental parameters are often inconsistent. The inconsistency lies largely in the wide variety of electrophoresis parameters which have frequently been reported (e.g. [125, 126]). It has been shown that the selection of a particular combination of electrophoresis parameters can have a dramatic impact on the assessment of diversity indices calculated from DGGE fingerprinting profiles (see chapter 4.4). This may be a result of the sub-optimal resolution of the DGGE banding pattern and lead to ambiguous results when the data are compared with other fingerprinting results. In chapter 4.4 it is

suggested that extended run times of the DGGE gel should be avoided, because this may lead to the instability of the denaturant gradient. These findings indicate that the unambiguous assessment of microbial diversity requires careful calibration of the experimental conditions.

5.3.2 A comparison of DGGE and some alternative methods for soil microbial community assessment

The investigation of complex environmental processes entails an understanding of temporal and spatial variability. This requires the processing of large numbers of samples.

Earlier studies that were based on cloning and cultivation experiments have provided valuable information on dominant microbial community phylotypes. They demonstrated for example that microbial communities in alpine systems displayed higher proteolytic activity soon after snow melt [103]. The advantages of such methods are their comparatively simple and inexpensive technical requirements. However, the interpretation of microbial diversity data from culturing experiments is often hampered by the fact that only a small fraction of the microbial community is actually culturable [127-129]. For this reason traditional culture dependent methods may be unable to reveal changes in specific microbial groups. Methods such as cloning, that employ sequence determination of ribosomal RNA are potentially capable of resolving the entire bacterial species richness in a particular soil sample [130]. Such approaches, however, involve considerable analysis efforts. Therefore such methods may only be carried out for a limited number of samples. The processing of a large number of samples using traditional methods i.e. cloning and culturing, is laborious and time intensive. This hampers investigators in their attempts to encompass the full scale of temporal and spatial variation in microbial community structure.

Genetic fingerprinting provides an alternative approach for the assessment of differences in microbial community structures between different soil samples and over time. These approaches are based on the polymerase chain reaction (PCR) which allows for the amplification of DNA using group-specific primers. Different sequence types are separated by

gel-electrophoresis and subsequently individually detected. The resulting genetic fingerprints represent the microbial community under investigation. The main advantage of genetic fingerprinting is that it provides a rapid assessment of the microbial community structure in a single analysis, therefore allowing the simultaneous processing and direct comparison of numerous samples in parallel. Disadvantages are related to the limited amount of phylogenetic information that can be inferred from genetic fingerprints and the less precise differentiation between microbial types as compared to sequence analyses [131].

RFLP (Restriction Fragment Length Polymorphism) is a popular fingerprinting method for the investigation of microbial diversity patterns. With the RFLP approach an amplified sequence segment is subjected to endonuclease digestion and the resulting fragments are resolved by size on agarose or polyacrylamid gels [132-134]. The underlying principle of this approach is that the presence and location of restriction sites on a particular DNA segment varies between different groups of organisms. RFLP provides an inexpensive and highly reproducible approach for microbial community analysis. However, in highly diverse microbial communities, the RFLP method often yields complex patterns that are difficult to resolve and analyze [135].

T-RFLP (Terminal Restriction Fragment Length Polymorphism) is an alternative fingerprinting method which is frequently used for the estimation of genetic diversity. T-RFLP is derived from the RFLP method [136]. The T-RFLP method employs specific labeled primers that have a fluorescent dye which is incorporated into DNA fragments during PCR amplification. Analysis of labeled terminal restriction fragments is achieved using a capillary sequencer, which potentially enables the separation of fragments whose lengths differ by as little as one base pair. Only the terminal restriction fragments carrying the fluorescent label are automatically detected and quantified. The advantage compared to RFLP

is that signal intensities obtained from labeled fragments are proportional to the number of fragments passing the detector, allowing for a more accurate quantification as compared to traditional gel staining procedures. Additionally, each measured fragment represents one specific phylotype since only one restriction fragment from each amplified sequence is detected, whereas in RFLP analysis, multiple fragments may belong to the same organism. T-RFLP fingerprinting method is recommended as a tool for the resolution of highly diverse microbial communities [135, 137, 138]. On the other hand in environments with many closely related organisms T-RFLP may fail to differentiate between them [131]. A further advantage of the T-TFLP method is the very high throughput of samples which is possible due to the automation of the process. However, automation also entails a considerable investment in specialized hardware.

The DGGE technique has a number of advantages. In comparison to culture dependant methods it provides a more complete picture of the community structure and it is relatively rapid and therefore allows for a high sample throughput. In comparison to RFLP it provides a higher phylogenetic resolution. In addition it has the advantage over both RFLP and T-RFLP that bands may be excised and subsequently sequenced to provide additional information about the organisms present. Furthermore, in comparison to the T-RFLP method the experimental setup is comparatively simple and inexpensive. However, sequence information obtained from band sequencing is limited by the length of the DNA-fragments that can be separated by DGGE, i.e. 200 - 700 bp [139].

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Curriculum Vitae

2001 – 2007	PhD at the Soil Biology Group, Institute of Biogeochemistry and Pollutant Dynamics (IBP), Swiss Federal Institute of Technology (ETH).
2001	Biologist for the Marine Institute Hydra-Kostanz.
2000	Diploma in Biology (Georg-August-Universität Göttingen, Germany).
1992 – 2000	Study of Biology (Georg-August-Universität Göttingen, Germany).
1992	Study of Political Science, Business Management and Journalism (Georg-August-Universität, Germany).
1992	Abitur (German School-Leaving Exam and University Entry Qualification).
1989 – 1992	High School at the Goetheschule (Gymnasium) in Kassel, Germany.
1978 – 1989	Primary-/ Middle- School (Realschule) at the Fasanenhofschule in Kassel, Germany.
1971	Born on August 13th in Rome, Italy.

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