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RESPONSES OF SENSITIVE ALPINE ECOSYSTEMS TO CLIMATE CHANGE

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Abstract

Mountains cover 25% of the world's land surface and are distributed across all latitudes and longitudes. Alpine ecosystems are largely characterized by low temperatures and comprise various habitats including soils, bare rocks, permafrost, glaciers and snow (Donhauser and Frey, 2018). Climate, vegetation and soil properties in alpine ecosystems vary greatly over short spatial distances and altitudinal gradients. For this reason, mountains are rich in biodiversity and the gross and net photosynthesis is generally low because of low temperatures and water-saturated surface conditions, which strongly limit decomposition and ecosystem respiration, resulting in a net influx and long-term net C accumulation in soils. Mountain ecosystems are among the most sensitive ecosystems to climate change and are being affected at a faster rate than other terrestrial habitats. During the last century, the European Alps are experiencing a strong climate warming, with an increase of the mean annual air temperature exceeding the global average of 0.6±0.2°C, with possible consequences on the CO_2 release into the atmosphere. Moreover, an upward migration and areal expansion of shrubs has been widely documented as a consequence of climate change. Since above-ground ecosystems are interlinked with the related below-ground components, the expected shifts in alpine plant communities due to climate change are expected to alter the release and composition of organic compounds into the soils and their decomposition rates, in turn altering the composition and functioning of soil microbial communities. As soil microorganisms are largely responsible for cycling of soil organic carbon and other nutrients, they have a key role in climate feedback, including production or consumption of greenhouse gases such as CO_2, CH_4 and N_2O.

Climate change may have both short-term effects on ecosystems, by modifying above- and below-ground linkages in current species assemblages, and longer-term impacts that include species redistribution such as the upward shifts along elevation gradients. In this light, this study focused on the Stelvio Pass, Italian Alps, where two sites at two different elevations were chosen, a subalpine and an alpine belt. The effects of simulated environmental changes were investigated in field experiments by the use of open-top chambers at the alpine belt. With this study we aimed *i*) to

characterize the composition and functional potential of the microbiome of alpine and subalpine soils and its expression in the time of the highest vegetation productivity; *ii*) to explore the potential short-term and long-term effects of increasing temperature on alpine soils. We found that the composition and functional potential of the microbiome was strongly differentiated under different types of vegetation. Prokaryotic and eukaryotic alpha diversity increased along the altitudinal gradient according to the greater environmental harshness characterizing high altitudes. Subalpine shrubland sites showed the highest share of fungi, that was correlated with higher amounts of carbohydrate-active enzymes, specific for degrading fungal biomass and recalcitrant plant biopolymers, such as pectin, cellulose, glucuroxylans. The subalpine vegetation shift seemed to cause a possible loss of species connected to the alpine soils, the shrubs expansion may accelerate the more recalcitrant C decomposition and decrease total ecosystem C storage, thereby increasing the efflux of CO_2 to the atmosphere and having a positive feedback to warming. On the other hand, the short-term warming effects studied by means of OTCs, did not show to determine significant differences in communities composition and functioning between experimental warming devices and the counterpart controls. Since five-years of experimental warming did not allow us to make bold conclusions, longer-term warming experiments are needed to investigate eventual effects on soil microbiome.

Finally, given the similarities between alpine and polar regions, such as the greening and shrub encroachment reported all over the Arctic, the bacterial communities associated to three different soil habitats in Western Greenland, representative of a vegetation increasing coverage, were investigated with the metabarcoding approach. The type of coverage resulted to be the main predictor of community composition and the shrub composition a fundamental parameter in vegetated plots. The results showed a high number of species unique of the simplest ecosystems, that are expected to be outcompeted with the expansion of shrubs, suggesting a possible loss of local diversity. The advent of high-throughput-sequencing methods allowed to have available large amounts of sequencing data on microbial soils from different terrestrial environments and may give us interesting results when combined. In the light of this, I collaborate to the creation of a global database which represents the

most comprehensive atlas of global fungal distribution occurrences from high-throughput-sequencing metabarcoding studies available to date. This database is of high importance to determine how environmental factors affect the diversity and distribution of fungal communities worldwide.

Chapter 1

Introduction

Alpine ecosystems represent one of the most important terrestrial systems and provide unique ecosystems within all the earth's climatic zones (Diaz et al., 2003; Barry, 2008). Climate, vegetation and soil properties in alpine ecosystems vary greatly over short spatial distances as well as along altitudinal gradients (McCain and Grytnes, 2010). For this reason, mountains are rich in biodiversity - about 40% of Europe's flora is present there (Sundseth, 2009) - and the gross and net photosynthesis is generally low because of low temperatures and water-saturated surface conditions, which strongly limit decomposition and ecosystem respiration, resulting in a net influx and long-term net C accumulation in soils. Mountain ecosystems are particularly sensitive to climate warming since their biota is largely determined by local temperature (Diaz et al., 2003; Vanneste et al., 2017; Donhauser and Frey, 2018; Pintaldi et al., 2020). In particular, the European Alps are experiencing a strong climate warming, with an increase of the mean annual air temperature (MAAT) exceeding the global average of 0.6±0.2°C (Wieser et al., 2019), with possible consequences on the CO_2 release into the atmosphere. An upward migration and areal expansion of shrubs has been also widely documented for them as a consequence of climate change (Cannone et al., 2007; Malfasi and Cannone, 2020). This "shrub encroachment" represents one of the most dramatic shifts in vegetation occurring worldwide, particularly in alpine and high-latitude regions (Cannone et al., 2007; Elmendorf et al., 2012; Grau et al., 2019; Collins et al., 2020; Canini et al., 2019, 2020).

Since aboveground ecosystem components are interlinked with the belowground ones, the expected shifts in alpine plant communities due to climate change are expected to alter the rates of supply of organic compounds into the soils and their composition thus altering the composition and functioning of soil microbial communities (Hobbie et al., 2007) with consequences for organic matter decomposition and other microbe-mediated processes. This is because soil microorganisms, regulating biogeochemical cycles, determine the turnover of soil organic matter pool provided by the

primary production of plants (Chen et al., 2017). The turnover of soil organic matter (SOM) is governed by an array of ecological processes that are responsible for the uptake and release of carbon and nutrients in various forms (Hagedorn et al., 2019). These include many specialist functions such as the production and uptake of methane, different nitrogen-containing compounds, or mineralization of carbon into inorganic forms (primarily CO_2). The understanding of microbial communities contribution to these soil ecological functions is crucial for predicting future changes in plant productivity, greenhouse gas production and the climate. Given the relevance of microorganisms for governing global biochemistry, characterizing these communities remains an important challenge for ecologists and earth system modelers (Crowther et al., 2019). Four distinct research areas have emerged to characterize global soil communities, describing patterns in (i) biomass and abundance, (ii) functional group composition, (iii) taxonomic diversity and composition, and (iv) involvement in ecosystem functions (Crowther et al., 2019).

1.1 Stelvio Pass

Centrally located in the European continent and densely populated for most of its parts, the European Alps constitute a dominant feature of the European landscape. Characterized by extensive lowlands, deeply incised valleys and peaking at an elevation of more than 4800 m, the Alps are subject to a strong topographic variability. In this thesis, we focus on an area located in the Italian central Alps, close to the Stelvio Pass (46°31’43.1’’N 10°27’11.2’’E; elevation 2230-2750 m a.s.l.), a high alpine site in the Stelvio National Park (Figure 1).

Air temperature and precipitation records for the period 1978–2015 were provided by the nearest available meteorological station at Cancano (located 9 km far from the study area at 46°31’02.2’’N, 10°19’14.7’’E; elevation 1948 m a. s. l.). The mean annual air temperature (MAAT) was 3.3 ± 0.75°C, with January and July as the coldest and warmest months, respectively (- 5.2 ± 1.8°C and 12.2 ± 1.6°C). Mean annual precipitation sum was 810 mm, 56% of which fell between May and September. Snow can fall at any time, but snow cover is continuously for 6 months, from mid-November to May. Vegetation is a mosaic of habitat types of the subalpine and alpine belts. Hereafter

the main plant communities of the study site will be briefly described, on the base of past and recent vegetation and floristic assessments (Giacomini and Pignatti, 1955; Cannone et al.,2007; Cannone and Pignatti, 2014). The higher-altitude alpine belt shows two different plant communities, namely an alpine grassland and a snowbed, which are characterized by the highest vulnerability to climate change impacts and are at the highest risk of regression also due to the ingression of species from neighbour community types (Cannone et al., 2007; Cannone and Pignatti, 2014). Alpine grasslandis mainly constituted by the *Caricion curvulae alliance*, with *Carex curvula* as dominant and representative species. Among the characteristic species, there are several graminoids (Graminaceae, Cyperaceae and to a less extent Juncaceae) including *Agrostis rupestris, Festuca halleri*, *Juncus trifidus*, *Oreochloa disticha* and many other vascular plants including *Hieracium glanduliferum*, *Minuartia recurva*, *Pedicularis kerneri*, *Phyteuma hemisphaericum*, *Primula daonensis*, *Senecio incanus*, *Veronica bellidioides*. Other frequent abundant species are *Leontodon helveticus* and *Trifolium alpinum*. The typical alpine grassland is the *Caricetum curvulae* association, mainly present at 2600 m asl in the study area, generally spanning from 2400 to 2800 m asl. Where the snow cover remains on the ground for at least 8 months, the alpine snowbed is present, constituted by the *Salicion herbaceae alliance*. Its characteristic species are *Salix herbacea*, *Alchemilla pentaphyllea* and *Soldanella pusilla* and, on the base of their relative abundance, *Salicetum salicetosum* and S. *alchemilletosum* variants can be distinguished. This association is present mainly between 2550 and 2750 m asl. At the lower altitude site a subalpine grassland (*Caricetum firmae*) and a shrubland dominated by *Rhododendron ferrugineum* occur (Figure 1). In the study area this shrublands have a patchy distribution, mainly located in gully and in area with enhanced snowdepth in order to protect leaves from winter frost damages, and forms mosaic with alpine grassland communities where *R Rhododendron ferrugineum* has been encroaching since 1953 in response to recent climate change. At the elevation of our study site, the only anthropogenic land use is extensive summer pasturing. Transhumance in this area is known since 1800 and practiced during the summer when cattle herds are taken to the highland, while in autumn they are brought back to the lowland.

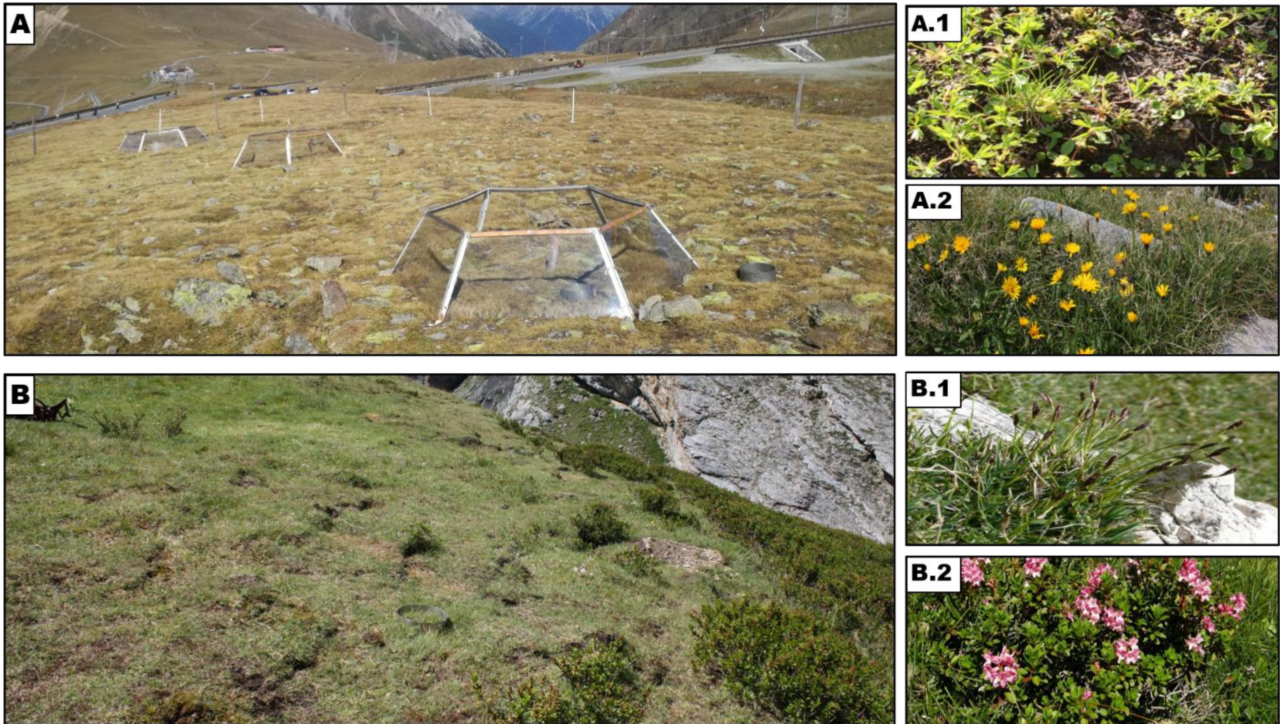

Figure 1: Vegetation types of the plots under study in the Stelvio Pass area. A) Alpine belt; A.1) Alpine snowbed; A.2) Alpine grassland; B) Subalpine belt; B.1) Subalpine grassland; B.2) Subalpine shrubland.

The effects of simulated environmental changes were investigated in field experiments by the use of open-top chambers (OTCs, Figure 2) at the alpine belt. OTCs were installed and maintained since 2014 according to the method of the International Tundra Experiment (ITEX). They passively warm up and their top openings enable precipitation to enter and reduce temperature extremes (Marion et al., 1997).

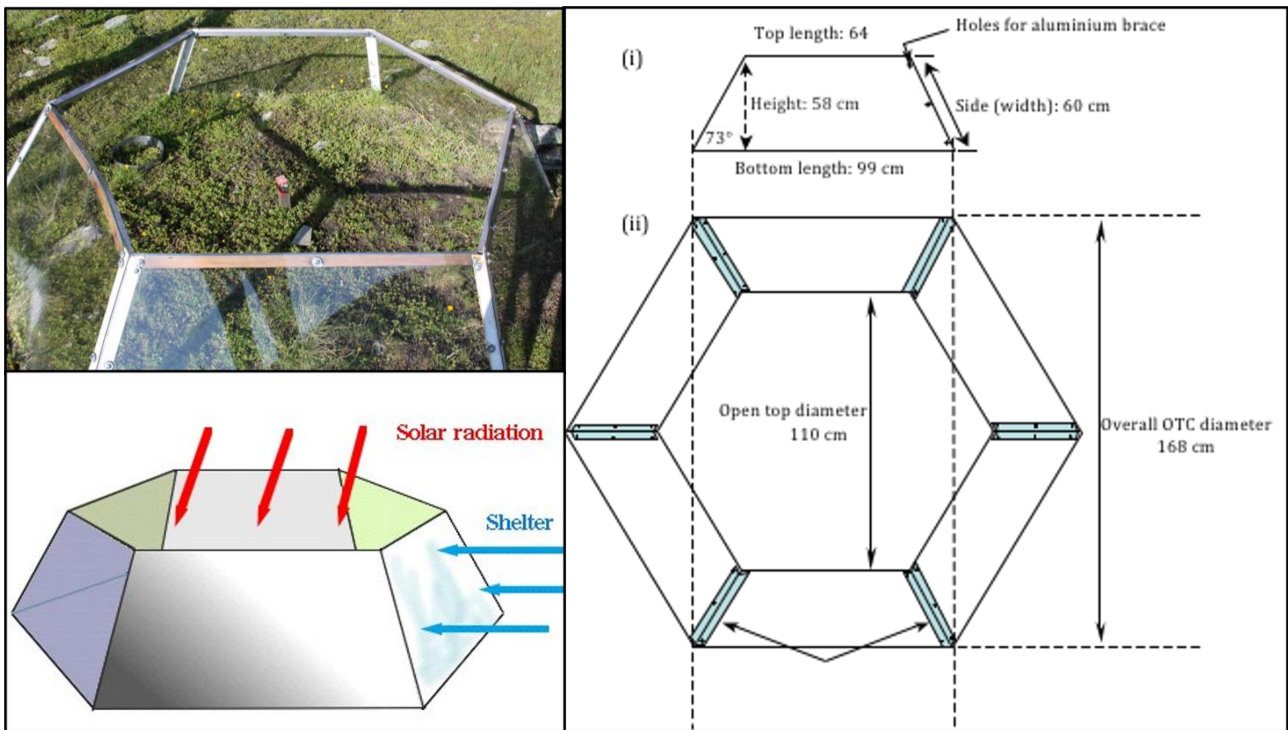

Figure 2: Open-top chamber (OTC) of ITEX design (International Tundra Experiment). Bottom left picture by Ulf Molau (Jägerbrand et al., 2005).

The ITEX research model combines long-term and short-term experimentation with monitoring and has the elegance and simplicity called for to understand ecosystem response and vulnerability to change. The experiment was designed to examine the effects of temperature change; maximize geographic representation, by minimizing technical and equipment requirements. The paired plots outside the OTCs acted as control plots which represented the current conditions of the site.

Furthermore, this area is suited for studies of “space-for-time” substitution along the elevation gradient. They assume that spatial changes in the structure and function of the ecosystem moving from low to high elevation, are similar to longer-term temporal trajectories of mountain ecosystems under climatic change. Indeed, the Stelvio Pass records a long history of botany surveys and a clear evidence of recent climate change effects, with areal changes of vegetation since 1953, consisting of shrub upward migration and encroachment at the expenses of the alpine grasslands and snowbeds (Cannone et al., 2007, Malfasi and Cannone, 2020). The shrub encroachment in this area dated back to 1860s, the end of the Little Ice Age, and is now involving also tree encroachment that started since the 1960s

and 1970s (Malfasi and Cannone, 2020). Therefore, the comparison between alpine and subalpine soils is used to assess the potential effects of the space-for-time substitution, due to the upward vegetation shift expected in the case of the future warming scenario RCP6.0 (Representative Concentration Pathway 6.0) by the end of this century (IPCC, 2013). While the alpine sites represent the current conditions in the higher alpine belt, subalpine sites may represent the future conditions of these alpine communities by the end of this century.

1.2 Greenland

Greenland is a land of extremes, located between 59° and 83° N, northeast of Canada and northwest of Iceland, between the Arctic Ocean and the North Atlantic Ocean. It is the largest island and the least densely populated country in the world covering about 2.17 million square kilometres (Lorentzen and Penninga, 2018). About 80% of the surface is covered by a permanent ice sheet, which measures around 3 kilometres at its thickness. The climate is arctic to subarctic: the average daily temperature of Nuuk, the capital, located in the southwestern part of the island, varies seasonally from −5.1 to 9.9 °C. The lowest mean annual temperatures, about −31 °C, occur on the north-central part of the north ice-sheet, and temperatures in the south are about −20 °C.

In the last years, the Arctic is experiencing rapid temperature increases at a rate two to three times higher than the global average over the past 150 years (IPCC, 2013), making climate dominant driver of ecosystem changes in this region (Smol et al., 2005, Post et al., 2009, Schuur et al., 2015).

Greenland provides perhaps the most striking recent example of rapid climate change in the Arctic, where it is estimated that a complete melting of the ice sheet could result in an increase of the length of ice-free periods, an increase of global see level of about 7 meters and in an overall greening of terrestrial areas (Goetz et al., 2005; Bhatt et al., 2010; Myers-Smith et al., 2011). In particular, recent studies reported significant increases in the cover and height of shrubs (e.g. *Betula nana* and *Salix pulchra*), combined with significant decreases in the coverage of bryophytes, lichens (Pattison and

Welker, 2014) and graminoid (Elmendorf et al., 2012), most likely due to the competitive exclusion by shrubs (Jägerbrand et al., 2009).

1.3 Alpine and arctic soils: similarities and differences

Arctic and alpine ecosystems cover about eleven million square kilometers, or 2 %, of the Earth's land surface (Figure 3), with about five million square kilometers in arctic tundra, three million square kilometers in alpine tundra, and the remainder in intermediate northern highlands (Virtanen et al. 2016). They differ in their ambient and soil temperatures. Even though there is greater seasonal variability in air temperatures in arctic than in alpine regions, the greater presence of permafrost in arctic soils tends to buffer soil temperatures from fluctuations, resulting in smaller seasonal fluctuations in soil temperatures in arctic regions than in mid-latitude alpine regions (Richardson et al. 2003). Gradients of environmental severity characterise both arctic and alpine environments, with conditions becoming increasingly harsh with latitude and elevation, respectively (Donhauser and Frey, 2018). Although both environments encompass a wide range of soil moistures, water is more often limiting in alpine soils than in low arctic soils (Richardson et al. 2003), because relative humidity tends to decrease with increasing elevation, which can exacerbate moisture stress in alpine environments.

Indeed, alpine regions are strongly influenced by elevation, slope angle and slope aspect, which are important topographic features in terms of temperature and moisture regimes. The differences in soil temperature and moisture create microclimates that vary significantly from zonal climatic conditions, and strongly affect soil biological and chemical processes (e.g. plant growth, microbial activities), thereby affecting organic matter input and mineralization (Zhao and Li, 2017). However, soils on steep slopes are prone to erosion while soils on gentle slopes rather experience deposition from steeper areas. In this light, soil stability largely depends on comprehensive vegetation cover forming a tight root network (Körner 2003).

Figure 3: Alpine (left) and Arctic (right) landscapes.

On a global scale, gross and net photosynthesis in arctic-alpine ecosystems is generally low because of low temperatures and water-saturated surface conditions that are the result of topography or permafrost (Strimbeck et al., 2019). These conditions strongly limit decomposition and ecosystem respiration, resulting in a net influx and long-term net C accumulation in soils. Thus, they are critical reservoirs in the global C cycle, with as much as 50 % of the global belowground C pool stored in organic soils and deeper organic deposits in permafrost regions alone (Tarnocai et al. 2009). The increase in shrub biomass, cover and abundance (colloquially termed *shrubification*) observed in many arctic and alpine tundra ecosystems over the past century with latitude and elevation respectively, is expected to have consequences on global climate and C cycle (Naito and Cairns, 2011, Cannone et al., 2007; Malfasi and Cannone, 2020; Collins et al., 2020). Several potential ecological consequences of an increase in deciduous shrub cover in tundra areas have been reported: *i*) taller and denser shrub patches reduce albedo, especially during spring snowmelt, which is accelerated as branches start to emerge from the snow; *ii*) taller canopies also trap more snow, which acts as insulation and raises winter soil temperatures (Vowles and Björk, 2019). Higher soil temperatures can in turn increase both winter and summer nitrogen (N) mineralization, litter decomposition and winter respiration rates (Strimbeck et al., 2019). Thus, an increase in deciduous shrub cover may trigger a number of processes that have the potential to accelerate C turnover in tundra ecosystems.

1.4 Exploration soil microbial communities: some classical and molecular approaches

In the past, our understanding of microbial communities was severely limited by our ability to grow microorganisms in the laboratory. The development and application of cultivation-independent techniques based on molecular analysis of marker genes and more recently meta-omics analysis approaches have provided alternative paths towards the exploration of the microbial ecosystems that encompass their entire complexity.

In particular, quantitative PCR, which analyses rRNA copy number variations across genomes, is typically used to estimate the size and share of bacterial and fungal microorganisms in soils (Frostegård et al., 2011). Additionally, thanks to its simplicity and minimal costs, metabarcoding represents the most common approach for investigating environmental diversity and describing the composition of microbial communities (Smith and Peay, 2014). It used to be the first high-throughput sequencing technology used to study the total microbial communities and which microorganisms are active there. It relies on the selection of taxonomic informative (barcode) genes for which universal primers are available to amplify short sequences, with sufficient phylogenetic signal and availability of comprehensive taxonomic reference databases that can be sequenced in parallel without the necessity for cloning. If this approach provides information about the microbial community composition and who is abundant under certain conditions, it does not answer the important question about what they do. To try to answer this question, it is essential to measure the rates of soil processes and provide their link to microbes. In this light, more recently, new advances resulting in the development of "omic" technologies seem to represent a feasible way to link microbial taxa with ecosystem processes. Metagenomics and metatranscriptomics refers to the sequencing of the entirety of microbial genomes and transcripts from a microbiota, representing useful tools for globally cataloguing microbial gene and transcript profiles and reflecting overall metabolic functions of microorganisms (Baldrian et al., 2019; Sugitha et al., 2020). Metagenomics is the study of the genomes in a microbial community and constitutes the direct approach to assess the potential taxonomic and functional profiles of a microbial community. Although whole-metagenome sequencing (WMS) provides a partial glimpse into the functional profile of a microbial community, it is better inferred using metatranscriptomics, which

involves sequencing of the complete transcriptome of the microbial community (Aguiar-Pulido et al., 2016). Metatranscriptomics provides the information on the expressed genes within the soil community as a whole. With the use of functional annotations of expressed genes, it is possible to infer the functional profile of a soil community at the sampling time.

Finally, another approach used to measure the soil process rates, is the enzymatic assays, that are considered attractive to soil ecologists, theoretically offering a cheaper and direct approach to link between soil organisms and biochemically well-defined reactions (Baldrian et al., 2019). To measure potential rates of soil extracellular enzyme activities, the measurement of soil extracellular enzymes are often used; synthetic substrates that are bound to a fluorescent dye are added to soil samples and the enzyme activity is measured as the fluorescent dye released from the substrate by an enzyme-catalyzed reaction, where higher fluorescence indicates more substrate degradation (Bell et al., 2013). Unfortunately, this approach measures potential rather than real enzyme activities because the use optimal conditions, including the use of synthetic and not limiting substrates (Nannipieri et al., 2012). Furthermore, assays for the detection of all enzymes are not available (Baldrian et al., 2019). For this reason, caution should be used when interpreting data from this analysis, and comparisons would be made with different temperatures, as in situ soil temperature can influence enzyme kinetics (Bell et al., 2013).

1.5 Aims of the thesis and chapters description

The main aim of this work was to provide a taxonomic and functional characterization of soil microbial communities of alpine and arctic environments, to understand how diversity, composition and functionality are affected by biotic and abiotic parameters. In particular the soil communities composition and functionality at different altitudes and under warming conditions, simulated by using OTCs, have been investigated in an alpine ecosystem (chapter 2 and 3). A better understanding of the patterns of geographic distribution and drivers of community assembly along environmental gradients of alpine ecosystems is fundamental for elucidating microbial processes, and for giving important

insights into the their shifts in a climate change context. An opportunity to study the long term effects of climate change on microbial ecosystems is given by the study of *shrubification* phenomena, which, occurring worldwide, may aid to describe its effects at the global scale, and to decide whether observed impacts are species- and site-specific. To achieve this, chapters 2 and 3 investigate the potential shrub expansion impacts into alpine ecosystems, while chapter 4 deals with the taxonomic and functional composition of bacterial communities in the Western Greenland, an arctic landscape. Finally, as individual studies on soil do not allow general conclusions on global scale distribution of microbial taxa due to the limited sampling efforts in space and time, new and more promising results can be obtained by comparative analysis of available data. The advent of high-throughput-sequencing methods allowed to have available large amounts of sequencing data on microbial soils from different terrestrial environments and may give us interesting results when combined. In the light of this, chapter 5 reports a global database creation which represents the most comprehensive atlas of global fungal distribution occurrences from high-throughput-sequencing metabarcoding studies available to date. This database is of high importance to determine how environmental factors affect the diversity and distribution of fungal communities worldwide.

Chapter 2

Microbial activity in alpine soils under climate change

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Abstract

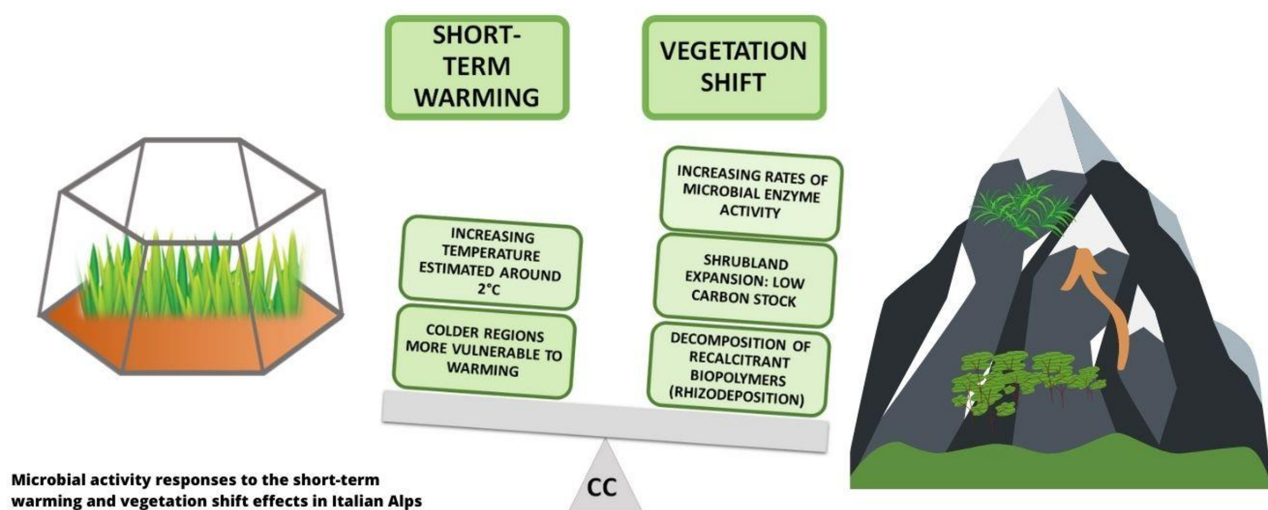
Soil enzymatic activity was assessed in the Stelvio Pass area (Italian Central Alps) aiming to define the possible effects of climate change on microbial functioning. Two sites at two different elevations were chosen, a subalpine (2239 m) and an alpine belt (2604-2624 m), with mean annual air temperature differing by almost 3 °C, coherent with the worst future warming scenario (RCP 8.5) by 2100. The lower altitude site may represent a proxy of the potential future situation at higher altitude after the upward shift of subalpine vegetation due to climate change. Additionally, hexagonal open top chambers (OTCs) were installed at the upper site, to passively increase by about 2°C the summer inner temperature to simulate short term effects of warming before the vegetation shift takes place. Soil physicochemical properties and the bacterial and fungal abundances of the above samples were also considered. The subalpine soils showed a higher microbial activity, especially for hydrolytic enzymes, higher carbon, ammonium and hydrogen ($p<0.001$) contents, and a slightly higher PO_4 content ($p<0.05$) than alpine soils. Bacterial abundance was higher than fungal abundance, both for alpine and subalpine soils. On the other hand, the short term effect, which increased the mean soil temperature during the peak of the growing season in the OTC, showed to induce scarcely significant differences for edaphic parameters and microbial biomass content among the warmed and control plots. Using the manipulative warming experiments, we demonstrated that warming is able to change the enzyme activity starting from colder and higher altitude regions, known to be more vulnerable to the rising temperatures associated with climate change. Although five-years of experimental warming does not allow us to make bold conclusions, it appeared that warming-induced upwards vegetation shift might induce more substantial changes in enzymatic activities than the short-term effects, in the present vegetation context.

Keywords: Alpine soils; Enzyme activity, Warming effect, Decomposition, Carbon storage; Vegetation shift.

Highlights:

- Subalpine soils showed a higher microbial activity than alpine soils;
- The vegetation shift, especially the shrubland expansion, may have a positive feedback to warming;
- Soil extracellular enzymes are more responsive to rising temperatures in colder and higher altitude regions;
- Vegetation shift effects on microbial enzyme activity may be stronger than those of short-term warming.

Graphical Abstract:



2.1 Introduction

During the last century, high altitude and latitude regions on Earth experienced rapid climate warming at rates much higher than other areas. The European Alps seem to be particularly affected, with an increase of the mean annual air temperature (MAAT) exceeding the global average of 0.6±0.2°C (Wieser et al., 2019). Many studies are documenting how changes in climate mean and extremes affect plant species distribution, composition, phenology, and forest structure (e.g.: Deb et al., 2018; Bjorkman et al., 2020; Dorji et al., 2020; Xu et al., 2020), alter the quality and quantity of plant litter and in turn the composition and functioning of soil microbial communities. An upward migration and areal expansion of shrubs has been widely documented for the European Alps as a consequence of climate change (Cannone et al., 2007), and further increases of temperatures in upcoming decades would deeply affect the extremely vulnerable high-elevation alpine ecosystems (Cannone and Pignatti, 2014; Malfasi and Cannone, 2020).

Warming is predicted to continue and two different extremes have been hypothesized by the end of this century, according to the best and the worst scenario (IPCC, 2013). The former involves an estimated MAAT increases of around 2 °C (Representative Concentration Pathway 2.6 (RCP2.6)) and the latter an increase from 2.6 to 4.8 °C (RCP8.5) (IPCC, 2013).

Soil microorganisms regulate the amount of organic carbon (C) stored in soil and how much is released back to the atmosphere. By producing extracellular enzymes (EEs), they decompose polymeric organic matter, including plant cell wall polymers such as cellulose, hemicelluloses and lignin, into smaller soluble molecules, and release in the environment micro- and macronutrients like nitrogen (N), phosphorus (P), sulphur (S) and other essential metals, which can be assimilated by plants and soil organisms (Cavicchioli et al., 2019). It follows that any disturbance in microbial activity will result in changes in both decomposition and nutrients released in an ecosystem (Margesin et al., 2014). Although microorganisms have a key role in maintaining a healthy global ecosystem, and changes in their diversity and activity will likely affect the resilience of all other organisms,

studies of microbial community at high altitude structure and function under climate change scenarios are mostly lacking (Cavicchioli et al., 2019).

Microbial activity in soil is affected by the vegetation, the amount and composition of the indigenous microbial community, the edaphic characteristics, the nutrient availability and the local climatic conditions (Lladó et al., 2017). Changes in these parameters may directly or indirectly affect the soil enzyme activities in complex ways which are difficult to predict. Relationship between vegetation, microbial activities, and soil properties are altitudinally defined, and a negative correlation between altitude and C and N contents, C/N and soil moisture was reported (Ma et al., 2004; Giri et al., 2007; Margesin et al., 2008).

Climate changes are expected to alter the quantity and composition of soil organic matter (SOM), the microbial soil enzymatic profiles and the related rate of decomposition of SOM components, and thus the efflux of CO_2 to the atmosphere (Bardgett et al., 2008; Wallenstein and Weintraub, 2008; Meng et al., 2020). Microbial decomposers play a key role in global C and nutrient cycles by driving the degradation and mineralization of organic matter (Manzoni, 2017). Colder regions store a large pool of soil organic matter and enzyme activities in these regions are more sensitive to temperature increases than those in warmer regions (Dong and Somero, 2009; Chen et al., 2015). Considerable losses of soil C are thus expected to occur in high altitude areas in response to rising temperature, driving a positive land carbon-climate feedback that could in turn accelerate climate change (Crowther et al., 2016). Several experimental studies, using warming devices (such as Open Top Chambers, OTC) found soil C losses (Melillo et al., 2017; Pries et al., 2017) caused by increasing both the turnover of the labile and readily accessible litter and SOM (Dorrepaal et al., 2009) and the depolymerization of chemically complex and recalcitrant C pools (Chen et al., 2020). Increases in the ratio of the ligninolytic to cellulolytic activity were observed under experimental warming, leading to faster degradation of chemically recalcitrant C pools, possibly exacerbating long-term C-climate feedbacks (Chen et al., 2020).

However, climate change may also affect microbial community composition and function and their enzymatic patterns indirectly by changing vegetation composition or stimulating plant growth, root exudates production and quality and quantity of litter deposition (Cheng et al., 2010; Burns et al., 2013). Greater rates of litter decomposition were recorded after the removal of different plant species in field experiments, highlighting the role of vegetation changes in the regulation of decomposition rates in high altitude regions (Ward et al., 2009).

Despite numerous studies in recent decades, the net effects of climate warming on soil C stocks are still unclear (Smith and Fang, 2010; Van Gestel et al., 2018) and the effects of warming on soil extracellular enzyme activities are highly variable across individual studies (Meng et al., 2020), since they do not follow simple biochemical rules (Baldrian et al., 2013). One of the biggest challenges is to understand the interactions between the complex biotic and abiotic components of soils and the rate of specific metabolic processes (Rousk and Bååth, 2011; Stein and Nicol, 2011) to identify the main drivers governing these activities, and how they alter the growth and activity of both primary producers and microorganisms (Treseder et al., 2012).

It is widely assumed that enzyme activity roughly doubles with a 10 °C increase in temperature; however, the accumulated evidence suggests a wide range in temperature sensitivities for different enzymes (Baldrian et al., 2013). Other parameters may drive enzyme production, even when temperature is more favourable. In general, extracellular enzymes activity is related to the quality of available organic matter and nutrients demands of the microbial biomass (Sinsabaugh et al., 2015). Other parameters, as climate, pH, and water content, directly and indirectly affect soil microbial metabolism by altering available substrate concentration and C, N, and P stoichiometry (Romanowicz et al., 2016).

In Arctic tundra, soil enzymes were produced while soils were frozen, and their activities did not increase during the summer; here, enzyme production seemed to be N-limited, even when temperature would drive higher enzyme activities (Wallenstein et al., 2009).

Based on these premises, the project *Responses of alpine sensitive ecosystems to climate change* (RESACC) aims to define and quantify the impacts of global warming on the sensitive high-altitude Italian Alps ecosystems, by a multidisciplinary approach (e.g. geology, plant phenology, microbiology and pedology). The present study focused on the microbial activity assessed as the production of extracellular enzymes in two different altitudinal belts, characterized by subalpine and alpine plant communities, respectively, and compared them with biotic and abiotic parameters, such as microbial biomass, soil properties, climate and dominating plant species.

With this study we aimed to: i) determine the potential effect on soil enzymatic patterns of the worst warming scenarios (RCP8.5) hypothesized by IPCC (2013) by 2100 and represented by the upward migration and encroachment of the subalpine shrubland in the alpine grassland (provided by the subalpine study site); ii) assess the impact of short-term warming on soil enzymatic pattern beneath an alpine vegetation mediated by OTC experimental warming devices (provided by the alpine study site) and representing the short-term effects that are about to happen until climate effects induce vegetation shifts. We hypothesized that short-term warming has little influence on enzyme production since high elevation soils may be nutrient limited. On the other hand, the vegetation upward migration and shrub encroachment should result in the increase of enzyme activity due to the higher plant root activity, since decomposition of recalcitrant biopolymers is primed by rhizodeposition (Dijkstra and Cheng, 2007; Kohout et al., 2018). Under this scenario, the C accumulated in high altitudes may be rapidly released into the atmosphere as soon as subalpine vegetation colonizes high altitude soils.

2.2 Materials and Methods

2.2.1 Sampling sites and design

The study area is located around Stelvio Pass, in Stelvio National Park, Italian Central Alps (46°31’ N, 10°25’ E). This area was selected due to the clear evidences of recent climate change, with areal changes of vegetation since 1953, implying shrub upward migration and encroachment at the expenses of the alpine grasslands and snowbeds (Cannone et al., 2007). The shrub encroachment in

this area was dated back to 1860s, the end of the Little Ice Age, and is now involving also tree encroachment, started since 1960s and 1970s (Malfasi and Cannone, 2020). Two sites at two different elevations were chosen, a subalpine (2239 m a.s.l.) and an alpine belt (2604-2624 m a.s.l.) respectively, experiencing a difference in MAAT of almost 3 °C from each other, coherent with the worst future warming scenario (RCP8.5) by 2100 (IPCC, 2013). Four sampling points (Table 1), characterized by two different vegetation patterns each, were chosen. The higher-altitude alpine site showed two different plant communities, an alpine grassland climax (*Caricetum curvulae*) and a snowbed (*Salicetum herbaceae*). These communities are characterized by the highest vulnerability to climate change impacts and are at the highest risk of regression also due to the ingression of species from neighbour community types (Cannone et al., 2007; Cannone and Pignatti, 2014). At the lower-altitude site a subalpine grassland (*Caricetum firmae*) and a shrubland dominated by *Rhododendron ferrugineum* occurred. In this study, an observational study and a manipulative experiment were conducted to assess the long-term and short-term effects, respectively, of warming on soil enzyme production. To study the long-term effects, the higher altitude alpine sites were compared to the lower altitude subalpine sites. Alpine sites represented the current conditions in the alpine belt, whereas subalpine sites represented the potential evolution of the alpine communities in a long-term warming scenario by the end of this century. Five samples were collected from each vegetation type in alpine belt (grassland and snowbed) and subalpine belt (grassland and shrubland), making n = 10 of total sampling units for each belt. To study the short-term effects, small (2.08 m diameter hexagonal) open top chambers (OTCs) were installed and maintained since 2014, to simulate warming at the alpine grassland and snowbed. OTCs, according to the method of the International Tundra Experiment (ITEX), passively increase summer ambient temperature inside them (Marion et al., 1997), coherent with the best future warming scenario (RCP2.6) by 2100 (IPCC, 2013). The paired plots outside the OTCs acted as control plots, representing current conditions.

Table 1. Location of the sampling sites and soil properties

Site	Phytosociological association	Altitude (m asl)	Coordinate	Water content (%)	pH	C (%)	N (%)	H (%)	$P\text{-}PO_4$ (ppm)	$N\text{-}NH_4$ (ppm)	Bacteria (rRNA gene copies/g dry soil)	Fungi (rRNA gene copies/g dry soil)
Alpine snowbed	*Salicetum herbaceae*	2624	46.53083°N; 10.44136°E	41.7 ± 4.6	5.3 ± 0.5	8.4 ± 1.9	0.8 ± 0.1	1.6 ± 0.3	1.9 ± 1.4	3.4 ± 0.8	$5.6\ 10^{12} \pm 1.4\ 10^{12}$	$8.4\ 10^{9} \pm 2.5\ 10^{9}$
Alpine grassland	*Caricetum curvulae*	2604	46.53157°N; 10.44066°E	38.7 ± 4.1	5.2 ± 0.2	8.2 ± 1.3	0.02 ± 0.03	1.4 ± 0.3	5.0 ± 1.8	5.5 ± 1.0	$4.5\ 10^{12} \pm 0.8\ 10^{12}$	$12.0\ 10^{9} \pm 5.2\ 10^{9}$
Subalpine grassland	*Caricetum firmae*	2239	46.522303°N; 10.408601°E	57.5 ± 6.8	5.7 ± 0.3	17.0 ± 4.2	0.5 ± 0.3	2.4 ± 0.7	6.0 ± 2.8	25.0 ± 6.5	$4.7\ 10^{12} \pm 0.7\ 10^{12}$	$8.5\ 10^{9} \pm 2.1\ 10^{9}$
Subalpine shrubland	*Rhododendron ferrugineum*	2239	46.522303°N; 10.408601°E	49.4 ± 11.4	5.8 ± 0.2	16.8 ± 4.7	0.8 ± 0.4	2.7 ± 0.3	4.4 ± 4.0	32.7 ± 6.3	$5.8\ 10^{12} \pm 2.3\ 10^{12}$	$15.0\ 10^{9} \pm 4.0\ 10^{9}$
OTC treatment												
Alpine snowbed OTC	*Salicetum herbaceae*	2624	46.53083°N; 10.44136°E	43.3 ± 1.0	5.4 ± 0.3	9.0 ± 0.6	0.7 ± 0.3	1.7 ± 0.08	1.0 ± 0.4	5.1 ± 1.9	$6.0\ 10^{12} \pm 0.9\ 10^{12}$	$6.1\ 10^{9} \pm 2.0\ 10^{9}$
Alpine grassland OTC	*Caricetum curvulae*	2604	46.53157°N; 10.44066°E	38.7 ± 6.1	5.3 ± 0.4 ±	7.1 ± 2.6	0.02 ± 0.02	1.2 ± 0.4	6.3 ± 4.2	6.3 ± 2.8	$3.9\ 10^{12} \pm 0.7\ 10^{12}$	$13.0\ 10^{9} \pm 8.0\ 10^{9}$

Samples were collected in July 2019, during the vegetation growing season. To have easily cross-comparable data, all samples were collected in the same day, avoiding differences based on daily variables, like precipitation events. For each sample, soil was collected with a sterilized spatula in at least three points up to 10 cm depth. After removal of surface soil and roots, it was passed through a 5 mm sterile mesh and mixed, to have a composite sample collected in a sterile Falcon tube. Each Falcon tube was transported to the laboratory under frozen conditions and stored at −20 °C until the analyses.

2.2.2 Soil physicochemical parameters

During the whole growing season, we measured the soil temperature in situ at 2 cm depth using thermistors (Hobo pro V2 2x U23-003, accuracy 0.2 °C, resolution 0.02 °C). Soil pH was measured by adding 20 ml of distilled water to 10 g of air-dried soil, shaking the soil and water mixture vigorously and measuring the pH after some minutes of sitting. Soil samples were oven dried at 105 °C for 24 hours. The water content was calculated by taking the difference between the fresh sample weight and that of the dried sample. All chemical analyses were carried out on the 2-mm soil fraction. Total C, total N and hydrogen (H) contents were determined with an automatic element analyser (Carlo Erba). To determine dissolved nutrient concentrations, briefly, c. 10 g of air-dried soil was extracted in 100 ml de-ionized water, shaken for 2 h, and filtered using glass microfiber filters (Whatman GF/D). The obtained extracts were analysed colorimetrically for $N\text{-}NH_4$ by the salicylate method, $N\text{-}NO_3$ by the cadmium reduction method, $P\text{-}PO_4$ by the molybdenum blue method using a continuous flow analyser (FlowSys, Systea, Rome, Italy).

2.2.3 DNA extraction and quantitative PCR

DNA was co-extracted with RNA using the DNA Elution Accessory Kit combined with the RNeasy PowerSoil Total RNA Kit (MoBio Laboratories). Three aliquots (3 × 1 g of material) were extracted per sample. Triplicate DNA extracts were pooled, and DNA was cleaned with a GeneClean Turbo Kit (MP Biomedicals) as described previously (Baldrian et al., 2012).

Bacterial and fungal rDNA copies were quantified by quantitative polymerase chain reaction (qPCR) using the 1108f and 1132r primers for bacteria (Wilmotte et al., 1993; Amann et al., 1995) and the FR1/FF390 primers for fungi (Prévost-Bouré et al., 2011) as described previously (Žifčáková et al., 2016). Amplifications were performed on a StepOnePlus cycler (Applied Biosystems) using optical grade 96-well plates. Each 20 µl reaction mixture contained 10 µl SYBR Green Master Mix (Applied Biosystems), 0.9 µl BSA (10 mg/ml), 1.35 µl of each primer, 1.5 µl of template and 6.1µl of water. The PCR cycling protocol was the same for fungal and bacterial DNA quantification, as follows: 56 °C for 2 min; 95 °C for 10 min; 95 °C for 15 s and 60 °C for 1 min (40 cycles). *Streptomyces lincolnensis* DNS 40335 and *Hypholoma fasciculare* CCBAS281 genomic DNA were used as standards. Fungal and bacterial abundances were expressed as number of copies of rDNA genes per gram of dry soil.

2.2.4 Enzyme assays

Enzyme activity was measured as described previously (Štursová and Baldrian, 2011). The activity of selected hydrolytic enzyme (Table 2) was assayed in soil slurries prepared by mixing 0.25 g of freeze-dried soil with 25 ml of 5 mM sodium acetate buffer (pH 5.0).

Table 2. Extracellular hydrolytic enzymes assayed and corresponding substrates

Enzyme	**EC (Enzyme Commission)**	**Substrate**
β-glucosidase	3.2.1.21	4-methylumbelliferyl-β-D-glucopyranoside
Exocellulase (cellobiohydrolase)	3.2.1.91	4-methylumbelliferyl-β-D-cellobioside
α-glucosidase	3.2.1.3	4-methylumbelliferyl-α-D-glucopyranoside
β-xylosidase	3.2.1.37	4-methylumbelliferyl-β-D-xylopyranoside
β-galactosidase	3.2.1.23	4-methylumbelliferyl-β-D-galactopyranoside
Lipase	3.1.1.3	4-methylumbelliferyl-caprylate
N-acetylglucosaminidase (chitinase)	3.2.1.14	4-methylumbelliferyl-N-acetylglucosaminide
Acidic phosphatase	3.1.3.2	4-methylumbelliferyl-phosphate

Samples were homogenized using an UltraTurrax (IKA Labortechnik, Germany) for 3 min at 8,000 rev min^{-1} in an ice bath and the homogenate was used as a sample in the assays using methylumbelliferone-based substrates. The activities of *laccase, Mn-peroxidase, endocellulase* and *endoxylanase* were determined in extracts because spectrophotometric measurements in soil homogenates are impossible. Freeze-dried soil samples were extracted using 160 mM phosphate buffer, pH 7 and desalted using Sephadex columns. Laccase was assayed using the oxidation of 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid, manganese peroxidase using 3-methyl-2-benzothiazolinone hydrazone and 3,3-dimethylaminobenzoic acid in the presence of Mn and hydrogen peroxide, endocellulase and endoxylanase were measured using azo-dyed carboxymethyl

cellulose and birchwood xylan (Megazymes). All enzyme assays were performed at pH 5 except for laccase where the pH of the buffer was 4.5. Enzyme activity was expressed in units of nmol of substrate converted per hour per g soil dry mass (nmol $h^{-1}g^{-1}$).

2.2.5 Statistical analysis

The null hypothesis of no difference on enzymatic activities between alpine and subalpine samples and, between vegetation types within each altitude, was tested using pairwise PERMANOVA (Anderson, 2001) through the wrapper function pairwise.adonis2() (Martinez Arbizu, 2020). This function performs multilevel pairwise comparisons using adonis2() from *vegan* package v. 2.5-6 (Oksanen et al., 2019) in R v. 3.6.2 (R Core Team, 2019). Hellinger transformed matrix of the enzyme activities was used as response matrix and PERMANOVA was conducted on Euclidean distances. The null hypothesis of no difference between OTC and control plots in alpine belt was tested using PERMANOVA analysis through adonis2() function in *vegan*. Hellinger transformed enzyme activities was used as response matrix and alpine vegetation type (grassland vs. snowbed), treatment (OTC vs. control) and their interactions were used as fixed explanatory factors. *P*-values were computed using 9999 permutations. To illustrate the PERMANOVAs, non-metric multidimensional scaling (NMDS) ordinations were conducted using the function metaMDS() from *vegan*. Similar to PERMANOVAs, NMDS ordinations were conducted on Euclidean distances of Hellinger transformed matrix of the enzyme activities. We used the envfit() function in *vegan* to fit edaphic variables (pH, water, C, N, P-PO_4, N-NH_4, H contents) and the bacterial and fungal abundance onto the NMDS ordination axes. We performed Pearson correlations between independent variables using the chart.Correlation() R function on *PerformanceAnalytics* package v. 1.5.3 (Peterson et al., 2018). The null hypothesis of no differences in soil parameters, enzyme activity and total bacterial and fungal abundance between alpine sites and subalpine sites was tested using one-way analysis of variance (*ANOVA*). The null hypothesis of no effect of OTC treatment on above characteristics in the grassland and snowbed of the alpine belt was tested using ANOVA followed by a *post hoc* pairwise multiple

comparison procedure (*Tukey's HSD*). The response variables were the same as above (i.e. soil parameters, enzyme activity and, fungal and bacterial abundance), and, alpine soil type (grassland vs. snowbed), treatment (OTC vs. control) and their interaction were used as fixed explanatory factors.

2.3 Results

2.3.1 The effect of vegetation shift on enzyme activity

To determine the potential effect of vegetation shift as a consequence of continuous warming, on soil enzyme activity, enzymatic and edaphic data were compared between samples of the subalpine (2239 m a.s.l., grouping grassland and shrubland sites) and alpine sites (2604-2624 m a.s.l., climax alpine grassland and snowbed), the former representing the vegetation that could settle in the present alpine belt due to prolonged climate warming.

Soils from the different altitudes exhibited significant differences in most of their physicochemical properties (Table 3); pH and water content were significantly higher in subalpine soils than alpine soils. Subalpine soils had 0.4 higher pH (5.3 vs 5.7) and near 12% higher water content compared to alpine soils (Table 3). Subalpine soils also had significantly higher contents of C, ammonium and H than alpine soils, and a slightly, yet significant, higher PO_4 content (Table 3). Bacterial abundance was higher than fungal abundance in both alpine and subalpine soils but differences between altitudes were not significant (Table 3).

Table 3. Soil properties, activities of extracellular enzymes and microbial biomass in alpine and subalpine soils

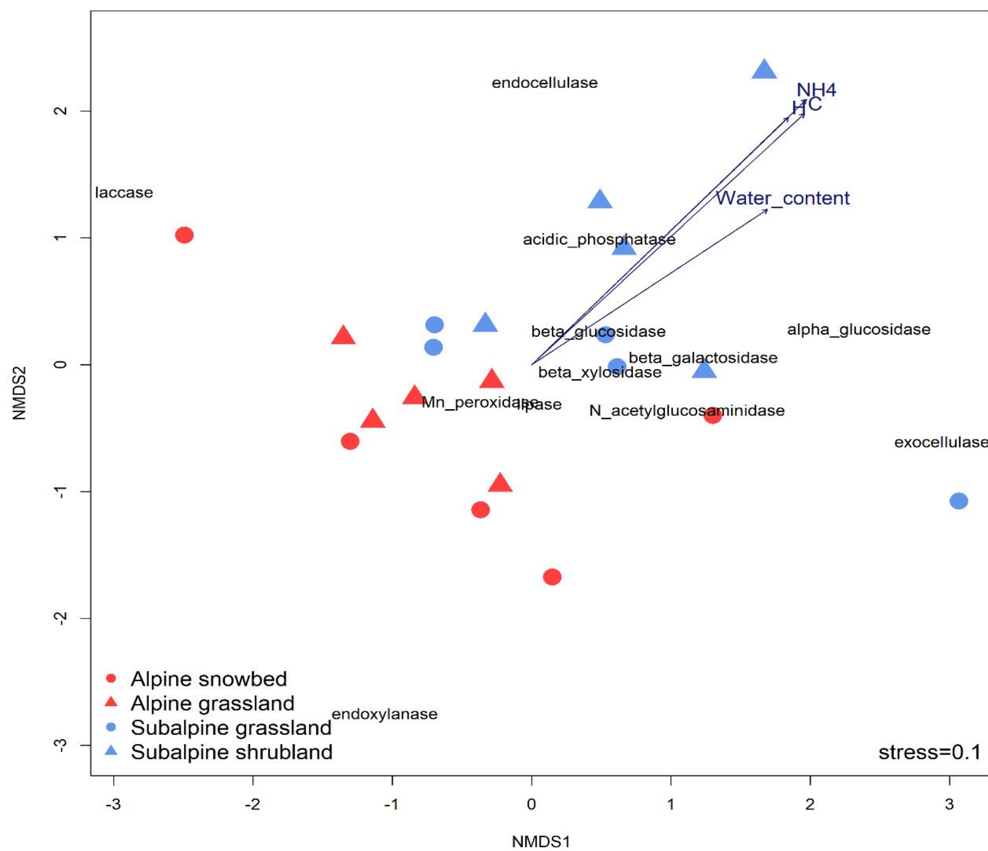
	Alpine soils (n=10)	Subalpine soils (n=10)	*p*
Water content (%)	40.2 ± 4.4	53.4 ± 9.8	**
pH	5.3 ± 0.4	5.7 ± 0.3	**
C (%)	8.3 ± 1.5	16.9 ± 4.2	***
N (%)	0.4 ± 0.4	0.6 ± 0.3	n.s
H (%)	1.5 ± 0.6	2.6 ± 0.6	***
P-PO_4 (ppm)	3.4 ± 2.3	5.2 ± 3.4	*
N-NH_4 (ppm)	4.4 ± 1.4	28.8 ± 7.3	***
β- glucosidase	6.3 ± 2.8	13.4 ± 4.4	***
Acidic phosphatase	50.3 ± 24.9	159.9 ± 73.5	***
β-xylosidase	2.5 ± 1.6	3.7 ± 1.3	n.s.
N-acetylglucosaminidase	0.7 ± 0.6	1.0 ± 0.7	n.s.
Exocellulase	0.2 ± 0.3	0.4 ± 0.4	n.s.
β-galactosidase	0.9 ± 0.7	2.1 ± 1.1	**
α-glucosidase	0.2 ± 0.2	1.0 ± 0.7	**
Lipase	198.7 ± 84.8	228.4 ± 93.4	n.s.
Laccase	3.5 ± 5.5	2.5 ± 2.6	n.s.
Mn-peroxidase	2.0 ± 0.8	2.0 ± 0.5	n.s.
Endocellulase	1.2 ± 1.9	2.9 ± 3.9	n.s.
Endoxylanase	0.8 ± 0.7	0.1 ± 0.3	*
Bacteria (rRNA gene copies/g dry soil)	$5.1\ 10^{12} \pm 1.2\ 10^{12}$	$5.3\ 10^{12} \pm 1.7\ 10^{12}$	n.s.

Fungi (rRNA gene copies/g dry soil)	$1.0\ 10^{10} \pm 0.4\ 10^{10}$	$1.1\ 10^{10} \pm 0.5\ 10^{10}$	n.s.

[a] The significance of the differences observed (p) is indicated as *** p<0.001, ** p<0.01, * p<0.05, n.s. non-significant

Alpine and subalpine soils had significantly different enzymatic activities (Fig. 1, Table 2S), except for the contrast between alpine snowbed and subalpine grassland (Table 2S); however, a single outlier in the alpine snowbed explains the non-significant result, since all other samples from snowbed clearly separated from subalpine samples (Fig. 1).

Fig. 1 Non-metric Multidimensional Scaling (NMDS) ordination plot of the differences (Euclidean distance) in enzyme activities composition among the sites studied. Legend: Red circles, alpine snowbed; red triangles, alpine grassland, blue circles, subalpine grassland; blue triangles, subalpine shrubland). Only the significant edaphic variables (water content, carbon (C), hydrogen (H), ammonium (NH_4)) have been fitted in the ordination. Enzymes names in the ordination are the centroids of specific enzymes, added as weighted averages.



According to soil parameters, only water content, C, $N\text{-}NH_4$ and H were significantly associated with the main patterns in enzymatic profile. These significant parameters were, in turn, strongly intercorrelated (Fig. 1S). The comparison between the microbial community enzyme profiles is reported in Figure 1. The NMDS diagram showed a gradient in enzymatic activity and soil parameters, along which samples tended to separate from subalpine shrubland (upper right), to subalpine grassland, alpine grassland, and finally alpine snowbed (lower left) (Fig. 1). Although the subalpine shrubland was not significantly different from subalpine grassland (Table 2S), it tended to be separated from this latter site in the ordination and was correlated with higher values of water content, C, $N\text{-}NH_4$ and H.

Subalpine sites tended to have higher activity of the majority of enzymes, as indicated by the placement of subalpine samples and most of enzyme centroids in the upper right part of the ordination. By contrast, alpine sites tended to have higher activities of endoxylanase, Mn-peroxidase and lipase. Furthermore, laccase seemed to be higher in one sample of snowbed and did not align with any of the alpine samples in the ordination (Fig. 1). Similar to PERMANOVA analysis, the enzymatic activities, analysed individually, did not vary among the four different plant communities of the two sites (data not shown), but many statistically significant differences were detected by comparing the grouped alpine and subalpine soils (Table 3). All the analysed enzymes were detected in the soil samples, but subalpine soils showed a significantly higher activity of β-glucosidase, acidic phosphatase, β-galactosidase, α-glucosidase, and endoxylanase (Table 3).

Table 4. Soil factors showing significant correlations with enzyme activities.

Enzyme	**Significant factors affecting**
β-glucosidase	**C**, bacteria
β-xylosidase	C
N-acetylglucosaminidase	water content, N

Exocellulase	water content, C, N
β-galactosidase	C
α-glucosidase	water content, **C**, N
Acidic phosphatase	**C**, N, **bacteria**, fungi
Lipase	N, bacteria
Mn-peroxidase	- temperature, water content, **N**
Endoxylanase	-Water content. -C. - P

[a]Normal script indicates slight correlation ($p<0.05$); bold script indicates strong correlation ($p<0.01$). The minus sign (-) indicates negative correlation.

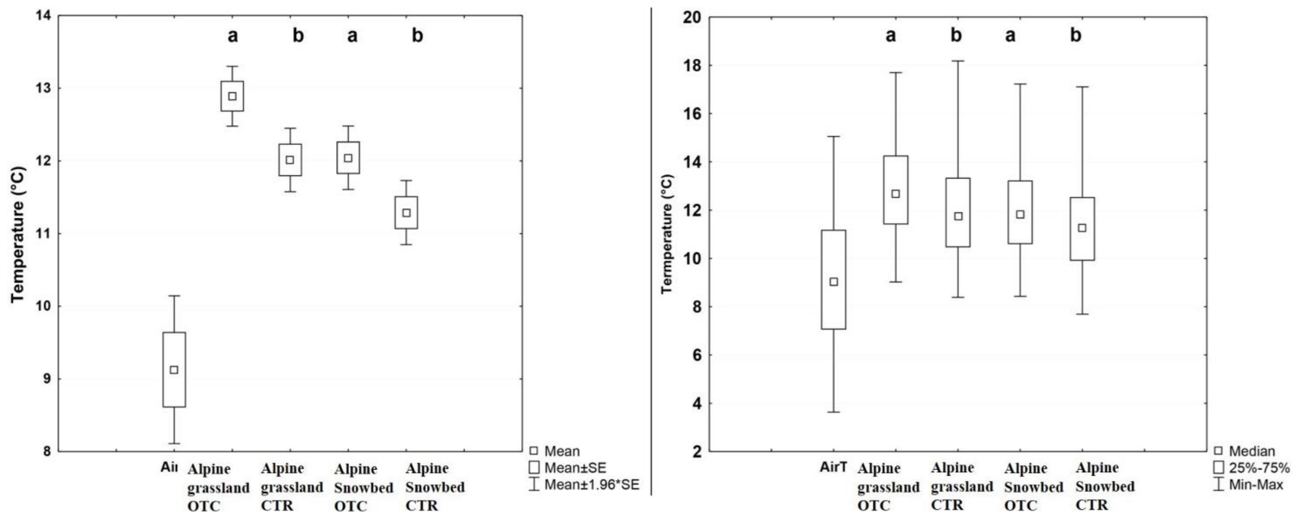
C, N and bacterial biomass were among the parameters most strongly correlated to enzyme activities (Table 4). β-glucosidase, α-glucosidase, and acidic phosphatase were strongly positively correlated with C, while Mn-peroxidase was the only enzyme to be strongly positively correlated with N content. Finally, acidic phosphatase was strongly positively correlated to bacterial biomass (Table 4).

2.3.2 Short-term warming effect on enzyme activity

To assess the short-term effects of climate change where warming is not yet accompanied by vegetation change, unmanipulated soils were compared to warmed soils in the OTCs. Soils within the OTCs did not show significant differences from the counterpart controls in all chemical parameters (Table 1S). The OTC effect increased significantly the mean soil temperature during the peak of the growing season in both elevations of the alpine belt (Fig. 2), with comparable temperature increases but different ranges, from 12 °C to 12.9 °C in the alpine grassland and from 11.3 °C to 12 °C in the snowbed (Table 1S). When considering the enzymes individually, neither snowbed nor grassland warming exerted significant effects on soil activity, neither significant differences were recorded for the edaphic parameters, except for P-PO_4 in alpine grassland. Finally, no significant

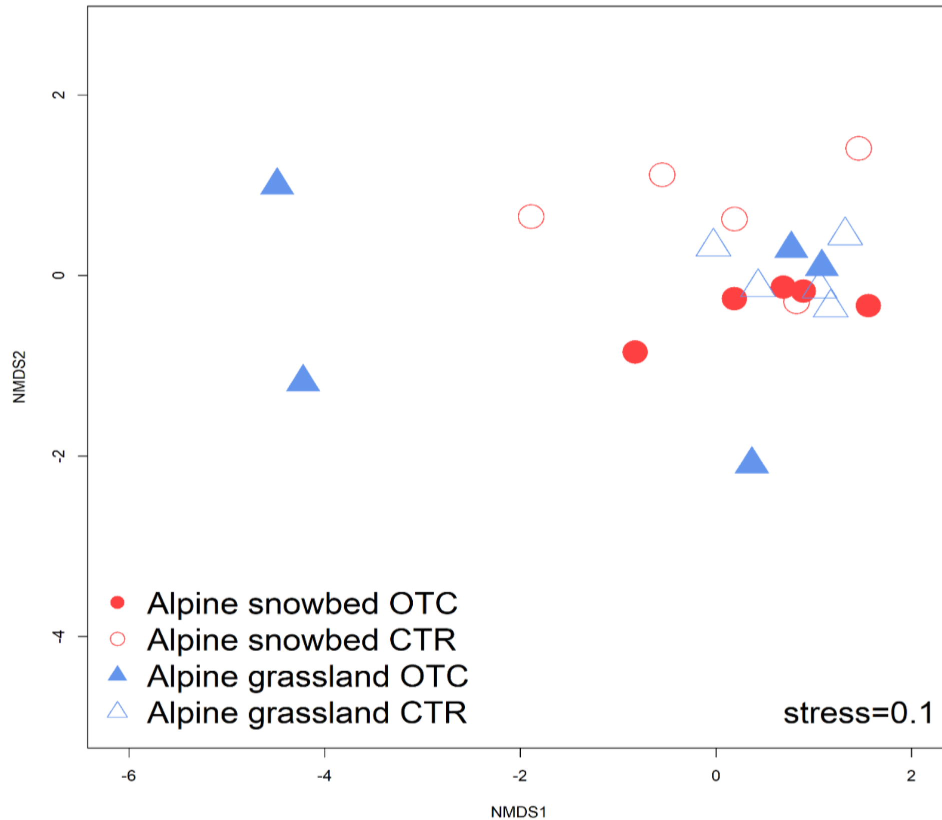
differences in microbial biomass content were recorded among the warmed and control plots (Table 1S).

Fig. 2 Air and soil temperature during the peak of the growing season (July) showing the effects of the experimental warming (OTC) respect to the non-manipulated plots (CTR) on the alpine snowbed and alpine grassland communities. Legend: A) mean ± SD; B) range, median and quartiles. Different letters indicate values significantly different from each other ($p<0.05$) by one-way analysis of variance (*ANOVA*). AirT= Air temperature.



When comparing the total enzyme patterns, the OTC treatment and the vegetation type, used as independent fixed explanatory factors, they were not significant to explain the variance between control and manipulated plots. However, the combined effects of the vegetation type and OTC treatment significantly explained the variance in enzyme composition (Table 3S). This significant interaction suggests that OTC treatment effect was linked to the vegetation type. Whereas the enzymatic composition of warmed and control plots tended to separate in the snowbed soils, they clustered together in the alpine grassland (Fig. 3).

Fig. 3 Non-metric Multidimensional Scaling (NMDS) ordination plot of the differences (Euclidean distance) in composition of enzyme activities between the treated (OTC) and control (CTR) soils at the higher altitude.
Legend: red full circles, alpine snowbed OTC; red empty circles, alpine snowbed CTR; blue full triangles, alpine grassland OTC; blue empty triangles, alpine grassland CTR.



Furthermore, the alpine snowbed OTCs clustered with alpine grassland controls (Fig. 3). A similar trend was recorded for the temperature; the mean soil temperature recorded for these two sites was not significantly different (Table 1S). None of the soil parameters significantly correlated with the enzymatic composition of the samples in the ordination (Fig. 3).

2.4 Discussion

The present work aimed to determine the effect of a warming-induced upwards vegetation shift and short-term warming impacts on soil enzymatic patterns. A number of significant differences was observed comparing data obtained for subalpine and alpine soils. Higher altitudes are generally associated with increasing environmental harshness, i.e. colder climate, lower nutrient availability, increasing acidity and lower plant biomass, which in turn influence microbial activities (Ma et al., 2004; Margesin et al., 2009). The same was recorded for our study area, where the two alpine soils had significantly lower contents of C, N-NH_4, H, P-PO_4, water content and pH (Table 3), and different

enzymatic patterns compared to subalpine soils (Fig. 1). Higher enzyme activity characterized the subalpine soils, as shown by the clustering of most enzymes and subalpine soil samples in the upper right corner in the NMDS (Fig.1), suggesting that they are positively driven mainly by higher C, ammonium, H and water contents. The higher C content of the subalpine soils may denote a bigger labile C availability for hydrolytic enzymes (Meng et al., 2020), and the higher water content may induce an increase of microbial activity. Enhanced microbial activity, CO_2 evolution and net N mineralization with water increases were reported also by Geisseler et al. (2011).

Unlike the subalpine soils, the alpine soils showed a negative correlation with soil parameters and only two oxidative enzymes of the whole dataset, namely *Mn-peroxidase* and *laccase,* overlapped on this site. *Laccase* was highest in one sample of alpine soils, but no physicochemical parameters and microbial biomasses explained this result as showed by any arrows correlated with this sample in Figure 1**.** Warming would favour the microbial communities functionality degrading old and recalcitrant C pools by oxidative enzymes, which are able to accelerate the breakdown of recalcitrant C sources (Chen et al., 2020). Their presence only in alpine soils could therefore suggest a greater sensitivity of higher sites to global warming. Furthermore, while most of the enzymes are produced from a wide heterogeneous group of microorganisms, *Mn-peroxidase* is a ligninolytic enzyme exclusively produced by a limited number of fungi, and also extracellular *laccase* is mostly of fungal origin (Baldrian, 2009). As shown by Margesin et al. (2003) fungi are characterized by lower optimum and maximum temperatures for growth and activity compared with bacteria and are thus well adapted to cold climatic conditions.

Between the two subalpine soils, the shrubland tended to separate from the grassland, suggesting a specific enzyme pattern. In addition, this site showed a stronger influence of soil parameters on enzymatic activity thanks to its stronger positive correlation with main soil drivers in the ordination. As reported by previous studies, shrubs exhibit different physiological and metabolic characteristics compared to grasses. The above-ground biomass and rates of C fixation and ecosystem respiration are highest in the shrub community, but the total ecosystem C storage is highest in grassland

community (Sørensen et al., 2018). Furthermore, as reported by Tape et al. (2006), shrubs warm the soil during winter by promoting the accumulation of the snow cover. Higher winter soil temperatures stimulate soil processes driven by microbial communities and enhance N mineralization that in turn stimulates shrub growth in high elevation and latitude systems (Hagedorn et al., 2019). Shrub encroachment influences the cycling of water and energy, increases oxygenation and nutrient content, as also shown in this study, in surface soils (Bragazza et al. 2015). According to their lower efficiency in C storage, their increase in the abundance and extent in tundra areas (Malfasi and Cannone, 2020) may increase the amount of atmospheric CO_2, implying positive feedback on climate change. Getting into the details of enzyme activities (Table 3, 4), most of them were significantly more abundant in subalpine than alpine soils. Some enzymes involved in breaking down carbohydrates and polysaccharides, as β-galactosidase, β-glucosidase and α-glucosidase, were higher in subalpine soils. Both glucosidase enzymes were strongly correlated with the C content, showing a higher activity with greater substrates availability (Sinsabaugh et al.,2008), while other studies reported a ß-glucosidase activity more sensitive to soil moisture (Zhang et al., 2011) or N availability (Wallenstein et al., 2009). Mn-peroxidase had a strong correlation with N content, in agreement with Sinsabaugh (2010) who observed a clear response to changes in oxidase activities in soil after N enrichment. Acidic phosphatase was strongly correlated with bacterial biomass and C content, slightly correlated with fungal biomass and N, and did not correlate with the P-PO_4 content. Margalef et al. (2017) reported the total N as the most important factor in predicting phosphatase activity, and the organic P, rather than available P, as the most important P fraction. According to this study, phosphatase activity reflected the total N in soil, because high N availability is required for organisms to start investing in the production of protein phosphatase (N-rich molecule), which in turn is often well correlated with microbial biomass. Finally, endoxylanase was the sole enzyme recorded with higher values in alpine than subalpine soils. This enzyme has been widely reported from psychrophilic organisms, having a lower thermostability and a higher specific activity at low temperatures (Van Petegem et al., 2003). These features may be the reason for its higher titres at the colder higher altitude

conditions. Decrease in fungal and bacterial biomass with altitude was not recorded (Table 3), although many previous studies observed a decrease in microbial biomass and activities with altitude and colder, higher climatic conditions (Ma et al., 2004; Margesin et al., 2009; Xu et al., 2014; Hofmann et al., 2016). Lower microbial abundances and enzyme activities were recorded in subalpine sites compared to submontane sites in South Tyrol in the Italian Alps, as well as in soils of the alpine belt compared with those of the snowbed belt in the Austrian Central Alps (Margesin et al., 2014; Hofmann et al., 2016).

Experimental warming treatments represent a useful tool aiding the understanding of the possible short-term consequences of global warming. Recent studies showed that artificial warming in various contexts significantly altered the microbial community functions, increased the activity of enzymes degrading recalcitrant C, changing the activity of enzymes involved in labile C degradation and N cycling, and accelerating P and S processes (Xue et al., 2016). Besides this, other studies reported no changes in soil enzyme activities (Bell et al., 2010), no effects on microbial biomass (Biasi et al., 2008), or on soil parameters (Wang et al., 2014, Li et al., 2011). Rinnan et al. (2007) did not see any effect of warming on fungal or bacterial biomass during 15 years of a long-term warming experiment. These contradictory results could be associated with variations in temperature, duration of warming and different altitudinal contexts as well as the diversity of ecosystems, therefore further researches are needed to draw general conclusions. Despite that, experimental warming devices (OTCs) have since long been considered suitable for studying the responses of the ecosystems to artificial warming (Aronson and McNulty, 2009). In this study, OTCs installed in the alpine belt induced a significant increase of soil temperature in both plant communities studied (Fig. 2), yet after five-years of artificial warming we did not observe relevant changes in soil parameters, microbial biomass or the activity of each individual enzyme in the alpine belt (Table 1S). The NMDS ordination, however, showed a clear separation of the overall enzyme activity patterns in the OTCs and controls in the snowbed, while the same shift was not equally detectable for grassland site (Fig. 3). In fact, due to a long-lasting snow cover and low soil temperature during the growing season, alpine snowbed habitats are possibly more

affected than alpine grassland communities by warming, while the latter seem to be more stable and, up to date, not affected by warmer conditions. The warming, increasing soil temperature and changing snow cover, is an important factor controlling soil enzyme production; changes of enzyme patterns induced by significant temperature increases, with strongest effect in colder and more vulnerable regions, have been reported in previous studies (Tscherko et al., 2001; Meng et al. 2020; Matteodo et al., 2016).

A recent research collecting data from 78 papers (Meng et al., 2020), has revealed that experimental warming stimulated oxidative enzymes, but had various effects on hydrolytic enzymes. This shift in enzyme activity degrading recalcitrant C sources may thus increase microbial accessibility of litter and SOM, leading to accelerated soil C loss with prolonged warming (Chen et al., 2020). Our study showed a shift in total enzyme pattern but in our case, the change of the ratio of hydrolytic and oxidative enzymes was not observed.

The ongoing studies of warming effects indicate that one or two decades of warming are necessary until pronounced shifts of soil parameters, enzyme activity and biomass become observable (Rinnan et al., 2007; Timling and Taylor, 2012; Domínguez et al., 2017). Unfortunately, the warming effect is extremely variable depending on its context, including temperature, moisture, plant community composition and other factors (Cowles et al., 2018). Overall, five years of experimental warming showed to slightly affect enzyme patterns of the microbial communities of snowbed site. With this in mind, even the slightest changes observed for this site represent an alarm bell and would require monitoring.

2.5 Conclusions

Climate change in the alpine regions will likely have short-term effects on ecosystems by modifying above- and belowground linkages, as well as long-term impacts with the expected migration of subalpine vegetation upwards. The migration may increase rates of microbial enzyme activity, increasing turnover of the labile and readily accessible soil organic matter but also may increase the

plant root activity through the rhizodeposition, which promotes the decomposition of recalcitrant biopolymers. Furthermore, the alpine soils undergoing the shrubland expansion may accelerate the decomposition and decrease total ecosystem C storage; thereby increasing the efflux of CO_2 to the atmosphere and having a positive feedback to warming. On the other hand, using the manipulative warming experiments, we demonstrated that warming, estimated around 2°C, is able to change the enzyme activity starting from the more vulnerable, colder and higher altitude regions. Although five-years of experimental warming do not allow us to make bold conclusions, it appears that vegetation shift induced by prolonged warming might be substantially more important in size compared to short-term effects in the present vegetation context.

Acknowledgments

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Supplementary material

Fig. 1S Cross-correlations between soil parameters. Legend: the significance of the correlations is indicated as *** p<0.001, ** p<0.01, * p<0.05.

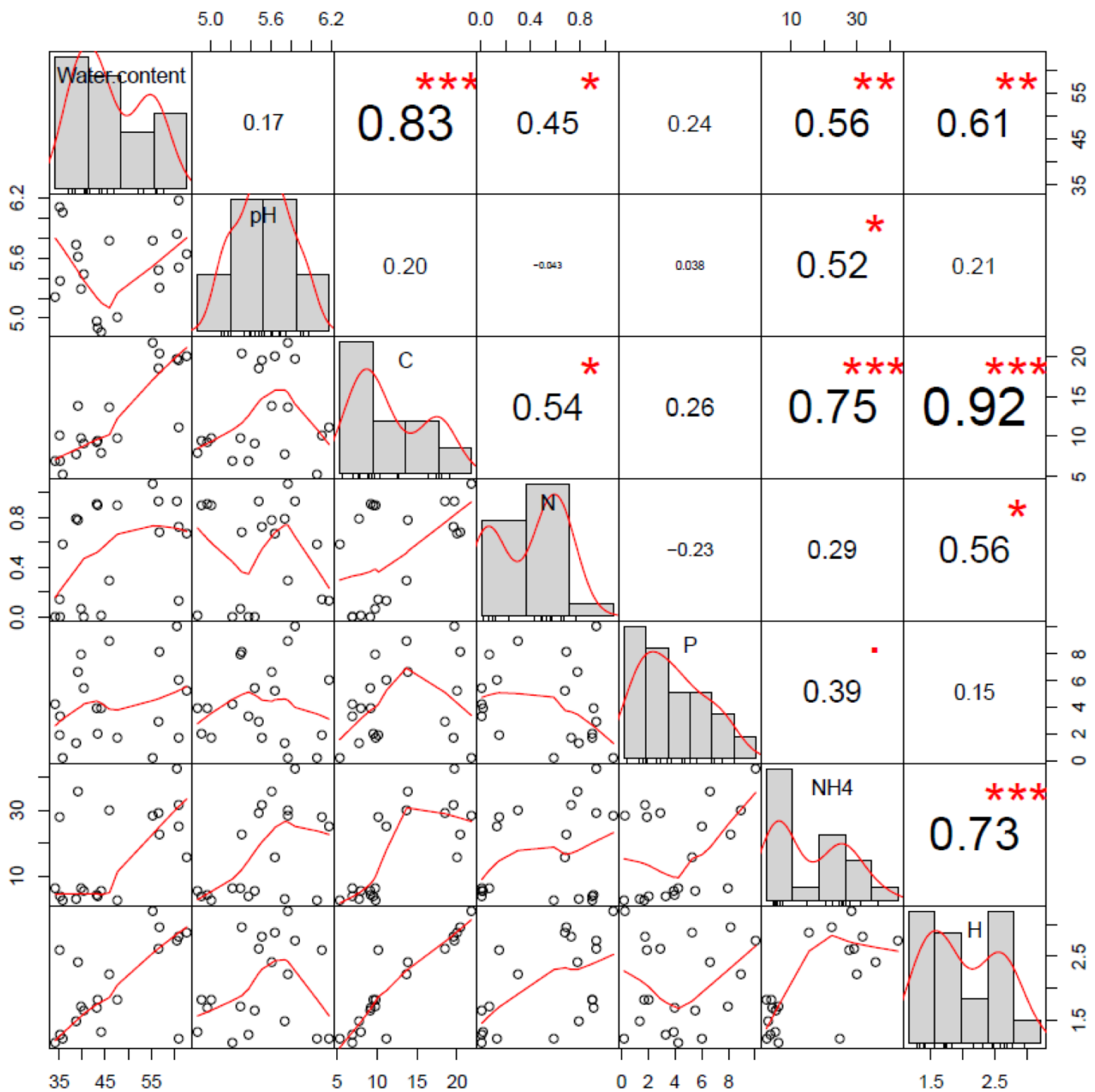

Table 1S. Comparison among alpine soils (snowbed and grassland) and edaphic, enzymatic and microbial parameters between warmed and control plots

	Alpine snowbed		Alpine grassland	
	warmed	control	warmed	control
Mean soil temperature (°C)	12.0 ± 2.1 a	11.3 ± 2.2 b	12.9 ± 2.0 c	12.0 ± 2.1 a
Water content (%)	43.3 ± 1.0 a	41.7 ± 4.6 a	38.7 ± 6.1 a	38.7 ± 4.1 a
pH	5.4 ± 0.3 a	5.3 ± 0.5 a	5.3 ± 0.4 a	5.2 ± 0.2 a
C (%)	9.0 ± 0.6 a	8.4 ± 1.9 a	7.1 ± 2.6 a	8.2 ± 1.3 a
N (%)	0.7 ± 0.3 a	0.8 ± 0.1 a	0.02 ± 0.02 b	0.02 ± 0.03 b
H (%)	1.7 ± 0.08 a	1.6 ± 0.3 a	1.2 ± 0.4 a	1.4 ± 0.3 a
P-PO_4 (ppm)	1.0 ± 0.4 a	1.9 ± 1.4 a	6.3 ± 4.2 b	5.0 ± 1.8 ab
N-NH_4 (ppm)	5.1 ± 1.9 a	3.4 ± 0.8 a	6.3 ± 2.8 a	5.5 ± 1.0 a
Bacteria (rRNA gene copies/g dry soil)	$6.0\ 10^{12} \pm 0.9\ 10^{12}$ a	$5.6\ 10^{12} \pm 0.8\ 10^{12}$ ab	$3.9\ 10^{12} \pm 0.7\ 10^{12}$ b	$4.5\ 10^{12} \pm 1.4\ 10^{12}$ ab
Fungi (rRNA gene copies/g dry soil)	$6.1\ 10^{9} \pm 2.0\ 10^{9}$ a	$8.3\ 10^{9} \pm 2.5\ 10^{9}$ a	$13.0\ 10^{9} \pm 8.0\ 10^{9}$ a	$12.0\ 10^{9} \pm 5.3\ 10^{9}$ a
β- glucosidase	5.7 ± 3.6 a	6.3 ± 2.2 a	11.2 ± 7.2 a	6.3 ± 3.5 a
Acidic phosphatase	33.6 ± 8.7 a	65.4 ± 24.9 a	74.4 ± 50.3 a	35.2 ± 14.5 a
β-xylosidase	3.4 ± 1.8 a	2.6 ± 2.1 a	4.7 ± 2.5 a	2.5 ± 1.1 a
N-acetylglucosaminidase	0.6 ± 0.4 a	1.1 ± 0.7 a	0.9 ± 0.7 a	0.4 ± 0.3 a
Exocellulase	0.1 ± 0.1 a	0.3 ± 0.4 a	0.3 ± 0.5 a	0.04 ± 0.08 a
β-galactosidase	0.6 ± 0.7 a	1.0 ± 0.8 a	2.4 ± 1.6 a	0.8 ± 0.8 a
α-glucosidase	0.1 ± 0.2 a	0.3 ± 0.4 a	0.6 ± 0.9 a	0.2 ± 0.2 a
Lipase	227.4 ± 60.9 a	256.1 ± 62.4 a	243.8 ± 140.6 a	141.3 ± 63.7 a

Laccase	8.9 ± 10.9 a	4.9 ± 7.3 a	27.2 ± 37.3 a	2 ± 3.2 a
Mn-peroxidase	2.0 ± 1.7 a	2.5 ± 0.7 a	1.8 ± 0.4 a	1.5 ± 0.4 a
Endocellulase	1.3 ± 2.2 a	1.6 ± 2.3 a	1.8 ± 1.7 a	0.8 ± 1.5 a
Endoxylanase	0.1 ± 0.1 a	1.0 ± 0.8 a	0.9 ± 0.3 a	0.6 ± 0.6 a

[a]Different letters within a row indicate that the mean values are significantly different from each other ($p<0.05$) according to ANOVA followed by the Tukey HSD test.

Table 2S. Results of the pairwise PERMANOVA analysis on enzymatic activities between sites

Contrast	***F***	***R*²**	***p***
Alpine snowbed vs. Alpine grassland	1.31	0.14	n.s.
Alpine snowbed vs. Subalpine grassland	1.23	0.13	n.s.
Alpine snowbed vs. Subalpine shrubland	2.84	0.26	*
Alpine grassland vs. Subalpine grassland	2.43	0.23	*
Alpine grassland vs. Subalpine shrubland	5.44	0.4	*
Subalpine grassland vs. Subalpine shrubland	0.85	0.1	n.s.

Table 3S. ANOVA table of the PERMANOVA analysis of the OTC experiment

Source	**Df**	***F***	R^2	***p***
Treatment	1	0.97	0.05	n.s.
Vegetation type	1	0.84	0.04	n.s.
Treatment:Vegetation type	1	2.68	0.13	*
Residual	16			

[a]The significance of the differences observed (*p*) is indicated as *** $p<0.001$, ** $p<0.01$, * $p<0.05$, n.s. non-significant. Treatment= OTC vs. control; Vegetation type= alpine grassland vs. alpine snowbed; Df= degrees of freedom; F= ratio of variance between groups to variance within groups; R^2= percentage variation explained.

Chapter 3

Composition and functioning of the soil microbiome in the highest altitudes of the Italian Alps and potential effects of climate change

Running title: Climate change effects on alpine microbiota

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Abstract:

The European Alps experienced a strong climate warming, with an increase of the mean annual air temperature exceeding the global average one of 0.6 ± 0.2°C. This study analyzed the soil microbiome composition and functioning at different altitudes and among different vegetation types at the Stelvio Pass aiming to characterize the composition and functional potential of the microbiome of high elevation soils and its gene expression in the time of highest vegetation productivity and to explore the potential short-term (using open top chambers devices) and long-term effects (space-for-time substitutions) of increasing temperature on alpine soil microbiome. Using a combination of metagenomic and metatranscriptomic approaches, we found that the functional potential of the soil microbiome and its expression largely differs among vegetation types that occur at colder and warmer climates. Prokaryotic and eukaryotic alpha diversity increased along the altitudinal gradient as a consequence of greater environmental harshness, characteristic from higher altitudes.

Shrubland showed the highest share of fungi that was correlated with higher amount of carbohydrate-active enzymes, specific for degrading fungal biomass and recalcitrant plant biopolymers, such as pectin, cellulose, glucuroxylans. According to our results, the subalpine vegetation shift may determine a possible loss of species connected to the alpine soils, the shrub encroachment may accelerate the more recalcitrant C decomposition and decrease total ecosystem C storage, increasing in turn the efflux of CO_2 to the atmosphere with a positive feedback to warming. On the other hand, the short-term warming effects, which increased the mean soil temperature during the peak of the growing season, recorded no significant changes in community composition and functioning between experimental warming devices and the counterpart controls. Since five-years of experimental warming did not allow us to make bold conclusions, longer-term warming experiments should be needed to investigate the effects of the temperature increase on soil microbiome.

Keywords: Alpine soils, Bacteria, Fungi, Shrubs expansion, Warming effect, Climate change, Decomposition, Nitrogen cycling.

3.1 Introduction

Mountains cover 25% of the global land surface and comprise unique ecosystems within all the earth's climatic zones (Diaz et al., 2003; Barry, 2008). The decrease of temperature with altitude in the mountains or with latitude in the Arctic, respectively, creates in both cases a gradient of ecosystems reflecting local temperature including specific ecosystems where the very low temperature or the short length of the warm season limit vegetation development and productivity (Donhauser and Frey, 2018). Soil temperature, always higher than air temperature, is strongly influenced by the presence of snow cover, which insulates the ground from air temperature fluctuations (Zhang et al., 2018). In coming decades, a warmer climate may increase the air temperature in high altitude regions, causing the snow covered period to shorten, the growing season to lengthen and soil temperatures to increase during the winter, spring and early summer, with consecutive effects on soil ecosystem processes (Mellander et al., 2007).

Compared to the Arctic, mountains are characterized by the combined effects of temperature and precipitation that lead to the development of stratified vegetation and soils belts specific for the climatic conditions at each elevation zone, developed on a small spatial scale. Due to this habitat heterogeneity, mountains exhibit high levels of biodiversity and provide multitude of ecosystem services including carbon (C) storage, water retention and nutrient accumulation (Pauli et al., 2015). Many studies suggest that high altitude environments, comprising various habitats as soils, bare rocks, permafrost, glaciers and snow, are among the most sensitive to the climatic change occurring on a global scale (Thompson, 2000; Diaz et al., 2003; Donhauser and Frey, 2018). In this context, Alps constitute a dominant feature of the landscape in Europe, with important economic and social implications (Gobiet et al., 2014). During the last century, global surface temperature has increased of about 0.6 ± 0.2°C and the Alps have experienced a warming well above the global average (Wieser et al., 2019). Many researches have documented how climate change in the mountains extends vegetation zones and causes upward migration of plants, with deep consequences for ecosystem functioning (Bjorkman et al., 2020; Xu et al., 2020). An upward migration and areal expansion of

shrubs has been widely documented for Alps as a consequence of climate change (Cannone et al., 2007), and further increases of temperatures in upcoming decades would deeply affect these extremely vulnerable high-elevation ecosystems (Cannone and Pignatti, 2014; Malfasi and Cannone, 2020).

Growing evidences show that the terrestrial ecosystem functioning depends on complex interactions between the biotic and abiotic components. Different plant species characterized by a wide range of ecophysiological traits can exert strong effects on belowground soil organic matter (SOM) (Singh et al., 2009). SOM is of central importance for climate feedback, as it represents a key reservoir for water and nutrients and a substrate for microbial communities. Indeed, all aboveground ecosystem components are interlinked with the belowground components; interactions and feedback between them drive ecosystem processes as biomass production, decomposition, and nutrient cycling (Freschet et al. 2013; Bragazza et al., 2015). In spite of the recognized role of soil microorganisms as contributors to many crucial ecosystem functions that affect and are affected by climate change (Cavicchioli et al., 2019, Guerra et al., 2020), belowground changes, where plant roots and microbial communities closely interact, are mostly unknown. Changes in vegetation type, edaphic characteristics, nutrient availability in the soil and the local climatic conditions may directly or indirectly affect the soil microbial communities structure and functioning (Lladó et al., 2017), leading to alterations in essential ecosystem cycles in complex ways which are difficult to predict. One fundamental yet hotly debated question is whether and how the networks in ecology, particularly microbial ecology, will change under future climate change scenarios (Yuan et al., 2021). In the future context of global warming which involves an increase in temperature and levels of CO_2, the role of microbes is crucial; indeed changes in microbial ecosystems can enhance ecosystem feedback to climate through increases of SOM decomposition rates and greenhouse gas emissions into the atmosphere (Rui-Chang et al., 2014).

Climate change may have both short-term effects on ecosystems, by modifying above- and belowground linkages in current species assemblages, and longer-term impacts that include species

redistribution such as the upward shifts along elevation gradients. The “shrub encroachment” represents one of the most dramatic shifts in vegetation occurring worldwide, particularly in alpine and high-latitude regions in response to climate change (Cannone et al., 2007; Elmendorf et al., 2012), with expected consequences on the soil microbial compartment (Canini et al., 2020; Collins et al., 2020).

The advent of high-throughput analysis of nucleic acids extracted from environmental samples has provided an unprecedented opportunity to assess the microbial composition and functioning. Metagenomics offers the picture of the composition and functional potential of microbial communities, while metatranscriptomics enables to investigate the potential expression of genes at the time of the sampling and helps to understand how microbes respond to environmental conditions (Carvalhais et al., 2012; Baldrian et al., 2019; Sugitha et al., 2020). Despite its potential, metatranscriptomic approaches have never been used to characterize the environmental conditions in high altitude soils and to predict the potential changes caused by warming.

The aims of the current study were *i*) to characterize the composition and functional potential of the microbiome of alpine soils and its expression in the time of the highest vegetation productivity; *ii*) to explore the potential short-term and long-term effects of increasing temperature on alpine soil microbiome. We have selected the study area of the Stelvio Pass in the Italian Alps with a long history of botany surveys and used the comparison of soil communities in alpine and subalpine soils to assess the potential effects of the upward vegetation shift that was recorded in this area in the past (Cannone et al., 2007; Malfasi and Cannone, 2020). Such a shift is expected in the case of the future warming scenario RCP6.0 (Representative Concentration Pathway 6.0) by the end of this century (IPCC, 2013). Furthermore, we have used open top chambers (OTC) to assess the effect of a local increase of temperature that does not in a short-term affect vegetation composition.

We hypothesized that (1) both prokaryotic and eukaryotic alpha diversity decreases along the altitudinal gradient as a consequence of greater environmental harshness characterizing high altitudes, mostly inhabited by a limited number of adapted microbes. Since several key soil functions (as for

ammonia oxidizer, diazotrophic, methanotrophic, phosphorus mineralizer, etc.) are provided by specialized microbes (Zhou et al., 2020), their absence in the harsh conditions of the highest elevations causes lower diversity of metabolic functions in the alpine soils. (2) Due to the higher dependence of fungi on vegetation compared to bacteria (Urbanová et al., 2015), the lower altitudes with higher plant production will host a larger share of fungi. This will be reflected by their higher share on ecosystem processes including decomposition, where the amount of carbohydrate-active enzymes (CAZY), specific for degrading fungal biomass and recalcitrant plant biopolymers, such as cellulose and lignin, will increase (Navarro et al., 2014). (3) The effect of experimental warming using OTCs on plants leads to an increase in net primary productivity (NPP). The enhanced C inputs into soil through rhizodeposition will lead to a stimulation of microbial SOM decomposition, which in turn may enhance gross N mineralization and microbial NH_4^+ consumption. Since N is a primary limiting nutrient in high altitude ecosystems, a stronger competition for it between plants and microorganisms could occur, with consequences for the N cycling, including, for example, higher N fixation. In turn, a decrease of N availability for plants could limit ecosystem productivity and C sequestration (Hungate et al., 2003).

3.2 Materials and Methods

3.2.1 Sampling sites and design

The study area is located around Stelvio Pass, in Stelvio National Park, Italian Central Alps (46°31’ N, 10°25’ E). This area was selected due to the clear evidences of recent climate change, with areal changes of vegetation since 1953, consisting of shrub upward migration and encroachment at the expenses of the alpine grasslands and snowbeds (Cannone et al., 2007). The shrub encroachment in this area was dated back to 1860s, the end of the Little Ice Age, and tree encroachment is observable since 1960s and 1970s (Malfasi and Cannone, 2020).

Two sites at two different elevations were chosen, a subalpine (2239 m a. s. l.) and an alpine belt (2604-2624 m a. s. l.) respectively, experiencing a difference in MAAT of almost 3 °C, coherent with

the prediction of the future warming scenario (RCP6.0) for the year 2100 (IPCC, 2013). The alpine belt showed two different plant communities, an alpine grassland climax (*Caricetum curvulae*) and an alpine snowbed (*Salicetum herbaceae*). These communities are high vulnerabile to climate change impacts and at high risk of regression due to the ingression of species from neighbour community types (Cannone et al., 2007; Cannone and Pignatti, 2014). At the subalpine belt, plant communities are represented by either a subalpine grassland (*Caricetum firmae*) or a shrubland dominated by *Rhododendron ferrugineum*. While the alpine sites represent the current conditions in the higher alpine belt, subalpine sites may represent the future conditions of these alpine communities by the end of this century.

To study the short-term effects (i.e., those not involving large vegetation shifts), hexagonal open top chambers (OTCs; 2.08 m diameter) were installed at the alpine site in 2014 and operated until the sampling in 2019, to simulate warming of alpine grassland and alpine snowbed. OTCs passively increase summer ambient temperature inside them (Marion et al. 1997) to reach temperatures predicted by the best future warming scenario (RCP2.6) by 2100 (IPCC, 2013). The paired plots outside the OTCs acted as control plots, representing current conditions.

The same samples of this study have already been used for enzyme activity analysis and qPCR (D'Alò et al., 2021). Five samples for each condition (elevation, vegetation type, warming simulation devices) were collected, making n=20 for alpine belt (grassland and snowbed, inside and outside OTCs) and n=10 for subalpine belt (grassland and shrubland). All samples were collected on the same day in July 2019, during the peak season of vegetation productivity. For each sample, soil was collected with a sterilized spatula in at least three points for each condition up to 10 cm depth. After removal of plant debris and roots, soil was passed through a 5 mm sterile mesh and mixed, to have a composite sample collected into sterile Falcon tubes. Each Falcon tube was immediately frozen in liquid N_2, transported to the laboratory under frozen conditions, and stored at −80 °C until the analyses.

3.2.2 Soil physicochemical parameters

During the whole growing season, in-situ soil temperatures were recorded during the whole growing season at 2 cm depth using thermistors (Hobo pro V2 2x U23-003). Soil pH was measured by adding 20 ml of distilled water to 10 g of air-dried soil, shaking the soil and water mixture vigorously, and waiting some minutes for sitting. Water content was calculated by taking the difference between the mass of the fresh samples and that of samples oven dried at 105 °C until constant mass. All chemical analyses were carried out on the 2-mm soil fraction. Total C, N and hydrogen (H) contents were determined with an automatic element analyser (Carlo Erba). To determine dissolved nutrient concentrations, 10 g of air-dried soil was extracted in 100 ml de-ionized water, shaken for 2 h, and filtered using glass microfiber filters (Whatman GF/D). The obtained extracts were analysed colorimetrically for ammonium ($N\text{-}NH_4$) by the salicylate method, nitrate ($N\text{-}NO_3$) by the cadmium reduction method, phosphorus ($P\text{-}PO_4$) by the molybdenum blue method using a continuous flow analyser (FlowSys, Systea, Rome, Italy).

3.2.3 Nucleic Acid extraction and libraries preparation

DNA and RNA were co-extracted using the DNA Elution Accessory Kit combined with the RNeasy PowerSoil Total RNA Kit (MoBio Laboratories). Three aliquots per sample (3 × 1 g of material) were homogenized using a mortar, pestle under liquid nitrogen, and extracted, and the triplicate extracts were pooled. Total DNA, cleaned with a GeneClean Turbo Kit (MP Biomedicals), was checked for quality and length distribution on an Agilent 2100 Bioanalyser (Agilent Technologies) and used to generate the metagenomic libraries using the TruSeq Nano DNA Library Preparation Kit. The library size-distribution was re-checked on an Agilent 2100 Bioanalyser (Agilent Technologies). Total RNA was purified using the OneStep PCR Inhibitor Removal kit (ZymoResearch) and DNA was removed using DNA 3wfree DNA Removal kit (Thermo Fisher Scientific) and checked by the negative PCR results with the bacterial primers 515F and 806R (Caporaso et al., 2012). This product was checked

for quality (RNA integrity number) and length distribution on an Agilent 2100 Bioanalyser (Agilent Technologies). Approximately 1 µg of RNA was treated with an equimolar mixture of RiboZero rRNA Removal Kits Human-Mouse-Rat and Bacteria (Epicentre) to remove both prokaryotic and eukaryotic rRNA. rRNA removal was checked on an Agilent 2100 Bioanalyser as described by Žifčáková et al. (2016). Reverse transcription was performed with SuperScript III (Thermo Fisher Scientific). The TruSeq Stranded Total RNA kit was used to generate the metatranscriptomic libraries with a final 14 cycles of amplification by FailSafe PCR Enzyme (Lucigen) and the library size-distribution was re-checked on an Agilent 2100 Bioanalyser (Agilent Technologies).

3.2.4 Quantification of bacterial and fungal gene copy numbers

For all samples, the quantitative PCR (Applied Biosystems StepOnePlus cycler) on bacterial and fungal rDNA copies was performed as described in D'Alò et al. (2021). The 1108f and 1132r primers were used for bacteria (Wilmotte et al., 1993; Amann et al., 1995) and the FR1/FF390 primers for fungi (Prévost-Bouré et al., 2011) as described previously (Žifčáková et al., 2016). Each 20 µl reaction mixture contained 10 µl SYBR Green Master Mix (Applied Biosystems), 0.9 µl BSA (10 mg/ml), 1.35 µl of each primer, 1.5 µl of template, and 6.1 µl of water. The qPCR cycling protocol was the same for fungal and bacterial DNA quantification, as follows: 56 °C for 2 min; 95 °C for 10 min; 95 °C for 15 s and 60 °C for 1 min (40 cycles). *Streptomyces lincolnensis* DNS 40335 and *Hypholoma fasciculare* CCBAS281 genomic DNA were used as standards. Fungal and bacterial abundances were expressed as number of copies of rDNA genes per gram of dry soil.

3.2.5 Sequencing and bioinformatics analysis

Samples of the metagenome (MG) and metatranscriptome (MT) were pooled in equimolar volumes and sequenced on an Illumina NovaSeq6000 SP (DS-150) with a 2 x 150 bp in an external laboratory (SEQme, Czech Republic). MG assembly and annotation were performed as described previously

(Žifčáková et al., 2017). Briefly, Trimmomatic 0.36 (Bolger et al., 2014) and FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) were used to remove adaptor contamination, trim low-quality ends of reads and omit reads with overall low quality (<30), sequences shorter than 50 bp were omitted. Combined assembly of all samples was performed using MEGAHIT 1.1.3 (Li et al., 2015). Metatranscriptome (MT) assembly and annotation were performed as described previously (Žifčáková et al., 2016). Trimmomatic 0.36 (Bolger et al., 2014) and FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) were used to remove adaptor contamination, trim low-quality ends of reads and omit reads with overall low quality (<30), sequences shorter than 50 bp were omitted. mRNA reads were filtered from the files using the bbduk.sh 38.26 program in BBTools (https://sourceforge.net/projects/bbmap/). Combined assembly was performed using MEGAHIT 1.1.3 (Li et al., 2015). Gene calling was performed using MG-RAST (Meyer et al., 2008) where the assemblies of MG and MT are deposited (mgm4901504.3 and mgm4901505.3, respectively). Taxonomic identification was performed in MG-RAST as well as using BLAST against all published fungal genomes available in January 2020. Of these two, taxonomic identification with a higher bit-score was used as the best hit. Functions of predicted genes were annotated with the hmmsearch function in HMMER 3.2.1 (Eddy, 2011) using the FOAM database as a source of HMMs for relevant genes (Prestat et al., 2014).

Genes encoding the carbohydrate-active enzymes (CAZymes) were annotated using the dbCAN HMM database V6. (Huang et al., 2018). CAZymes were grouped based on their participation in the utilization of distinct C sources based on their classification to known CAZyme families (Table 1S) (López-Mondéjar et al., 2020). Based on KEGG level 3 classification, genes involved in nitrogen cycling were assigned to individual processes and genes encoding transporters were grouped according to their targeting source (Table 2S, 3S). All other dominant KEGG pathways were based on the annotation at KEGG level 2.

The contribution of various organisms to total metagenome and metatranscriptome was based on the relative abundances of all reads and reads of genes encoding ribosomal proteins. Since ribosomes are

produced during cell division (Lladó et al., 2019), these values should reflect the growth of the organisms.

3.2.6 Statistical analyses

All analyses were performed using R software v. 4.0.3 (R Core Team, 2020). MG and MT total genes matrices were Hellinger-transformed (i.e. standardization to relative abundances followed by square root transformation) (Legendre and Gallagher, 2001) prior to the analysis. Then, the transformed MG and MT tables of all samples were submitted to two-dimensional non-metric multidimensional scaling (NMDS) ordination based on Bray Curtis dissimilarity, using the function metaMDS() from *vegan* package v. 2.5-6 (Oksanen et al., 2019). Homogeneity of multivariate dispersions was tested before the Adonis analysis using the betadisper() function (Anderson and Walsh, 2013) in the *vegan* package (Oksanen et al., 2019).

In the long-term observational study, the null hypothesis of no effect of each combination of altitude and vegetation types (explanatory factor with four levels: alpine grassland, alpine snowbed, subalpine grassland, subalpine shrubland) on total genes in MG and MT, was tested using PERMANOVA analysis (Anderson, 2001) through adonis2() function in *vegan* package v. 2.5-6 (Oksanen et al., 2019). Bray-Curtis dissimilarities of Hellinger-transformed MG and MT tables were used as response matrices and the combinations of altitude and vegetation type as explanatory factor. *P*-values were computed using 999 permutations. The null hypothesis of no effect of each combination of altitude and vegetation types on physicochemical properties, phyla composition, alpha diversity, C and N activities, transporters and KEGG at level 2 pathways, was tested using ANOVA followed by a *post hoc* pairwise multiple comparison procedure (*Tukey's HSD*).

Regarding short-term warming OTC experiment, to test the null hypothesis of no effect of warming on total genes in MG and MT, PERMANOVA was again used. Hellinger-transformed MG and MT matrices were used as response and the alpine vegetation type (factor with two levels: alpine grassland, alpine snowbed), OTC treatment (factor with two levels: OTC and control) and their

interaction as explanatory factors. The effect of OTC warming in above reported properties was tested using linear mixed effect models, as implemented in lme() function of *nlme* package v. 3.1-147 (Pinheiro et al., 2020). In this case, alpine vegetation type, OTC treatment and their interaction were included as fixed factors. Since the OTCs and controls were paired within plots, the blocking effect of plots was included as random intercept in the mixed models (random = ~1|plot).

3.3 Results

3.3.1 Soil physicochemical properties and microbial biomass

Soils from all four vegetation types exhibited significant differences in most of their physicochemical properties (Table 1). In brief, pH was acidic in all soils, without significant differences, except for the difference between the alpine and the subalpine grassland. In general, water content, total C, total H, N-NH_4, potassium (K) levels were significantly lower at higher altitude, without differences between soils of the same vegetation belt. While bacterial biomass did not differ among vegetation types, fungal biomass was significantly lower in alpine snowbed compared to alpine grassland and subalpine shrubland. The OTC treatment increased significantly the mean soil temperature during the peak of the growing season in both alpine vegetation belts (Table 1), with comparable temperature increases but different ranges: from 12 to 12.9 °C in the grassland and from 11.3 to 12 °C in the snowbed. However, soils within the OTCs did not show significant differences from the counterpart controls in physicochemical parameters and microbial biomass, except for C/N which was higher in alpine snowbed OTC compared to the counterpart control.

Table 1. Sampling site characteristics. The values represent means and standard errors, and letters are reported only for significant differences in one-way ANOVA post-hoc Tukey's HSD test ($p<0.05$) for vegetation types and in mixed effect models ($p<0.05$) for OTC experiment.

	Comparison among vegetation types				Comparison between treatments and controls			
Parameters	**Alpine snowbed**	**Alpine grassland**	**Subalpine grassland**	**Subalpine shrubland**	**Alpine snowbed OTC**	**Alpine snowbed CTR**	**Alpine grassland OTC**	**Alpine grassland CTR**
Phytosociological associations	*Salicetum herbaceae*	*Caricetum curvulae*	*Caricetum firmae*	*Rhododendron ferrugineum*	*Salicetum herbaceae*	*Salicetum herbaceae*	*Caricetum curvulae*	*Caricetum curvulae*
Altitude (m asl)	2624	2604	2239	2239	2624	2624	2604	2604
Coordinate	46.53083°N; 10.44136°E	46.53157°N; 10.44066°E	46.522303°N; 10.408601°E	46.522303°N; 10.408601°E	46.53083°N; 10.44136°E	46.53083°N; 10.44136°E	46.53157°N; 10.44066°E	46.53157°N; 10.44066°E
Mean soil temperature (°C)	-	-	-	-	12.0 ± 2.1 **a**	11.3 ± 2.2 **b**	12.9 ± 2.0 **a**	12.0 ± 2.1 **b**
Water content (%)	42.5 ± 3.3 **bc**	39.1 ± 5.2 **c**	49.4 ± 11.4 **ab**	56.6 ± 6.5 **a**	43.3 ± 1.0	41.7 ± 4.6	39.5 ± 5.8	38.7 ± 5.2
pH	5.4 ± 0.4 **ab**	5.2 ± 0.3 **b**	5.8 ± 0.2 **a**	5.6 ± 0.2 **ab**	5.4 ± 0.3	5.3 ± 0.5	5.3 ± 0.4	5.1 ± 0.3
C (%)	8.5 ± 1.3 **b**	7.5 ± 2.0 **b**	17.0 ± 4.4 **a**	16.7 ± 4.0 **a**	8.8 ± 0.5	8.2 ± 1.7	7.7 ± 2.7	7.3 ± 1.3
N (%)	0.5 ± 0.1 **bc**	0.5 ± 0.1 **c**	1.1 ± 0.2 **a**	0.7 ± 0.1 **b**	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1
H (%)	1.6 ± 0.2 **b**	1.4 ± 0.3 **b**	2.7 ± 0.4 **a**	2.4 ± 0.7 **a**	1.6 ± 0.1	1.6 ± 0.2	1.4 ± 0.4	1.3 ± 0.2
$P\text{-}PO_4$ (ppm)	1.5 ± 1.1 **b**	5.5 ± 3.1 **ab**	4.4 ± 4.0 **ab**	8.5 ± 6.3 **a**	1.0 ± 0.4	1.9 ± 1.4	5.7 ± 3.1	5.3 ± 3.5
$N\text{-}NH_4$ (ppm)	4.2 ± 1.7 **c**	6.1 ± 2.1 **c**	32.7 ± 6.3 **a**	24.2 ± 6.7 **b**	5.1 ± 1.9	3.4 ± 0.8	6.3 ± 2.2	5.8 ± 2.2
$N\text{-}NO_3$ (ppm)	1.5 ± 0.7	1.2 ± 1.2	2.3 ± 1.1	1.4 ± 0.9	1.6 ± 0.9	1.4 ± 0.5	1.2 ± 1.6	1.2 ± 0.9
K (ppm)	8.9 ± 2.6 **b**	24.2 ± 11.6 **b**	60.8 ± 45.5 **a**	66.9 ± 34.0 **a**	10.2 ± 2.4	7.5 ± 2.3	29.6 ± 14.3	18.9 ± 5.0
C/N	18.4 ± 2.6 **c**	14.6± 1.8 **b**	15.5 ± 1.3 **bc**	23.7 ± 1.3 **a**	16.5 ± 1.3 **b**	20.3 ± 2.3 **a**	14.7 ± 2.4	14.5 ± 1.1
C/P	9.5 ± 7.5	1.6 ± 0.6	20.2 ± 36.3	3.3 ± 2.8	10.0 ± 4.6	9.0 ± 10.2	1.5 ± 0.3	1.8 ± 0.8
N/P	0.5 ± 0.4	0.1 ± 0.0	1.3 ± 2.3	0.1 ± 0.1	0.6 ± 0.3	0.4 ± 0.4	0.1 ± 0.03	0.1 ± 0.1
Bacteria (rRNA gene copies/g dry soil)	5.8 10^{12} ± 8.3 10^{11} **a**	4.2 10^{12} ± 1.1 10^{12} **b**	4.7 10^{12} ± 7.4 10^{11} **b**	5.8 10^{12} ± 2.3 10^{12} **a**	6 10^{12} ± 8.8 10^{11}	5.6 10^{12} ± 8.4 10^{11}	3.9 10^{12} ± 6.6 10^{11}	4.5 10^{12} ± 1.4 10^{12}
Fungi (rRNA gene copies/g dry soil)	7.2 10^9 ± 2.4 10^9 **b**	12.8 10^9 ± 2.5 10^9 **a**	8.6 10^9 ± 2.1 10^9 **ab**	15 10^9 ± 4.0 10^9 **a**	6.1 10^9 ± 2.0 10^9	8.4 10^9 ± 2.5 10^9	13 10^9 ± 8.0 10^9	12 10^9 ± 5.3 10^9

3.3.2 Functional potential of the soil microbiome

Bacteria represented the most dominant domain with relative abundances of assigned metagenome reads >85% in all soil types, followed by Fungi with a share around 12%. Archaea and nonfungal Eukaryota showed very low abundances in all soils, with values < 1%; the highest proportion of Archaea was observed in alpine snowbed (Table 4S).

Proteobacteria was the most dominant phylum, with abundances >43.5% in all soil types. The highest abundance was detected in subalpine shrubland, significantly different from alpine belt soils. Actinobacteria and Acidobacteria were among the more abundant bacterial phyla in all habitats, while Ascomycota was the dominant fungal phylum (Figure 1A, Table 4S).

Higher share of Basidiomycota and Chitridiomycota reads was observed in the alpine soils while Planctomycetes and expecially Verrucomicrobia were high in subalpine soils. Snowbed was characterized by significant higher abundances of Mucoromycota, Cyanobacteria and Firmicutes while shrubland by lower values of Mucoromycota, Chloroflexi and Acidobacteria. Finally, Ascomycota reported the significantly lowest abundance in subalpine grassland. The taxon richness was calculated as the number of genera giving best hits to predicted genes in each habitat; subalpine soils showed significantly lower values of this diversity estimate compared to alpine soils (Figure 1B).

Figure 1: Composition and diversity of microbial communities in alpine and subalpine soils. A) Relative abundance of reads of microbial phyla in the soil metagenomes. B) Taxonomic richness of soil communities expressed as the number of microbial species that had best hits to genes present in each habitat. The values represent means and standard errors for vegetation types. Letters indicate significant differences in one-way ANOVA followed by post-hoc Tukey's HSD test (p<0.05).

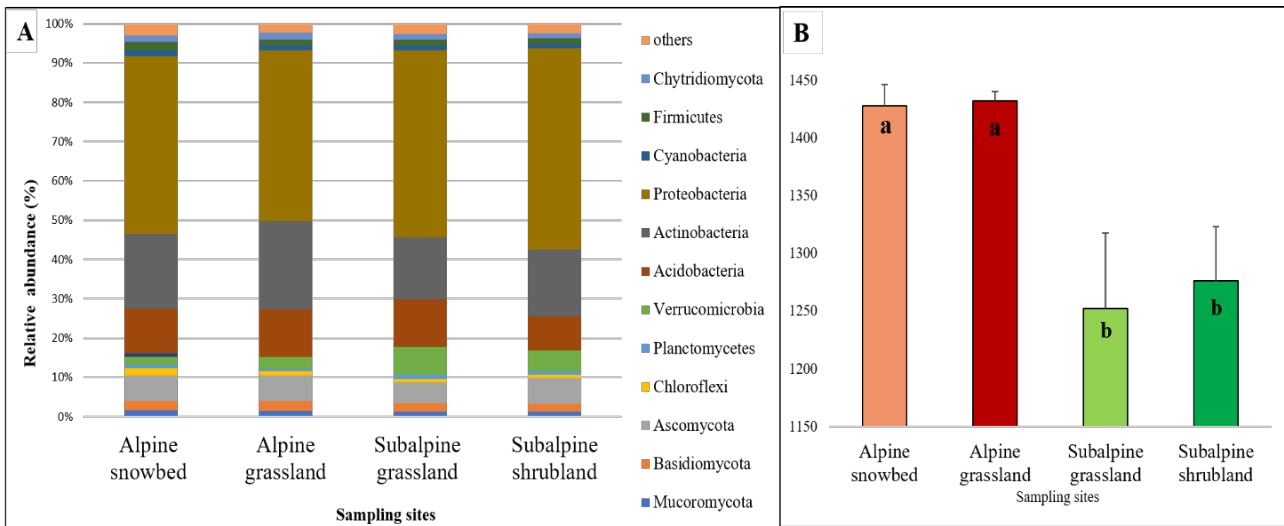
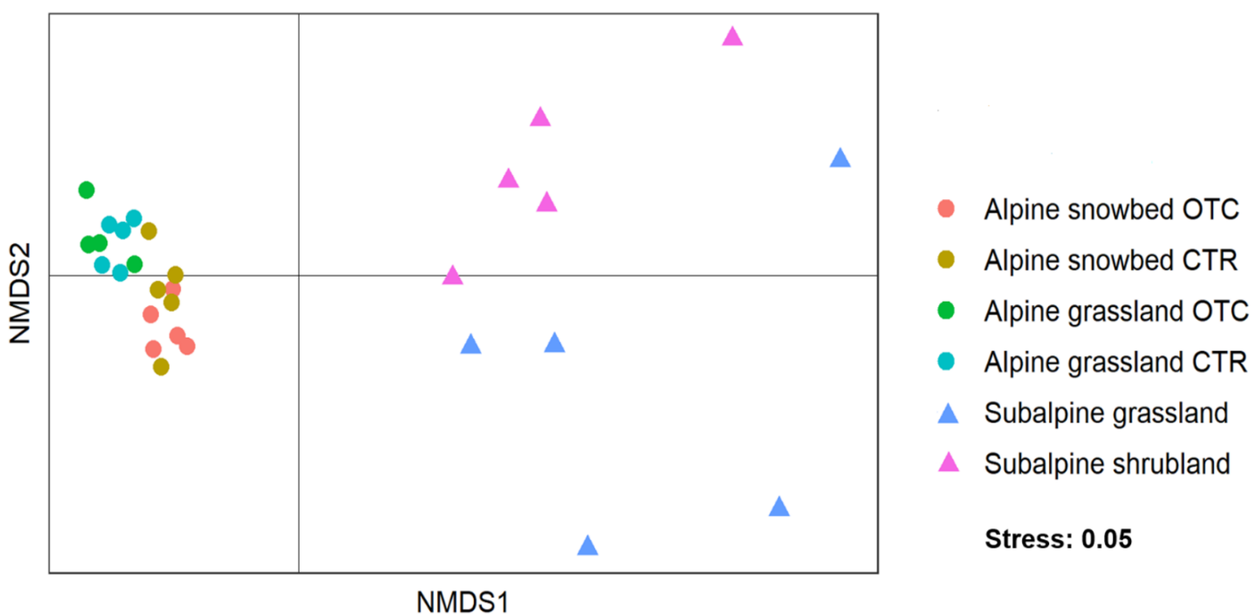


Figure 2: Non-metric multidimensional scaling of gene pools of the alpine and subalpine soil metagenomes.



Composition of gene pools of all habitats in NMDS showed a distinction of alpine and subalpine soils as well as differences among vegetation types within altitudes; the gene pools of the subalpine soils were much more heterogeneous (BETADISPER, $F = 34.6$, $p = 0.001$) (Figure 2). Accordingly, the effect of combined altitude and vegetation types was highly significant (PERMANOVA, $R^2 = 0.467$, $p < 0.001$). On the other hand, the effect of OTC treatment was not significant as main effect (PERMANOVA, p = 0.304) nor as interacting with alpine vegetation type (PERMANOVA, p = 0.214).

CAZymes involved in the degradation of α-glucans were the most common in all habitats (31-37% of all CAZYmes), yet significantly higher in both subalpine soils. CAZymes targeting cellulose, cello/xylobiose and pectin were more abundant in alpine soils, while those targeting galactans/arabinogalactans and β-glucans were more abundant in subalpine and alpine grasslands, respectively. Subalpine shrubland showed a high abundance of genes for lignin degradation (Figure 3).

Figure 3: Composition of the pools of genes encoding for carbohydrate-active enzymes (CAZymes) and N-cycling enzymes in alpine and subalpine soil metagenomes. The data represent relative share of each group in percent of all CAZymes and N-cycling genes, respectively. The values represent means for vegetation types. Letters indicate significant differences in one-way ANOVA followed by post-hoc Tukey's HSD test ($p<0.05$).

	Alpine snowbed		Alpine grassland		Subalpine grassland		Subalpine shrubland	
CARBON SOURCES DEGRADATION								
Alpha glucans	32.71	b	31.55	b	36.68	a	35.81	a
Glucuroxylans	19.37		19.80		19.54		20.04	
Peptidoglycan	9.09	ab	8.70	b	8.66	b	9.97	a
Cello/Xylobiose	8.43	a	8.59	a	6.56	b	6.98	b
Pectin	6.74	a	7.04	a	5.17	b	4.77	b
Lignin	5.47	b	5.81	ab	5.88	ab	6.73	a
Chitin	4.51		4.71		4.84		4.50	
Mannans	4.78	ab	4.33	ab	5.04	a	4.00	b
Glycoprotein/Glucosaminglycan	3.39		3.59		3.19		3.15	
Cellulose	2.96	a	2.73	a	1.73	b	1.80	b
Beta Glucans	1.90	b	2.32	a	1.57	bc	1.56	c
Galactans/Arabinogalactans	0.65	b	0.82	b	1.14	a	0.70	b
NITROGEN PATHWAYS								
Ammonia transversion	27.48	b	26.18	b	30.55	a	31.97	a
Low NH4 assimilitation	28.33	b	31.31	a	28.92	ab	30.66	ab
High NH4 assimilitation	18.41	a	18.10	b	16.75	ab	16.06	b
Assimilatory nitrite red.	7.57	b	9.11	a	6.64	b	6.78	b
Dissimilatory nitrate red.	4.35	a	3.89	a	2.51	b	3.04	b
Denitrification	3.70	b	2.99	c	4.54	a	3.85	bc
Assimilatory nitrate red.	2.51		2.46		3.07		2.16	
Nitrification	0.14	a	0.05	ab	0.00	b	0.05	ab
Nitrogen Fixation	0.02		0.00		0.05		0.03	
Dissimilatory nitrite red.	0.02	ab	0.01	b	0.13	a	0.08	ab

Among nitrogen cycling pathways, ammonia transversion and high/low NH_4^+ assimilations were the most abundant processes in the ecosystems, ranging from 16 to 32% of all N cycling genes in the sample metagenomes (Figure 3). Among N cycling processes, ammonia transversion and denitrification were higher in subalpine soils while dissimilatory nitrate reduction was higher in

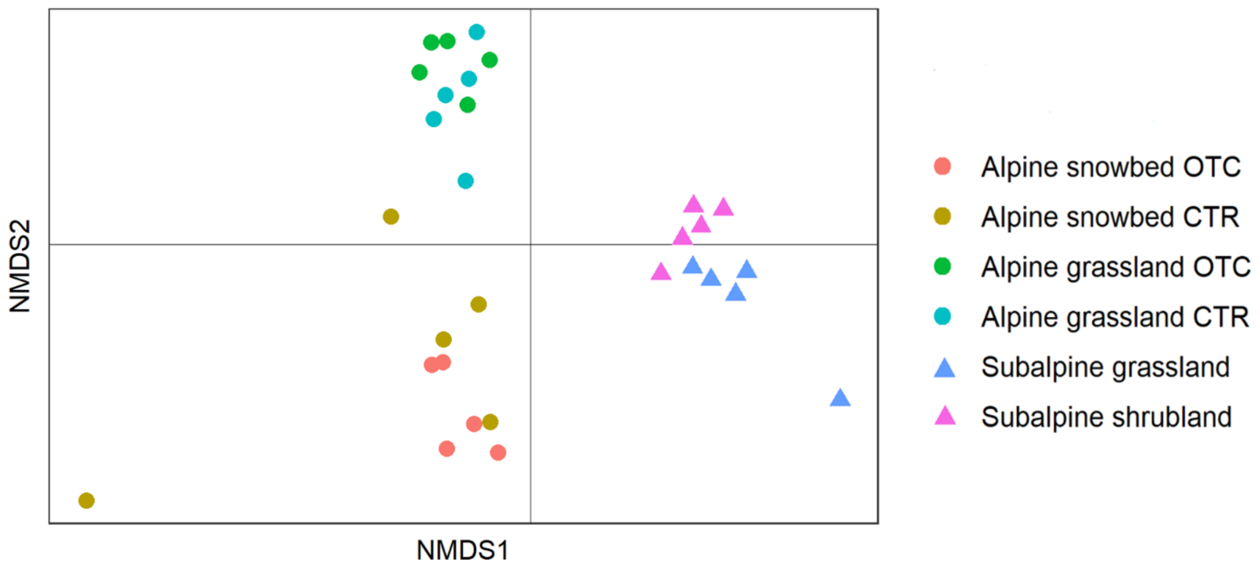
alpine soils; the highest share of genes involved in assimilatory nitrite reduction was found in alpine grassland. The genes involved in nitrification, nitrogen fixation and dissimilatory nitrite reduction represented just a minor fraction of the identified N-cycling genes (Figure 3). The importance of the N cycling in the soils of study is demonstrated by the high share of amino acid transporters that represent between 66% and 67.5% of all transporter genes (Table 5S). Considering other functions (Table 5S), subalpine soils showed significantly higher share of genes involved in cell growth and death, folding, sorting and degradation, transcription, energy and nucleotide metabolisms. Shrubland showed higher levels of environmental adaptation. The biosynthesis of other secondary metabolites, lipids and cofactors and vitamins metabolisms, and xenobiotics biodegradation and metabolism were higher in alpine soils, with higher values in alpine grassland compared to snowbed.

The OTC effect increased significantly the mean soil temperature during the peak of the growing season in both alpine vegetation belts (Table 1), with comparable temperature increases but different ranges, from 12 to 12.9 °C in the grassland and from 11.3 to 12 °C in the snowbed. When considering the microbiome composition, neither snowbed nor grassland warming exerted significant effects on the functional potential of the soil microbiome or on microbial biomass content (Table 1).

3.3.3 Microbiome functioning at peak plant productivity

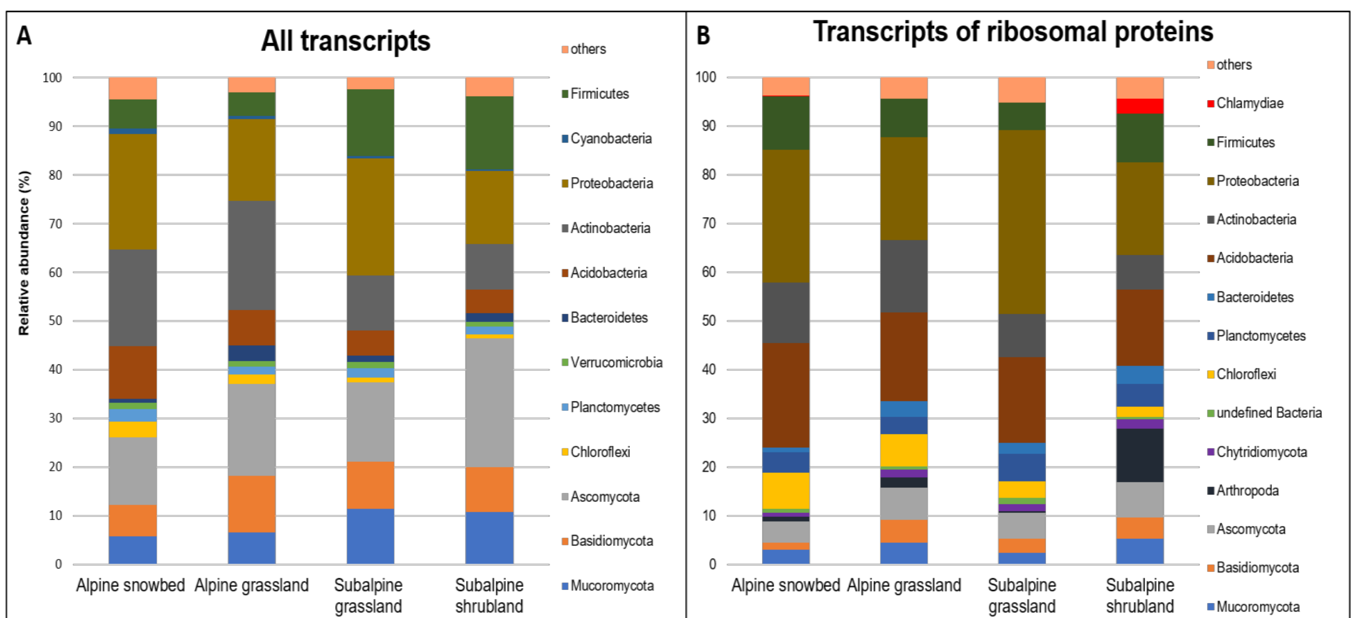
The share of bacterial and fungal activity expressed as their contribution to total transcription differed largely among habitats. Composition of gene pools of all habitats in NMDS separated samples from alpine and subalpine soils as well as those from different vegetation types within altitudes (Figure 4).

Figure 4: Nonmetric multidimensional scaling of transcript pools of the alpine and subalpine soil metatranscriptomes.



The combination of altitude and vegetation types had a strong effect on total genes in the metatranscriptome (PERMANOVA, $R^2 = 0.366$, $p < 0.001$). As with functional potential, OTC warming had no significant effect on MT composition as independent fixed explanatory factor (PERMANOVA, $p = 0.306$) nor interacting with alpine vegetation type (PERMANOVA, $p = 0.171$). While bacterial share on expression in the alpine snowbed was as high as 71%, in the subalpine shrubland it was only 51%, almost equivalent to the fungal share (48%, Figure 5A, Table 4S).

Figure 5: A) Composition and diversity of microbial communities in alpine and subalpine soils. A) Relative abundance of all transcripts of microbial phyla in the soil metatranscriptome; B) Microbial growth rates, expressed as relative abundance of all mRNA of genes encoding ribosomal proteins.



The activity of Archaea was highest in the snowbed where their share was 2 to 3-fold compared with the grasslands and the shrubland. In all habitats, most fungal transcripts classified to *Ascomycota* and most bacterial ones to *Proteobacteria* (Figure 5A; Table 4S). Both alpine soils were enriched in transcripts of *Actinobacteria*, while *Chloroflexi* and *Acidobacteria* were high in alpine snowbed. Subalpine soils showed high share of transcripts of *Firmicutes*, approximately threefold compared to alpine soils and high transcription of *Mucoromycota*. When looking at the expression of ribosomal proteins that may represent a proxy of growth, *Proteobacteria*, *Acidobacteria*, *Actinobacteria* and *Firmicutes* were the most active phyla in all habitats (Figure 5B, Table 6S). In subalpine soils, grassland differed from shrubland for a significantly higher abundance of *Proteobacteria* and lower of *Mucoromycota*; subalpine shrubland also exhibited higher share of ribosomal protein transcripts of *Arthropoda* (10.9%) and *Chlamydiae* (3.0%) than other habitats.

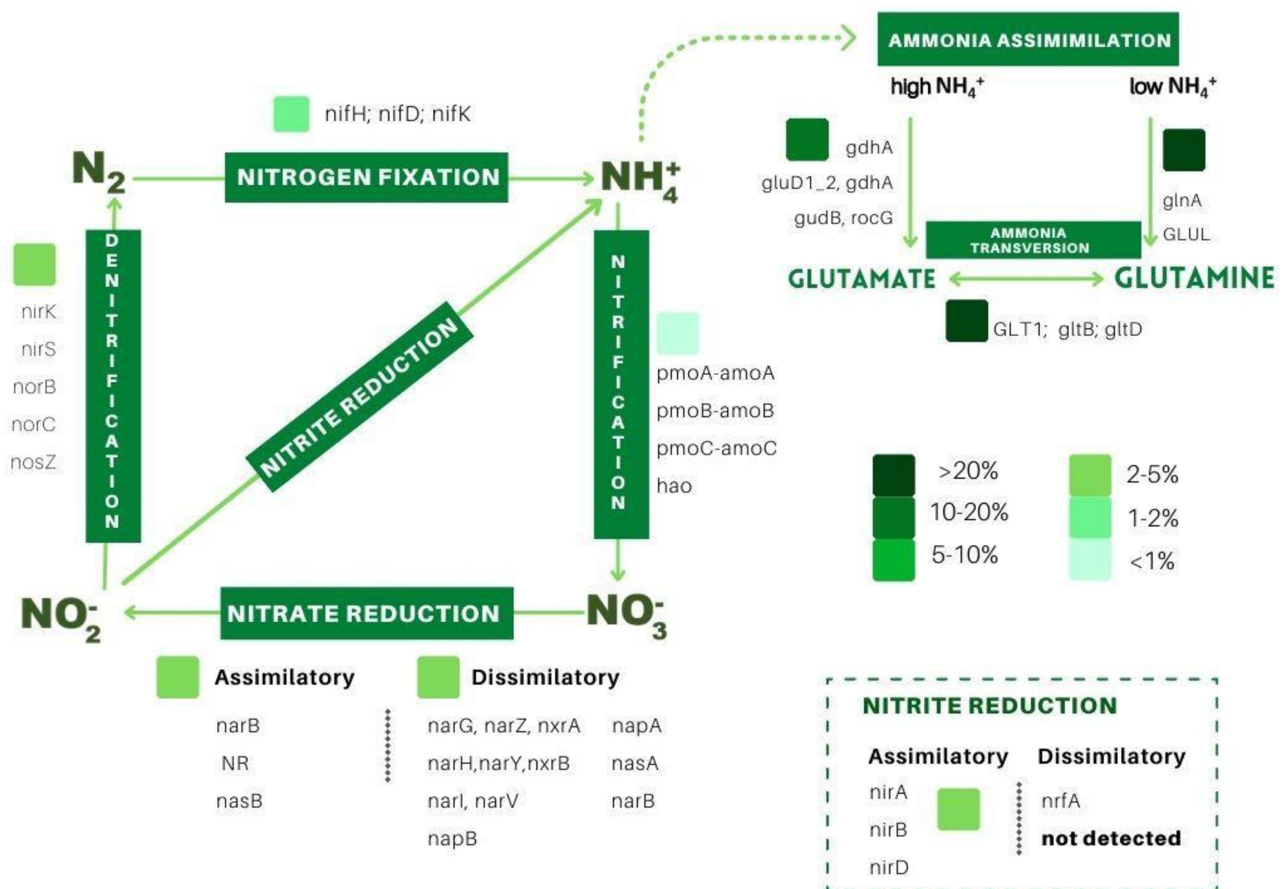
Transcription of CAZyme groups targeting various substrates was comparable across habitats, except for genes targeting α-glucans, peptidoglycan, pectin, cellulose and glucuroxylans (Figure 6). The subalpine shrubland was rich in transcripts targeting the plant cell wall components cellulose and pectin while the share of α-glucan degradation was comparatively low; peptidoglycan-targeting genes were most expressed in the alpine snowbed.

Transcripts involved in NH_4^+ assimilation were the most abundant in all conditions, followed by transcripts of ammonia transversion (Figure 6, 7).

Figure 6: Composition of the pools of genes encoding for carbohydrate-active enzymes (CAZymes) and N-cycling enzymes in alpine and subalpine soil metatranscriptomes. The data represent relative share of each group in percent of all CAZymes and N-cycling genes, respectively. The values represent means for vegetation types. Letters indicate significant differences in one-way ANOVA followed by post-hoc Tukey's HSD test ($p<0.05$).

	Alpine snowbed		Alpine grassland		Subalpine grassland		Subalpine shrubland	
CARBON SOURCES DEGRADATION								
Alpha glucans	27.86	a	25.12	a	23.48	ab	11.02	b
Peptidoglycan	16.92	a	5.76	b	14.96	ab	1.91	b
Lignin	7.96		14.92		8.45		7.10	
Glycoprotein/Glucosaminglycan	7.19		5.41		12.80		5.79	
Cello/Xylobiose	5.44		8.37		11.54		5.46	
Pectin	6.56	b	5.48	b	1.56	b	19.05	a
Beta glucans	8.71		10.21		5.80		6.76	
Cellulose	9.44	ab	7.64	b	6.31	b	21.12	a
Glucuroxylans	5.98	b	8.36	ab	5.70	b	16.46	a
Galactans/Arabinogalactans	2.02		3.70		3.03		0.00	
Chitin	1.19		3.82		6.36		5.33	
Mannans	0.58		1.22		0.00		0.00	
NITROGEN PATHWAYS								
Low NH4 assimilitation	44.23		49.07		52.06		52.84	
High NH4 Assimilation	19.35	a	21.32	a	10.13	b	20.68	a
Ammonia transversion	21.62		20.09		21.80		19.37	
Assimilatory nitrate red.	2.24		2.07		1.13		1.13	
Assimilatory nitrite red.	1.91		4.59		2.17		3.86	
Dissimilatory nitrate red.	4.69	a	1.94	b	2.61	ab	1.18	a
Denitrification	2.56	ab	0.26	b	7.41	a	0.00	b
Nitrification	1.43		0.00		0.00		0.19	
Nitrogen fixation	0.80		1.15		2.70		0.46	

Figure 7: Nitrogen cycle and ammonia assimilation pathways occurring in soils. The percentage of relative abundance for each pathway is represented by colored box.



These pathways did not show any significantly differences between soils, except for high affinity NH_4^+ assimilation which was significantly lower in subalpine grassland. In alpine soils, genes involved in dissimilatory nitrate reduction were significantly higher in snowbed than grassland soils. Share of transcription of genes involved in denitrification was higher in alpine snowbed and subalpine grassland, at lower levels in alpine grassland, and not detected in subalpine shrubland. The transcription of nitrification genes was not detected in alpine and subalpine grasslands while N_2 fixation occurred in all habitats. While the transcription of amino acid transporters was high in all habitats, transporters of mono/oligosaccharides increased while P transporters decreased in alpine soils (Table 7S).

Carbohydrate, amino acid, and energy metabolisms and translation were the most frequently transcribed metabolic pathways recorded in all soils, with values ranging from 7.3 to 13.9% (Figure

8, Table 7S). Except for translation, all showed significant differences between the alpine grassland and snowbed soils.

Figure 8: Relative abundance of transcript of dominant KEGG pathways in the alpine and subalpine soil metatranscriptomes. The values represent means for vegetation types. Letters indicate significant differences in one-way ANOVA followed by post-hoc Tukey's HSD test ($p<0.05$).

KEGG PATHWAYS	Alpine snowbed		Alpine grassland		Subalpine grassland		Subalpine shrubland	
Carbohydrate metabolism	13.40	a	11.83	b	12.80	ab	10.92	b
Amino acid metabolism	9.56	a	7.96	b	10.23	a	7.26	b
Energy metabolism	10.82	b	13.93	a	12.47	ab	12.84	ab
Nucleotide metabolism	5.57		4.63		5.32		4.34	
Metabolism of cofactors and vitamins	3.66	a	3.25	a	3.74	a	2.45	b
Lipid metabolism	4.85	a	3.75	bc	4.21	ab	3.04	c
Xenobiotics biodegradation and metabolism	5.07	a	4.62	a	4.75	a	3.29	b
Membrane transport	4.84		4.69		4.25		4.24	
Signal transduction	6.01	b	6.40	b	6.39	b	8.14	a
Metabolism of other amino acids	2.94		2.47		2.83		2.35	
Translation	8.23		8.64		8.55		10.08	
Folding, sorting,degradation	6.24		6.58		5.57		6.09	
Cell growth/death	4.34	ab	4.55	ab	3.91	b	5.08	a
Replication and repair	1.72	a	1.33	bc	1.54	ab	0.99	c
Biosynthesis of other secondary metabolites	1.48		1.35		1.51		1.23	
Metabolism of terpenoids and polyketides	1.99	a	1.56	b	2.03	a	1.37	b
Glycan biosynthesis and metabolism	0.73		0.71		0.60		0.66	
Transcription	2.84		2.76		2.62		2.70	
Transport and catabolism	1.90	c	2.64	b	1.89	c	3.97	a
Cell motility	0.90	b	0.83	b	1.15	b	1.84	a
Environmental adaptation	2.92	c	5.51	ab	3.62	bc	7.03	a
Signaling/interaction molecules	0.01	b	0.01	b	0.02	b	0.09	a

Subalpine shrubland showed specific transcription profile, where higher transcription in five pathways – signal transduction, transport and catabolism, cell motility, environmental adaptation, signaling and interaction molecules – and lower transcription of xenobiotics biodegradation and metabolism were recorded. No significant difference in transcription was recorded between experimentally warmed plots and controls.

3.4 Discussion

The present work aimed to characterize soil community composition and functioning along an alpine climosequence and to point out the mechanistic link between plant and soil microecosystem functions. Currently, there is a considerable interest in understanding the forces driving the relationship between soil

microbiota and plant community, and soil microorganisms are accounted as important sensitive indicators of changes in soil environment and nutrients (Tang et al., 2020). Some studies found a significant direct effect of plant community changes on related soil microbial community structure (Carney and Matson, 2005; Wang et al., 2013), while others suggest the elevation as the main controlling factor, indirectly affecting soil microbial community (Tang et al., 2020).

Significant differences in enzyme activities among the same alpine and subalpine soil samples have been previously reported (D'Alò et al., 2021). In accordance to these results, we have here demonstrated profound differences in microbial community composition in soils with different types of vegetation. Lower altitude soils, characterized by higher water, C, H, N-NH_4, and K contents, showed lower alpha diversity of the soil microbiome. Limited soil microbiome diversity at high elevations is generally linked to the increase in environmental harshness (Margesin et al., 2009; Donhauser and Frey, 2018). Nevertheless, some studies already reported an opposite trend. A meta-analysis study, where the effects of global change on soils were simulated by C and nutrients additions, highlighted negative effects on microbial richness (Zhou et al., 2020). Bacterial diversity observed in previous comparisons among soils of differing complexity showed to be lower in plots with higher vegetation complexity (Tam et al., 2001; Kumar et al., 2016).

Bacteria and fungi represented the major important groups in the studied soils, much more abundant than Archaea and other nonfungal Eukaryota. The ratio of fungi to bacteria has been extensively used in soil ecology as indicator of ecosystem functioning (Bardgett et al., 1996; Strickland and Rousk, 2010; Malik et al., 2016; Wagg et al., 2019).

The higher F/B value recorded in shrubland soil is in agreement with other studies (Strickland and Rousk, 2010; Urbanová et al., 2015) and is possibly determined by the combination of a greater recalcitrant plant detritus (Lauber et al., 2008; Li et al., 2017) and the wider fungal enzymes capabilities in their transformation and stabilization (Malik et al., 2016) that makes this habitat more suitable for fungi than the soils with other vegetation types.

Archaea were rare both in the gene and transcript pool in all samples. Their higher abundances in snowbed may be due to their ability to adapt to low temperatures, as already reported by Cavicchioli

et al. (2000) for Antarctic soils. Indeed, snowbed has a harsher soil, being characterized by long-lasting snow cover, longer periods of low soil temperature and short growing season.

Looking to the taxonomic composition of the communities, *Proteobacteria* was the most abundant phylum, with 50% share on the metagenome relative abundance in all soils. *Proteobacteria* have been typically recorded as dominant in soils (Janssen, 2006, Spain et al., 2009) being able to encompass an enormous level of morphological, physiological and metabolic diversity, and to play a pivotal role in global C and N cycling (Kersters et al., 2006). Nevertheless, our expression analysis showed similar rates of three microbial phyla, namely *Proteobacteria, Actinobacteria* and *Ascomycota*. *Actinobacteria*, in particular, were significantly more abundant in both alpine soils, which indicates that they have the capacity to effectively regulate the decomposition and synthesis of SOM and significantly improve the fertility in harsher soils (Liu et al., 2017). *Ascomycota* were the dominant fungal phylum, thanks to the high number of genes related to stress-tolerance, competitive abilities and resource uptake, allowing them to colonize a wide range of environments (Egidi et al., 2019).

Alpine snowbed soils were typical with a high transcription of *Chloroflexi* and *Acidobacteria*, confirming previous reports that suggest their association with oligotrophic soils (Costello and Schmidt, 2006; Kielak et al., 2016). The former phylum, including photosynthetic and stress-tolerant microorganisms, is able to take part in the primary production and nitrogen balance, playing an active biogeochemical role in cold and extreme environments (Rasran, 2004; Costello and Schmidt, 2006; Canini et al., 2020). The latter phylum, considered to consist of k-strategist with lower growth rates, has been reported to play a beneficial role to soil nutrient cycling and plant growth in high altitudinal soils (Huang et al., 2015; Kielak et al., 2016). The *Firmicutes* were most active at lower elevation where they were responsible for 14-15% of transcriptional activity. One of the possible explanations can be the extensive summer pasturing vocation of the area (Malfasi and Cannone, 2020) since the members of this phylum have been frequently associated with pasture areas and ruminant gut microbiomes (Rodrigues et al., 2013; Jami et al., 2014). Finally, the high activity of *Mucoromycota* in subalpine soils could be due to their frequent association with decomposition of fungal biomass or

their involvement in wood decomposition facilitating the breakdown of complex sugars (Fukasawa et al., 2011; Brabcová et al., 2016; Algora Gallardo et al., 2021).

Considering the rate of the ribosomal proteins transcription as a proxy of microbial growth (Lladó et al., 2019), the considerably higher share of bacteria compared to fungi may suggest faster turnover of bacterial biomass compared to fungi. High growth *Arthropoda* were recorded in subalpine shrubland. Structurally complex habitats, as shrub-dominated plant communities, have been reported to provide more favorable conditions for *Arthropoda* than open shrubless tundra, where they are generally more abundant in the summer growing season (Döbel and Denno, 1994; Rich et al., 2013). Exploring the repertoire of carbohydrate-degrading enzymes indicated that CAZymes degrading α-glucans represented the largest share of the metagenome and metatranscriptome, indicating the importance of the use of reserve C compounds including starch and glycogen. The same was earlier recorded for soils of the north-American prairies (Mackelprang et al., 2018). In MT results, unlike all soils, shrubland showed lowerα-glucans degradation, along with an increase of pectin and, at a less significant extent, of cellulose and glucuroxylans. These last substrates are much more recalcitrant compared to α-glucans (Xue et al., 2016), suggesting the possible degradation of old recalcitrant C sources in subalpine shrubland and, thus, a potential positive feedback to climate warming with the predicted shrubs expansion.

Peptidoglycans have been reported as indicators of bacterial turnover rates (López-Mondéjar et al., 2020). Accordingly, abundance of transcripts targeting these substrates showed the same trends as overall bacterial expression. The highest share of peptidoglycan degradation was recorded in snowbed, where C is limiting, and it could be linked with the ability of microbes to produce more enzymes degrading that substrate in both N and C limiting conditions (Mori, 2020).

Regarding nitrogen cycling, both MT and MG results showed high abundances of potential and real expressed transcripts involved in microbial ammonia incorporation into glutamine and glutamate organic molecules, which are important nitrogen donors in nitrogenous compounds metabolism in microbial cells (Pengpeng and Tan, 2013). This is in accordance with previous studies of Tláskal et

al. (2021) and Mackelprang et al. (2018), on microbial wood decomposition in forest and in north-American tall grass prairie soils, respectively. N is generally a limiting primary nutrient in high altitude ecosystems (Rütting and Andersen, 2015), resulting in a strong competition between plants and microorganisms. The high values for these pathways here recorded seem to suggest a certain ability of microorganisms in ammonium sequestration in all soils.

In alpine soils, snowbed was characterized by higher abundances of enzymes involved to dissimilatory nitrate reduction (MG and MT) and denitrification (MG) compared to grassland. Morse et al. (2015) reported higher dissimilatory nitrate reduction and denitrification rates at high elevation plots in northern hardwood forest, and during transitions between seasons, particularly in the snowmelt period. At the same way, snowbed characterized by long-lasting snow cover are here characterised by higher rates of dissimilatory nitrate reduction and denitrification, which directly converts NO_3^- to NO_2^-,followed by their sequential reduction to gaseous end products (NO, N_2O, and N_2) (Braker et al., 2010), suggesting an increase of N losses in this site.

Genes and mRNA encoding transporters could be used to investigate the sources request of soil microorganisms (Bei et al., 2019). The largest share of transporter genes and transcripts belonged to amino acid/oligopeptides in all soils. In alpine and polar regions, where bio-availability of nutrients is limited, proteins are the main N input and amino acids and oligopeptides are thought to represent a key N source for both plants and soil microorganisms, bypassing the need to take up inorganic N that is not present (Broughton et al., 2015; Datta et al., 2017). Here, the high share of organic N containing compounds transporters along with the high share of ammonia incorporation into biomolecules clearly confirm that organic N recycling is a major pathway in alpine soils. This source is partly supplemented by N fixation. The second more abundant were the mono/oligosaccharides transporters, which is not surprising, since oligo- and monosaccharides represent the most readily available energy and C sources (Gunina and Kuzyakov, 2015). Another essential element to many soil biological processes is P, for nucleic acid synthesis, respiration, and enzymatic activity. Although soils often contains large quantities of P, most of P is not available for plants, forcing them to develop

different strategies to acquire it, including the establishment of symbiotic associations with soil microbes (Kafle et al., 2019). In our study, despite P transporters genes were significantly less abundant in shrubland, their transcription was higher in both subalpine soils. Indeed, compared to alpine soils, a stronger competition for P between plants and microorganisms could occur in subalpine soils, where higher is the vegetation productivity. For this reason, microbes enhance their P uptake by increasing the P transporters, to contrast the higher vegetation demand.

KEGG database provides information for understanding high-level functions and utilities of a biological system. As for transporters, the most abundant KEGG metabolisms in both MG and MT were energy, carbohydrate and amino acids metabolisms in all soil samples, providing the necessary energy and organic sources for microbial growth, mobility and material exchange. Subalpine shrubland was associated with an higher abundance of genes and transcripts for environmental adaptation, meaning a great functional performance and an expected more stable and resilient ability to environmental perturbations.

No significant differences in communities composition and functioning between treatments and controls in both alpine vegetation types were recorded, despite the increase of temperature recorded within the OTCs. One should, however, consider the level of such change in the context of annual variation of climatic conditions that are typically much more pronounced. In this light, pronounced shifts of soil parameters, microbial composition and functioning are to be expected to occur only over longer periods of time, such as one or two decades of warming, as demonstrated in other warming experiments (De Angelis et al., 2017, Romero-Olivares et al., 2019). Similar conclusions were reached in a previous work in the same area, in which the enzymatic activities were studied by a spectrophotometric approach (D'Alò et al., 2021). We here confirm the need to conduct longer-term experiments to be able to explore the effect of warming on C and N cycling.

3.5 Conclusions

The soil microbiome community composition and functioning has been investigated, and a complex network of plant-soil interactions has been reported, highlighting how difficult may be to predict feedbacks of rapidly changing climate on alpine ecosystems, particularly on the global C cycle, where soil microbes play an integral role. We found that the composition and functional potential of the microbiome was strongly differentiated under different type of vegetation. In contrast with our first hypothesis, prokaryotic and eukaryotic alpha diversity increases along the altitudinal gradient as a consequence of greater environmental harshness characterizing high altitudes. Moreover, how hypothesized, fungi were more dependent on vegetation compared to bacteria; all soils, except for snowbed, hosted a larger share of fungi. In particular, shrubland showed the highest share, possibly correlated with higher amount of carbohydrate-active enzymes, specific for degrading fungal biomass and recalcitrant plant biopolymers, such as pectin, cellulose, glucuroxylans. There is evidence suggestive of a possible loss of species connected to the subalpine vegetation shift at the expense of alpine soils; moreover, the shrubs expansions may accelerate the more recalcitrant C decomposition and decrease total ecosystem C storage; thereby increasing the efflux of CO2 to the atmosphere and having a positive feedback to warming.

On the other hand, no significant differences in communities composition and functioning were reported between experimental warming devices and the counterpart controls. In this light, pronounced shifts of soil parameters, microbial composition and functioning are to be expected to occur only over longer periods of time, such as one or two decades of warming,, suggesting the need of long-term warming experiments.

Supplementary materials

Table 4S: Relative abundances of reads mapping to microbial taxa in the alpine and subalpine soils. Only the relative abundance at the phylum level higher than 1% in at least one of four soil habitats are showed. The values represent means and standard errors for vegetation types. Letters indicate significant differences in one-way ANOVA followed by post-hoc Tukey's HSD test (p<0.05). MG: metagenome; MT: metatranscriptome.

		Alpine snowbed	Alpine grassland	Subalpine grassland	Subalpine shrubland
Domain					
Archaea	MG	0.43 ± 0.08 **a**	0.31 ± 0.03 **b**	0.30 ± 0.02 **b**	0.27 ± 0.02 **b**
	MT	0.45 ± 0.12 **a**	0.24 ± 0.07 **b**	0.14 ± 0.03 **b**	0.15 ± 0.03 **b**
Bacteria	MG	85.93 ± 0.96 **c**	86.24 ± 0.47 **bc**	88.22 ± 0.39 **a**	87.18 ± 0.49 **ab**
	MT	71.18 ± 8.35 **a**	60.59 ± 6.57 **b**	61.06 ± 5.90 **ab**	50.65 ± 8.22 **b**
Fungi	MG	13.64 ± 0.88 **a**	13.44 ± 0.44 **ab**	11.47 ± 0.38 **c**	12.54 ± 0.50 **bc**
	MT	27.91 ± 8.25 **b**	38.80 ± 6.61 **a**	38.63 ± 5.87 **ab**	48.08 ± 7.80 **a**
Other Eukaryota	MG	0.01 ± 0.00 **b**	0.01 ± 0.00 **a**	0.01 ± 0.00 **b**	0.01 ± 0.00 **b**
	MT	0.46 ± 0.39 **b**	0.38 ± 0.17 **b**	0.18 ± 0.09 **b**	1.14 ± 0.63 **a**
Fungi/Bacteria	MG	0.16 ± 0.01 **a**	0.16 ± 0.01 **ab**	0.13 ± 0.00 **c**	0.14 ± 0.01 **bc**
	MT	0.41 ± 0.21 **b**	0.66 ± 0.19 **b**	0.64 ± 0.16 **b**	0.99 ± 0.30 **a**
Phylum					
Ascomycota	*MG*	6.50 ± 0.50 **a**	6.30 ± 0.40 **a**	5.30 ± 0.30 **b**	6.60 ± 0.40 **a**
	MT	13.85 ± 4.89 **b**	18.86 ± 8.06 **ab**	16.21 ± 6.84 **ab**	26.45 ± 6.51 **a**
Basidiomycota	*MG*	2.50 ± 0.20 **a**	2.60 ± 0.30 **a**	2.10 ± 0.10 **b**	2.00 ± 0.10 **b**
	MT	6.42 ± 3.77 **b**	11.63 ± 4.81 **a**	9.74 ± 3.97 **ab**	9.24 ± 1.79 **ab**
Mucoromycota	*MG*	1.60 ± 0.20 **a**	1.4 ± 0.10 **b**	1.4 ± 0.10 **bc**	1.30 ± 0.03 **c**
	MT	5.83 ± 2.10 **c**	6.64 ± 1.89 **bc**	11.41 ± 5.84 **a**	10.75 ± 1.87 **ab**
Chytridiomycota	*MG*	1.70 ± 0.10 **a**	1.70 ± 0.04 **a**	1.50 ± 0.03 **b**	1.40 ± 0.04 **b**
	MT	< 1%	< 1%	< 1%	< 1%
Proteobacteria	*MG*	45.70 ± 3.80 **b**	43.50 ± 3.30 **b**	47.50 ± 2.60 **ab**	51.30 ± 2.90 **a**

	MT	23.84 ± 5.81 **a**	16.76 ± 2.40 **ab**	24.14 ± 8.61 **a**	14.95 ± 3.67 **b**
Actinobacteria	*MG*	19.10 ± 3.10 **ab**	22.40 ± 3.30 **a**	15.90 ± 2.90 **b**	17.00 ± 2.30 **b**
	MT	19.74 ± 4.38 **a**	22.38 ± 3.5 **a**	11.32 ± 2.63 **b**	9.40 ± 4.60 **b**
Acidobacteria	*MG*	11.60 ± 1.20 **a**	12.20 ± 1.20 **a**	12.10 ± 3.20 **a**	8.70 ± 1.80 **b**
	MT	10.80 ± 3.32 **a**	7.31 ± 1.44 **b**	5.07 ± 1.31 **b**	4.94 ± 1.24 **b**
Verrucomicrobia	*MG*	2.00 ± 0.30 **c**	3.00 ± 0.50 **c**	7.10 ± 1.50 **a**	5.20 ± 1.20 **b**
	MT	1.06 ± 0.35	1.35 ± 0.39	1.19 ± 0.39	1.04 ± 0.21
Firmicutes	*MG*	2.20 ± 0.40 **a**	1.60 ± 0.10 **b**	1.50 ± 0.10 **b**	1.40 ± 0.10 **b**
	MT	6.03 ± 1.22 **b**	4.70 ± 0.95 **b**	13.73 ± 6.00 **a**	14.95 ± 3.40 **a**
Cyanobacteria	*MG*	1.50 ± 0.2 **a**	1.20 ± 0.10 **b**	1.20 ± 0.10 **b**	1.10 ± 0.10 **b**
	MT	1.03 ± 0.3 **a**	0.74 ± 0.24 **ab**	0.39 ± 0.06 **c**	0.41 ± 0.12 **bc**
Chloroflexi	*MG*	2.00 ± 0.60 **a**	1.10 ± 0.20 **a**	0.90 ± 0.10 **a**	0.80 ± 0.10 **b**
	MT	3.17 ± 1.02 **a**	1.92 ± 0.76 **b**	0.98 ± 0.27 **b**	0.86 ± 0.31 **b**
Planctomycetes	*MG*	0.80 ± 0.10 **b**	0.70 ± 0.03 **c**	1.01 ± 0.08 **a**	0.90 ± 0.07 a
	MT	2.57 ± 0.59 **a**	1.66 ± 0.28 **b**	2.03 ± 0.4 **ab**	1.56 ± 0.77 **b**
Bacteroidetes	*MG*	< 1%	< 1%	< 1%	< 1%
	MT	0.90 ± 0.21	3.24 ± 4.14	1.39 ± 0.11	1.64 ± 0.47

Table 5S: Composition of the pools of genes encoding for nutrient transporters and KEGG level 2 gene classes in alpine and subalpine soil metagenomes. The data represent relative share of each group in percent of each class of functions. Letters indicate significant differences in one-way ANOVA followed by post-hoc Tukey's HSD test ($p<0.05$).

	Alpine snowbed	**Alpine grassland**	**Subalpine grassland**	**Subalpine shrubland**
Transporters grouped by targeting substrates				
Amino acids/oligopeptides	66.10 ± 1.68	65.03 ± 1.4	65.06 ± 1.5	67.58 ± 12.28
Mono/oligosaccharides	13.89 ± 0.74 **a**	14.71 ± 0.61 **a**	13.62 ± 1.25 **b**	13.61 ± 1.45 **b**
K	5.98 ± 0.43 **b**	6.42 ± 0.42 **b**	7.74 ± 0.5 **a**	6.36 ± 0.17 **b**
P	5.35 ± 0.43 **ab**	5.47 ± 0.4 **a**	5.66 ± 0.26 **a**	4.84 ± 0.41 **b**
Fe	4.19 ± 0.29	4.32 ± 0.36	3.86 ± 0.38	4.05 ± 0.24
Mo	2.76 ± 0.18 **a**	2.57 ± 0.11 **a**	2.05 ± 0.31 **b**	2.08 ± 0.17 **b**

S	0.73 ± 0.1 **b**	0.67 ± 0.08 **b**	1.04 ± 0.07 **a**	0.77 ± 0.12 **b**
Ca	0.19 ± 0.05	0.21 ± 0.05	0.17 ± 0.1	0.18 ± 0.05
Na	0.36 ± 0.1 **a**	0.24 ± 0.09 **a**	0.22 ± 0.08 **b**	0.18 ± 0.08 **b**
Ni	0.27 ± 0.05 **ab**	0.25 ± 0.08 **ab**	0.33 ± 0.12 **a**	0.15 ± 0.13 **b**
Zn	0.18 ± 0.06	0.10 ± 0.03	0.23 ± 0.11	0.21 ± 0.07
Urea	0.00 ± 0.01	0.00 ± 0.00	0.01 ± 0.03	0.00 ± 0.00
KEGG pathways				
Carbohydrate metabolism	16.41 ± 0.17 **ab**	16.62 ± 0.14 **a**	16.20 ± 0.32 **bc**	15.84 ± 0.42 **c**
Amino acid metabolism	14.22 ± 0.3 **a**	14.20 ± 0.16 **a**	13.68 ± 0.21 **b**	14.03 ± 0.16 **ab**
Energy metabolism	9.37 ± 0.18 **b**	9.13 ± 0.11 **c**	9.90 ± 0.11 **a**	9.80 ± 0.26 **a**
Nucleotide metabolism	5.71 ± 0.08 **b**	5.50 ± 0.07 **c**	6.52 ± 0.26 **a**	6.28 ± 0.22 **a**
Metabolism of cofactors and vitamins	5.72 ± 0.17 **b**	5.88 ± 0.09 **a**	5.30 ± 0.08 **c**	5.32 ± 0.13 **c**
Lipid metabolism	5.59 ± 0.07 **b**	5.76 ± 0.06 **a**	5.21 ± 0.03 **c**	5.29 ± 0.11 **c**
Xenobiotics biodegradation and metabolism	5.65 ± 0.15 **a**	5.81 ± 0.09 **a**	5.15 ± 0.11 **c**	5.39 ± 0.17 **b**
Membrane transport	5.38 ± 0.31	5.15 ± 0.26	5.39 ± 0.22	5.48 ± 0.11
Signal transduction	5.18 ± 0.11 **a**	5.12 ± 0.07 **a**	5.12 ± 0.11 **ab**	4.96 ± 0.06 **b**
Metabolism of other amino acids	4.76 ± 0.11 **b**	4.89 ± 0.04 **a**	4.56 ± 0.07 **c**	4.71 ± 0.13 **bc**
Translation	3.82 ± 0.11 **ab**	3.72 ± 0.07 **b**	3.89 ± 0.1 **a**	3.89 ± 0.11 **a**
Folding, sorting, degradation	2.86 ± 0.08 **c**	2.81 ± 0.04 **c**	3.39 ± 0.16 **a**	3.17 ± 0.12 **b**
Cell growth/death	2.74 ± 0.06 **b**	2.65 ± 0.07 **c**	2.97 ± 0.06 **a**	2.99 ± 0.06 **a**
Replication and repair	2.79 ± 0.08	2.76 ± 0.06	2.77 ± 0.11	2.72 ± 0.07
Biosynthesis of other secondary metabolites	2.34 ± 0.02 **b**	2.46 ± 0.03 **a**	2.22 ± 0.12 **c**	2.18 ± 0.1 **c**
Metabolism of terpenoids and polyketides	2.36 ± 0.05 **b**	2.51 ± 0.06 **a**	2.26 ± 0.04 **c**	2.29 ± 0.05 **bc**
Glycan biosynthesis and metabolism	1.42 ± 0.02 **b**	1.48 ± 0.03 **a**	1.36 ± 0.06 **bc**	1.30 ± 0.07 **c**
Transcription	1.15 ± 0.09 **b**	0.94 ± 0.04 **c**	1.79 ± 0.2 **a**	1.62 ± 0.23 **a**
Transport and catabolism	0.94 ± 0.02 **a**	0.92 ± 0.02 **ab**	0.86 ± 0.02 **c**	0.90 ± 0.02 **b**
Cell motility	0.86 ± 0.1	0.89 ± 0.07	0.80 ± 0.05	0.81 ± 0.02
Environmental adaptation	0.72 ± 0.09 **b**	0.78 ± 0.01 **b**	0.65 ± 0.04 **b**	1.05 ± 0.19 **a**
Signaling/interaction molecules	0.00 ± 0.00 **a**	0.00 ± 0.00 **ab**	0.00 ± 0.00 **ab**	0.00 ± 0.00 **b**

Table 6S: Relative abundance of mRNA of genes encoding ribosomal proteins of soil organisms in alpine and subalpine soil metatranscriptomes. The values represent means and standard errors for vegetation types. Letters indicate significant differences in one-way ANOVA followed by post-hoc Tukey's HSD test ($p<0.05$).

Phylum	Alpine snowbed	Alpine grassland	Subalpine grassland	Subalpine shrubland
Mucoromycota	3.03 ± 1.37 **ab**	4.43 ± 1.30 **ab**	2.29 ± 1.34 **b**	5.29 ± 3.03 **a**
Basidiomycota	1.47 ± 1.01	4.65 ± 3.86	3.02 ± 1.77	4.28 ± 2.86
Ascomycota	4.36 ± 1.70	6.66 ± 2.85	5.21 ± 2.96	7.36 ± 2.41
Arthropoda	0.90 ± 1.53 **b**	2.10 ± 1.90 **b**	0.37 ± 0.51 **b**	10.90 ± 9.22 **a**
Chytridiomycota	0.84 ± 0.63	1.62 ± 1.19	1.44 ± 1.35	2.01 ± 1.30
Undefined Bacteria	0.80 ± 0.37 **ab**	0.74 ± 0.50 **ab**	1.38 ± 0.92 **a**	0.40 ± 0.30 **b**
Proteobacteria	27.31 ± 5.43 **ab**	21.20 ± 2.75 **b**	37.82 ± 17.73 **a**	18.97 ± 6.60 **b**

Acidobacteria	21.40 ± 4.17	18.26 ± 3.35	17.61 ± 4.41	15.71 ± 3.06
Actinobacteria	12.47 ± 2.76 **ab**	14.82 ± 2.79 **a**	8.91 ± 2.23 **bc**	7.19 ± 4.37 **c**
Firmicutes	11.02 ± 2.40	7.92 ± 2.57	5.60 ± 2.26	10.06 ± 11.03
Chloroflexi	7.44 ± 3.16 **a**	6.61 ± 2.22 **ab**	3.28 ± 1.90 **bc**	2.15 ± 1.10 **c**
Planctomycetes	4.16 ± 1.61	3.46 ± 1.33	5.74 ± 2.04	4.69 ± 3.16
Bacteroidetes	1.03 ± 0.60	3.23 ± 2.79	2.18 ± 1.70	3.63 ± 1.89
Chlamydiae	0.04 ± 0.11 **b**	0.02 ± 0.07 **b**	0.00 ± 0.00 **b**	2.98 ± 3.99 **a**

Table 7S: Composition of the pools of genes encoding for nutrient transporters and KEGG level 2 gene classes in alpine and subalpine soil metatranscriptome. The data represent relative share of each group in percent of each class of functions. The values represent means and standard errors for vegetation types. Letters indicate significant differences in one-way ANOVA followed by post-hoc Tukey's HSD test (p<0.05).

	Alpine snowbed	**Alpine grassland**	**Subalpine grassland**	**Subalpine shrubland**
Transporters grouped by targeting substrates				
Amino acid oligopeptides	47.41 ± 7.99	47.42 ± 6.91	50.76 ± 9.84	42.49 ± 16.45
Mono/oligosaccharides	36.61 ± 6.73 **ab**	38.79 ± 8.67 **a**	27.76 ± 7.63 **ab**	26.42 ± 7.38 **b**
P	4.44 ± 1.66 **c**	4.92 ± 2.10 **bc**	10.32 ± 6.37 **a**	10.16 ± 5.04 **ab**
K	4.99 ± 2.67	2.37 ± 2.34	3.43 ± 2.44	3.67 ± 3.16
Ca	2.30 ± 1.86	2.82 ± 1.94	2.41 ± 3.6	13.49 ± 19.65
Fe	2.02 ± 1.12	2.02 ± 2.10	3.05 ± 3.37	2.78 ± 2.69
S	0.53 ± 0.59	0.18 ± 0.27	1.15 ± 2.25	0,09 ± 0.21
Mn	0.08 ± 0.27	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Mo	0.57 ± 0.49	0.79 ± 0.73	0.66 ± 1.47	0.22 ± 0.49
Na	0.44 ± 0.66	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Ni	0.16 ± 0.28	0.29 ± 0.58	0.00 ± 0.00	0.00 ± 0.00
Urea	0.18 ± 0.48	0.28 ± 0.47	0.47 ± 1.05	0.00 ± 0.00
Zn	0.27 ± 0.50	0,13 ± 0.27	0.00 ± 0.00	0,68 ± 0.65
KEGG pathways				
Energy metabolism	10.82 ± 1.38 **b**	13.93 ± 1.59 **a**	12.47 ± 3.49 **ab**	12.84 ± 2.13 **ab**
Carbohydrate metabolism	13.40 ± 1.22 **a**	11.83 ± 0.77 **b**	12.80 ± 0.52 **ab**	10.92 ± 2.05 **b**
Amino acid metabolism	9.56 ± 1.22 **a**	7.96 ± 0.66 **b**	10.23 ± 1.71 **a**	7.26 ± 1.40 **b**
Translation	8.23 ± 1.22	8.64 ± 0.70	8.55 ± 1.41	10.08 ± 2.67
Signal transduction	6.00 ± 0.58 **b**	6.40 ± 0.63 **b**	6.39 ± 0.96 **b**	8.14 ± 1.12 **a**
Folding, sorting, degradation	6.24 ± 1.00	6.58 ± 0.78	5.57 ± 0.78	6.09 ± 0.63
Environmental adaptation	2.92 ± 1.46 **c**	5.51 ± 1.46 **ba**	3.62 ± 1.82 **bc**	7.03 ± 3.15 **a**
Nucleotide metabolism	5.57 ± 0.87	4.63 ± 0.52	5.32 ± 1.27	4.34 ± 0.73
Xenobiotics biodegradation and metabolism	5.07 ± 0.49 **a**	4.62 ± 0.63 **a**	4.75 ± 0.40 **a**	3.29 ± 1.10 **b**
Cell growth/death	4.34 ± 0.56 **ab**	4.55 ± 0.47 **ab**	3.91 ± 1.17 **b**	5.08 ± 0.51 **a**
Lipid metabolism	4.85 ± 0.35 **a**	3.75 ± 0.47 **bc**	4.21 ± 0.91 **ab**	3.04 ± 0.83 **c**
Membrane transport	4.84 ± 0.26	4.69 ± 0.59	4.25 ± 0.64	4.24 ± 0.70
Metabolism of cofactors and vitamins	3.66 ± 0.44 **a**	3.25 ± 0.39 **a**	3.74 ± 0.61 **a**	2.45 ± 0.80 **b**

Transport and catabolism	1.90 ± 0.48 **c**	2.64 ± 0.44 **b**	1.89 ± 0.52 **c**	3.97 ± 1.00 **a**
Metabolism of other amino acids	2.94 ± 0.56	2.47 ± 0.38	2.83 ± 0.69	2.35 ± 0.55
Transcription	2.84 ± 0.77	2.76 ± 0.28	2.62 ± 0.72	2.70 ± 0.37
Metabolism of terpenoids and polyketides	1.99 ± 0.22 **a**	1.56 ± 0.17 **b**	2.03 ± 0.49 **a**	1.37 ± 0.46 **b**
Replication and repair	1.72 ± 0.39 **a**	1.33 ± 0.20 **bc**	1.54 ± 0.31 **ab**	0.99 ± 0.20 **c**
Biosynthesis of other secondary metabolites	1.48 ± 0.31	1.35 ± 0.23	1.51 ± 0.25	1.23 ± 0.31
Cell motility	0.90 ± 0.21 **b**	0.83 ± 0.19 **b**	1.15 ± 0.30 **b**	1.84 ± 0.59 **a**
Glycan biosynthesis and metabolism	0.73 ± 0.40	0.71 ± 0.21	0.60 ± 0.32	0.66 ± 0.17
Signaling/interaction molecules	0.01 ± 0.01 **b**	0.01 ± 0.01 **b**	0.02 ± 0.03 **b**	0.09 ± 0.06 **a**

Chapter 4

Expansion of shrubs could result in local loss of soil bacterial richness in Western Greenland

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Abstract

Climate warming in Greenland is facilitating the expansion of shrubs across wide areas of tundra. Given the close association between plants and soil microorganisms and the important role of soil bacteria in ecosystem functioning, it is of utmost importance to characterize microbial communities of arctic soil habitats and assess the influence of plant edaphic factors on their composition. We used 16S rRNA gene amplicons to explore the bacterial assemblages of three different soil habitats representative of a plant coverage gradient: bare ground, biological soil crusts dominated by mosses and lichens, and vascular vegetation dominated by shrubs. We investigated how bacterial richness and community composition were affected by the vegetation coverage, and soil pH, moisture, and carbon (C), nitrogen (N) and phosphorus (P) contents. Bacterial richness did not correlate with plant coverage complexity, while community structure varied between habitats. Edaphic variables affected both the taxonomic richness and community composition. The high number of Amplicon Sequence Variants (ASVs) indicators of bare ground plots suggests a risk of local bacterial diversity loss due to expansion of vascular vegetation.

Keywords: Arctic**,** environmental filtering, climate change, edaphic parameters, metabarcoding, 16S rRNA gene.

4.1 Introduction

In many Arctic environments, rising temperatures are causing an increased melting of glacial ice and an expansion of shrubs (Sturm *et al.* 2001; Tape *et al.* 2006) at the expense of vegetation dominated by bryophytes and lichens (Normand *et al.* 2013; Cahoon *et al.* 2016; Vowles and Björk 2019). These trends are particularly apparent in Greenland, one of the regions in the Arctic most affected by climate change (Howat and Eddy 2011; Bevis *et al.* 2019).

Soil microbial communities and vegetation are linked by a variety of direct and indirect interaction: plants provide photosynthetically fixed carbon and low molecular weight root exudates that are used as energy sources by soil microorganisms, whose community composition is in turn shaped by their ability to metabolize different compounds and to resist different antimicrobial metabolites present in the exudates (Marschner *et al.* 2004; Berg and Smalla 2009; Berendsen *et al.* 2012; Schulz-Bohm *et al.* 2018). On the other hand, rhizosphere microorganisms can strongly influence plant growth and health. Arctic tundra is one of the most nitrogen limited environments on Earth and the input from biological nitrogen fixation, mainly contributed by Cyanobacteria, is critical to the development of these ecosystems (Solheim *et al.* 2006). Additionally, the mobilization of nitrogen from organic matter is an important source for plant growth, and is at least partly influenced by bacterial activity (Chen *et al.* 2014; Leff *et al.* 2015). Furthermore, plant growth promoting bacteria may improve resource acquisition, modulate plant hormones secretion or act antagonistically against pathogens (Glick 2012; Berendsen *et al.* 2012). This close association between plants and microorganisms have led to the concept of plants as 'superorganisms' that partly rely on their microbiome interactions for specific functions and traits (Mendes *et al.* 2013).

The reported decline of mosses and lichens due to climate change and their replacement by vascular plants, particularly shrubs (Normand *et al.* 2013; Cahoon *et al.* 2016; Vowles and Björk 2019), can be expected to affect soil bacteria. Changes in the composition and structure of soil bacterial communities in the Arctic have been recently documented in simulations of long-term warming (Deslippe *et al.* 2012), higher nutrient availability (Koyama *et al.* 2014; Männistö *et al.* 2016), and

altered precipitation regimes (Ricketts *et al.* 2016), as well as in natural successions in glacier forelands (Kwon *et al.* 2015; Kim *et al.* 2017) and along permafrost thaw gradients (Deng *et al.* 2015; Frank-Fahle *et al.* 2014). These responses of bacterial communities to climate changes, and their impact on ecosystem functionality are of utmost scientific relevance. Despite this importance, our understanding of the warming-driven changes in Arctic soil habitats remains uncomplete. For example, the potential effects of the shrub expansion on the structure and functioning of associated bacterial communities are challenging to accurately forecast, since the effects of vegetation type on soil communities in Arctic tundra are not well known (Krab *et al.* 2019). Differences have been observed across Alaskan tundra vegetation types (Wallenstein *et al.* 2007), suggesting that plant communities influence bacterial communities via the quantity and quality of the litter supply, and by modifying the soil physical environment. In soils of Eastern Greenland (Ganzert *et al.* 2014) abiotic parameters, related to different habitats, shaped microbial communities. In a Canadian low Arctic tundra system Denaturing Gradient Gel Electrophoresis analyses revealed that vegetation coverage plays a key role in shifting bacterial communities (Chu *et al.* 2011). A more recent study in the Canadian Arctic, using 16S rRNA gene pyrosequencing, suggested that soil responses to warming would be vegetation-specific, likely due to the differences in the structure of microbial communities associated with different plants (Shi *et al.* 2015). However, other works in Finnish (Männistö *et al.* 2007) and Canadian tundra ecosystems (Buckeridge *et al.* 2010), did not show any differences in bacterial communities among vegetation types.

Given the wide metabolic capabilities of bacteria, a deeper knowledge of the relationships between biotic and abiotic factors shaping soil bacterial community structure and function may be useful to predict the effects of global change on the vast and highly vulnerable Arctic soil carbon stocks (Crowther *et al.* 2016). Shrub encroachment, especially of deciduous species, such as *Betula nana*, may produce more labile compounds from leaf litter, increasing the turnover of soil C (Weintraub and Schimel 2005; Wookey *et al.* 2009). The impact of warming will depend on how efficiently plant-derived carbon is incorporated into microbial biomass or converted to carbon dioxide and released to

the atmosphere (Cotrufo *et al.* 2013). In addition, plant litter and root exudates can facilitate the activity of microorganisms with enhanced decomposition abilities for old stocks of organic matter, in a process known as priming (Fontaine *et al.* 2003; Kuzyakov 2002; Walker *et al.* 2015).

In our study, we selected three different habitats in Western Greenland, representing a gradient of vegetation complexity: bare ground (BG), biological soil crusts (BSCs) dominated by mosses and lichens, and vascular vegetation (VV) dominated by shrubs, e.g. *Empetrum*, *Vaccinium*, *Betula*, and *Salix*. The main aims were: i) to characterize the diversity and composition of soil bacterial communities in this Arctic region; ii) determine whether the diversity and community composition of soil bacteria are related to edaphic parameters; iii) identify the abiotic parameters most closely associated with variation in bacterial communities. Additionally, focusing only on the vascular vegetation plots, we aimed to test for correlations between relative cover of different shrub genera and the composition of soil bacterial communities, to assess the impact of shrub expansion on these patterns.

4.2 Materials and methods

4.2.1 Sampling

Sampling was carried out in July 27-31, 2017 in the area of Kobbefjord, Nuuk, West Greenland (64°08' N, 51°23' W). The climate of the area is classified as low Arctic (Jonasson *et al.* 2000). The mean annual air temperature in the years 2008-2010 was 0.7 °C, with the mean air temperature of the warmest month, July, 10.7 °C. Over the same period annual precipitation ranged between 838 and 1,127 mm, with an average of 25–50 % of the total annual precipitation falling as snow during the winter period (Søndergaard *et al.* 2012). Samples were collected in the area close to the NERO line, a permanent vegetation transect established in 2007 to monitor changes in vegetation species composition (Bay *et al.* 2008).

In total, 20 plots ($2m^2$), representing three habitat types, were sampled: 5 in bare ground (BG), 6 in biological soil crusts (BSC) and 9 in soils covered with vascular vegetation (VV). Exact coordinates,

elevation, and shrub genera composition for each plot are listed in Supplementary Table 1. In each plot, three replicate soil samples (up to 10 cm depth) were collected aseptically, after removing the top of the soil, plant litter in vascular vegetated plots and the superficial coverage of mosses and lichens in BSC plots. Samples were transported in sterile bags and stored at -20 °C at University of Tuscia, Italy, until further processing.

4.2.2 Edaphic parameters

Gravimetric soil water content was measured on 5 grams subsamples dried at 105 °C (Reynolds 1970). Measurements were repeated until no variation in weight was observed. pH was measured in a 1:2.5 suspension of dried soil in deionized water, with a HI9321 pH meter (Hanna Instruments Woonsocket, Rhode Island, United States). For each sample, soil moisture and pH were measured in independent triplicates. Phosphorus (P), carbon (C) and nitrogen (N) content were analysed at Eger Innovations Nonprofit Kft. (Eszterházy Károly University, Eger, Hungary). P content was measured by Microwave Plasma Atomic Emission Spectrometry (MP-AES) and C and N content by CNS elemental analyser.

4.2.3 DNA extraction, amplification and sequencing

For each sample, DNA was extracted from 0.5 g of soil using DNEasy Powersoil kit (QIAGEN, Hilden, Germany), according to the manufacturer's protocol. The V4 hypervariable region of the 16S rRNA gene was amplified using 515F (Parada et al., 2016) and 806R (Apprill *et al.* 2015) primers; libraries were prepared following the protocol of Minich et al. (2018). The equimolar pool of uniquely barcoded amplicons was paired-end sequenced (2 × 300 bp) on an Illumina MiSeq platform at the Vincent J. Coates Genomics Sequencing Laboratory at University of California, Berkeley.

4.2.4 Bioinformatic analyses

Bcl files were converted to Fastq files, demultiplexed and primer removed using bcl2fastq (v 2.18). Dual-matched 8-bp indexes were used to eliminate the occurrence of "barcode bleed" (or tag-switching) between samples.

Demultiplexed 16S rRNA gene sequences were processed with the QIIME2 (Quantitative Insights Into Microbial Ecology, v. 2018.11; Bolyen *et al.* 2018) platform. 3,179,253 starting sequences were denoised, trimmed to length 160 bp, merged and clustered in Amplicon Sequence Variants (ASVs), using DADA2 (Callahan *et al.* 2016), which includes phiX reads removal and chimera detection. We obtained 12,143 quality filtered ASVs, each with at least two reads in the total dataset. Taxonomy was assigned with the *q2-feature-classifier* within the database Greengenes v. 13_8 (99% OTUs from 515F/806R region of sequences). Chloroplasts, mitochondrial, chimeric and low identity ASVs (less than 80% identity to other prokaryotic 16S rRNA sequences) were removed, retaining 10,980 ASVs. The dataset was normalized for subsequent analyses, rarefying the number of reads per sample to the lowest reads obtained (14,251 reads) using the *rrarefy* function in the *vegan* package v. 2.5-2 (Oksanen *et al.* 2018) in R v. 3.5.2 (R Core Team, 2018), retaining a total of 10,578 ASVs. Sequences of ASVs were submitted to NCBI gene bank (BioProject PRJNA550020).

4.2.5 Statistical analyses

Unless otherwise specified, all statistical analyses were carried out with the *vegan* package v. 2.5-2 (Oksanen *et al.* 2018) in R v. 3.5.2 (R Core Team, 2018). Total bacterial richness (including all the ASVs retrieved), as well as relative richness (proportion of ASVs in a sample belonging to each group) and relative abundance (proportion of total reads in each sample assigned to each group) of most abundant phyla and classes among the three habitats were compared using ANOVA and Tukey's HSD test. Linear regression analyses were used to examine relationships between edaphic factors (pH, soil moisture, C, N and P content, and C/N ratio) and bacterial phyla and classes relative richness and abundances

We performed Non-Metric Multidimensional Scaling (NMDS) of the weighted Bray-Curtis distances of Hellinger transformed matrix of the bacterial community. We used the *envfit* R function to project edaphic variables (pH, soil moisture, C, N and P content, and C/N ratio) and the relative abundance values of the shrub genera (*Betula*, *Empetrum*, *Salix*, and *Vaccinium*) or of different taxonomic groups onto the NMDS ordinations. In addition, we tested whether bacterial communities were statistically different among habitat types using the multi response permutation procedure (MRPP). We determined preferences of unique ASVs for each habitat using indicator species analyses on the Hellinger transformed matrix of the bacterial community (Dufrêne and Legendre 1997) in PC-ORD v. 6.0 (McCune *et al.* 2002).

Permutational multivariate analysis of variance (PerMANOVA; Anderson 2001) was carried out on Hellinger transformed Bray-Curtis distance matrix to determine the effect of each soil physicochemical parameter on the observed variance of the total community and of dominant phyla. Significant variables obtained from this analysis, were considered in a model to determine the combined effect of soil parameters on the variance of the community. The same approach was used taking into account only VV plots (27 samples in total) in order to assess the effect of the relative abundance of the four dominant shrub genera (*Salix*, *Betula*, *Vaccinium* and *Empetrum*), in combination with edaphic parameters, on the variance of the total community and of the different phyla.

4.3 Results

4.3.1 Bacterial richness and abundance patterns

The filtered and rarefied dataset of the 16S rRNA gene amplicons contained 10,578 bacterial ASVs. The proportions of ASVs found exclusively in VV plots was the highest (41.6%) compared to BSC and BG plots (12.1 and 18.9%, respectively), whereas the BG samples showed the highest number of indicator ASVs (407 ASVs, compared with 86 and 114 for VV and BSC samples, respectively). Only 759 ASVs (7.2%) out of 10,578 were present in all the habitats.

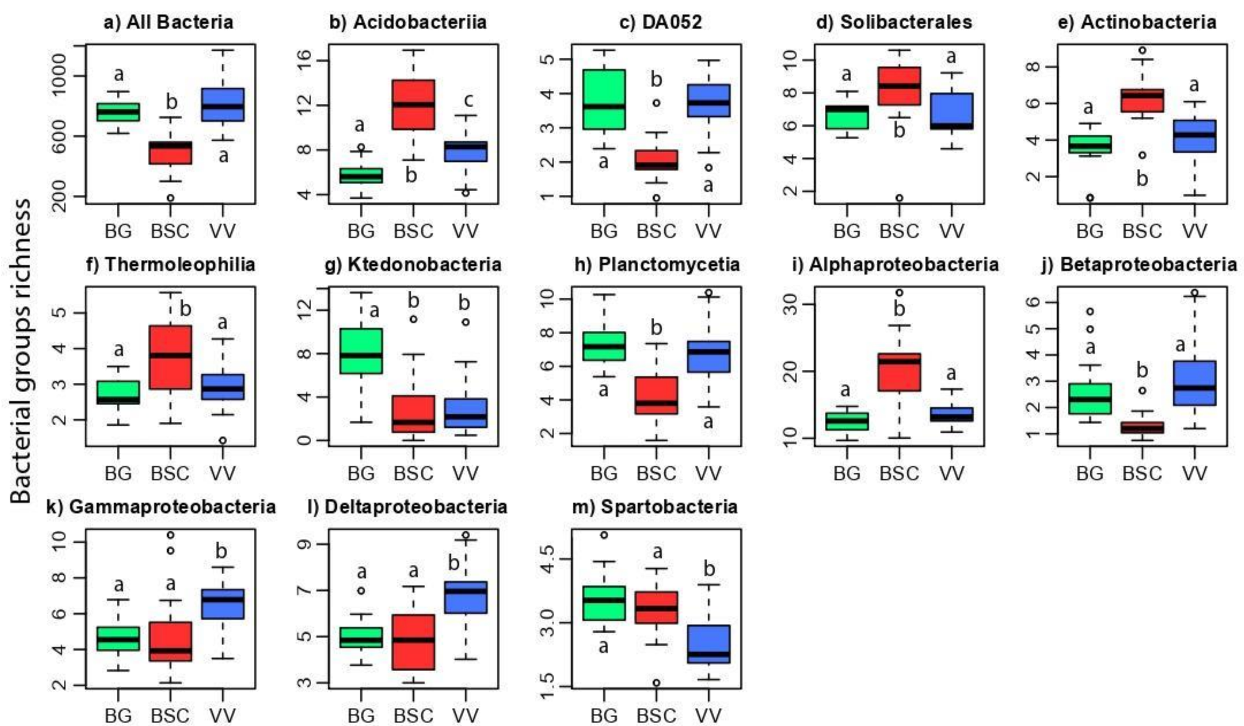
The total richness of the bacterial communities was higher in VV and BG samples compared to BSC, with the two former not significantly different from each other (Figure 1a). We selected 10 most abundant phyla of 35 identified (encompassing both Bacteria and Archaea) and 12 most abundant classes of 103 retrieved for further analyses. The 10 phyla selected represented more than 80% of total reads identified and more than 95% in many samples. The 12 classes were representative of more than 65% of reads identified at this level in all the samples.

Among the most representative classes and phyla studied, those that had the highest richness in BG plots, were Ktedonobacteria (Chloroflexi), Spartobacteria (no statistical significance with BSC; Verrucomicrobia), and the phylum Cyanobacteria (Figure 1; Supplementary Figure 1).

Classes with the highest richness in BSC plots were Acidobacteriia and Solibacterales (Acidobacteria), while DA052 within the same phylum showed the lowest richness and abundance in this habitat. Classes Actinobacteria and Thermoleophilia (Actinobacteria) had higher richness and abundance in BSC plots, compared to BG and VV. The same was for the class Alphaproteobacteria and the phylum Armatimonadetes, that had higher richness for this habitat type. Instead, Planctomycetia (Planctomycetes), the class Betaproteobacteria, and the phylum Chlamydiae showed lower richness in BSC plots (Figure 1; Supplementary Figure 1).

Gammaproteobacteria and Deltaproteobacteria (Proteobacteria), as well as Bacteroidetes had the highest richness in VV plots (Figure 1; Supplementary Figure 1). The same general trends were observed in the relative abundances of individual taxonomical groups (Supplementary Figure 2 and 3).

Figure 1. Richness of the total bacterial community and relative richness of the 12 dominant bacterial classes in each habitat (green, Bare Grounds plots; red, Biological Soil Crusts plots; blue, Vascular Vegetation plots). Letters indicate significant differences in one-way ANOVA post-hoc Tukey's HSD test (P <0.05).



4.3.2 Effect of environmental parameters on richness and abundance of different groups

Soil moisture, P and N content of soil samples showed an increase from BG plots to vegetated plots (BSC + VV), while pH decreased. C content and the C/N ratio also increased from BG plots to the vegetated plots, but with higher values in BSC compared to VV plots (Figure 2). C content and the C/N ratio were the main predictors of total bacterial richness, with a negative correlation (slope=-4.06 and r^2=0.105 for C content; slope=-10,01 and r^2=0.160 for C/N ratio), while N content was only marginally significant (slope=-72.89 and r^2=0.035), and both soil moisture and pH were positively correlated (slope=2.14 and r^2=0.052 for soil moisture; slope=101.38 and r^2= 0.058 for pH; Supplementary Figure 4).

Richness and abundance of the dominant phyla and classes were significantly related to both C and N content and C/N ratio. Correlations were positive for both richness and abundance of Acidobacteria, Actinobacteria (N content not significant for richness), Armatimonadetes and

Bacteroidetes and with the richness of Proteobacteria and Verrucomicrobia. Conversely, these three parameters were negatively correlated with both the richness and abundance of Chloroflexi, with the richness of Cyanobacteria and with the abundance of Planctomycetes and Verrucomicrobia (Figure 3, Supplementary Figures 5). pH significantly influenced phyla richnesses and abundances. There were negative correlations with both the richness and the abundance of Acidobacteria, Actinobacteria, Armatimonadetes and Bacteroidetes and with the richness of Proteobacteria and Verrucomicrobia; and positive correlations with both richness and abundance of Chloroflexi, with the richness of Cyanobacteria and with the abundance of Verrucomicrobia (Figure 3, Supplementary Figures 5). Soil moisture and P content had a significant effect on a smaller number of the dominant taxonomic groups. Soil moisture was positively correlated with the richness and abundance of Bacteroidetes, Chlamydiae and Proteobacteria and negatively with Chloroflexi (Figure 3, Supplementary Figures 5). P content was positively correlated with both richness and abundance of Bacteroidetes, the richness of Proteobacteria and Verruomicrobia and the abundance of Actinobacteria. It was also negatively correlated with both richness and abundance of Chloroflexi, with the richness of Cyanobacteria and with the abundance of Verrucomicrobia (Figure 3, Supplementary Figures 5). The trends were similar for the classes analysed within these phyla (see Supplementary Table 2 for all statistical details).

4.3.3 Community composition

Bacterial communities structure was well differentiated between the habitat types (Figure 4a, b; MRPP p=0.001, A=0.132). The same differentiation was also apparent when analysing the phyla-level composition (Supplementary Figure 6). Among variables fitted to the ordinations, all soil parameters, relative abundances of the four main shrub genera (see Supplementary Table 1) and of the different phyla, were significant, except for the relative abundances of *Betula*, *Empetrum* and Cyanobacteria (Supplementary Table 3).

Table 1. Proportion of variation in bacterial community composition explained by habitat (categorical) and soil variables (continuous), based on permutational multivariate analyses of variance. Variables with significant results in individual analyses (Table S4, Supporting Information) were added sequentially in reverse order on explained variance to a combined model. Variables that remained significant in the combined model are in bold.

All Bacteria			**Acidobacteria**			**Actinobacteria**			**Armatimonadetes**		
Variable	Variance (%)	*p*	Variable	Variance (%)	*p*	Variable	Variance (%)	*p*	Variable	Variance (%)	*p*
habitat	**26.730**	**0.0001**	habitat	**33.178**	**0.0001**	habitat	**25.249**	**0.0001**	habitat	**14.178**	**0.0001**
pH	**4.400**	**0.0001**	pH	**4.937**	**0.0001**	pH	**4.275**	**0.0009**	pH	**3.288**	**0.0004**
C/N ratio	**3.217**	**0.0027**	C/N ratio	**3.323**	**0.0030**	C/N ratio	**4.223**	**0.0006**	C/N ratio	1.718	0.2851
C	1.439	0.2206	C	1.251	0.2634	C	1.799	0.1010	Soil moisture	1.643	0.3552
N	**2.965**	**0.0068**	N	**2.789**	**0.0109**	N	**3.579**	**0.0019**	C	1.311	0.7322
P	1.335	0.2886	P	1.197	0.2934	Soil moisture	1.594	0.1640	N	1.997	0.1165
Soil moisture	1.669	0.1225	Soil moisture	1.579	0.1255	P	1.340	0.2904	P	1.405	0.6362
Residuals	58.244		Residuals	51.747		Residuals	57.941		Residuals	74.460	
Bacteroidetes			**Chlamydiae**			**Chloroflexi**			**Cyanobacteria**		
Variable	Variance (%)	*p*	Variable	Variance (%)	*p*	Variable	Variance (%)	*p*	Variable	Variance (%)	*p*
habitat	**21.732**	**0.0001**	habitat	**6.754**	**0.0001**	habitat	**20.075**	**0.0001**	habitat	**9.266**	**0.0001**
pH	**4.253**	**0.0001**	Soil moisture	**2.946**	**0.0006**	pH	**3.496**	**0.0016**	C/N ratio	**2.819**	**0.0114**
C/N ratio	**2.456**	**0.0199**	N	2.010	0.1504	C/N ratio	**3.428**	**0.0015**	pH	**2.498**	**0.0370**
Soil moisture	**3.158**	**0.0032**	pH	**2.444**	**0.0100**	C	1.536	0.2857	C	1.549	0.5352
C	1.537	0.2281	P	1.603	0.7579	Soil moisture	**3.149**	**0.0034**	Soil moisture	**3.127**	**0.0027**
N	1.849	0.1051	C	2.037	0.1321	N	1.790	0.1519	N	**4.227**	**0.0002**
P	1.487	0.2678	C/N ratio	**2.389**	**0.0152**	P	1.327	0.4641	P	1.510	0.5799
Residuals	63.527		Residuals	79.816		Residuals	65.199		Residuals	75.003	

Planctomycetes			Proteobacteria			Verrucomicrobia		
Variable	Variance (%)	*p*	Variable	Variance (%)	*p*	Variable	Variance (%)	*p*
habitat	**20.573**	**0.0001**	habitat	**25.647**	**0.0001**	habitat	**29.161**	**0.0001**
C/N ratio	**3.591**	**0.0010**	pH	**4.157**	**0.0003**	pH	**5.334**	**0.0002**
pH	**3.342**	**0.0027**	C/N ratio	**3.320**	**0.0027**	C/N ratio	**2.973**	**0.0003**
C	1.499	0.2898	C	1.285	0.3321	C	1.734	0.0774
N	**2.307**	**0.0338**	N	**3.379**	**0.0013**	N	**2.761**	**0.0103**
P	1.163	0.6371	Soil moisture	1.800	0.0912	P	1.531	0.1374
Soil moisture	1.339	0.4134	P	1.431	0.2318	Soil moisture	1.506	0.1771
Residuals	66.187		Residuals	58.983		Residuals	55.001	

When the effects of the habitat type (BG, BSC or VV) and single edaphic parameters were tested on both the total community composition and the phyla-level composition, all the variables were significant, with the habitat type always explaining the highest proportion of variation (26% for the total community; Supplementary Table 4). Conversely, when the variables were combined additionally in a model, only the type of habitat, the pH and the C/N ratio had an independent effect on the observed variance for both the total community and all the phyla considered, with the only exception of Armatimonadetes (Table 1).

Figure 2. Differences in soil parameters across the three habitats (green, Bare Ground plots; red, Biological Soil Crusts plots; blue, Vascular Vegetation plots). Letters indicate significant differences in one-way ANOVA post-hoc Tukey's HSD test (significant for $P<0.05$).

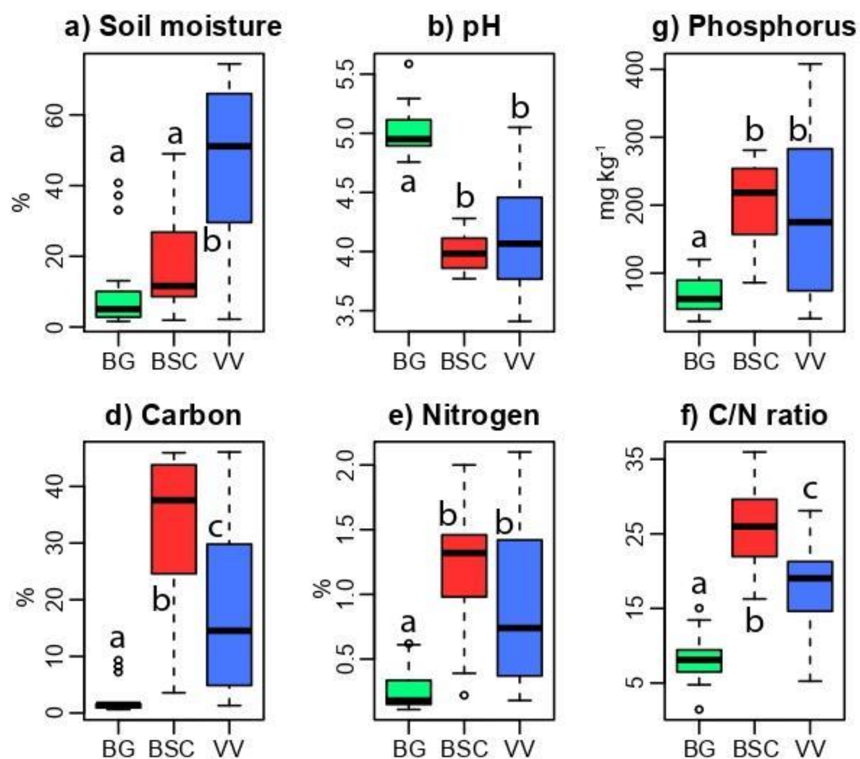


Figure 3. Summaries of linear regression models for the variation of richness of 9 dominant bacterial phyla in relation to soil parameters: soil moisture, pH, P content, C and N content and C/N ratio. The significance of the regressions is indicated as *** $P<0.001$, ** $P<0.01$, * $P<0.05$. Only significant regressions are reported. See Figure S4 (Supporting Information) for individual plots with data points shown.

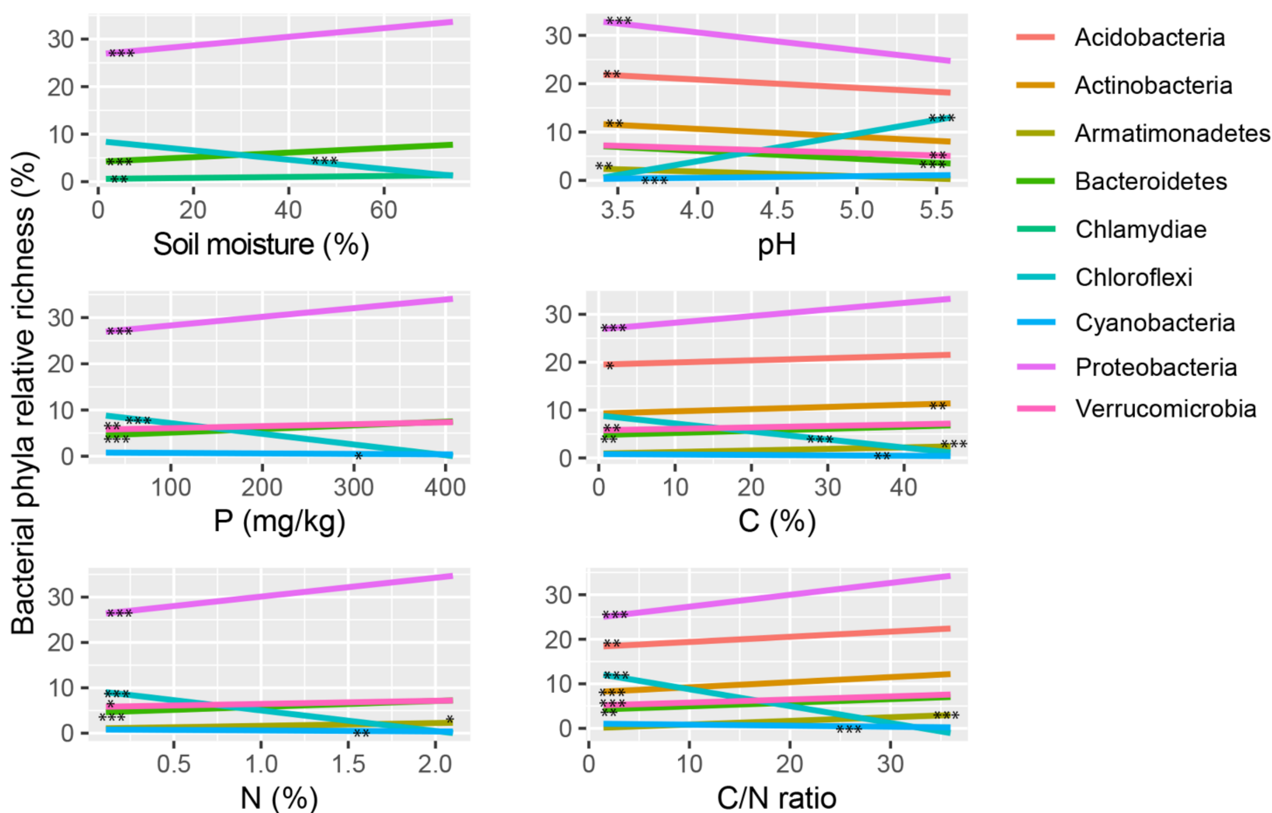
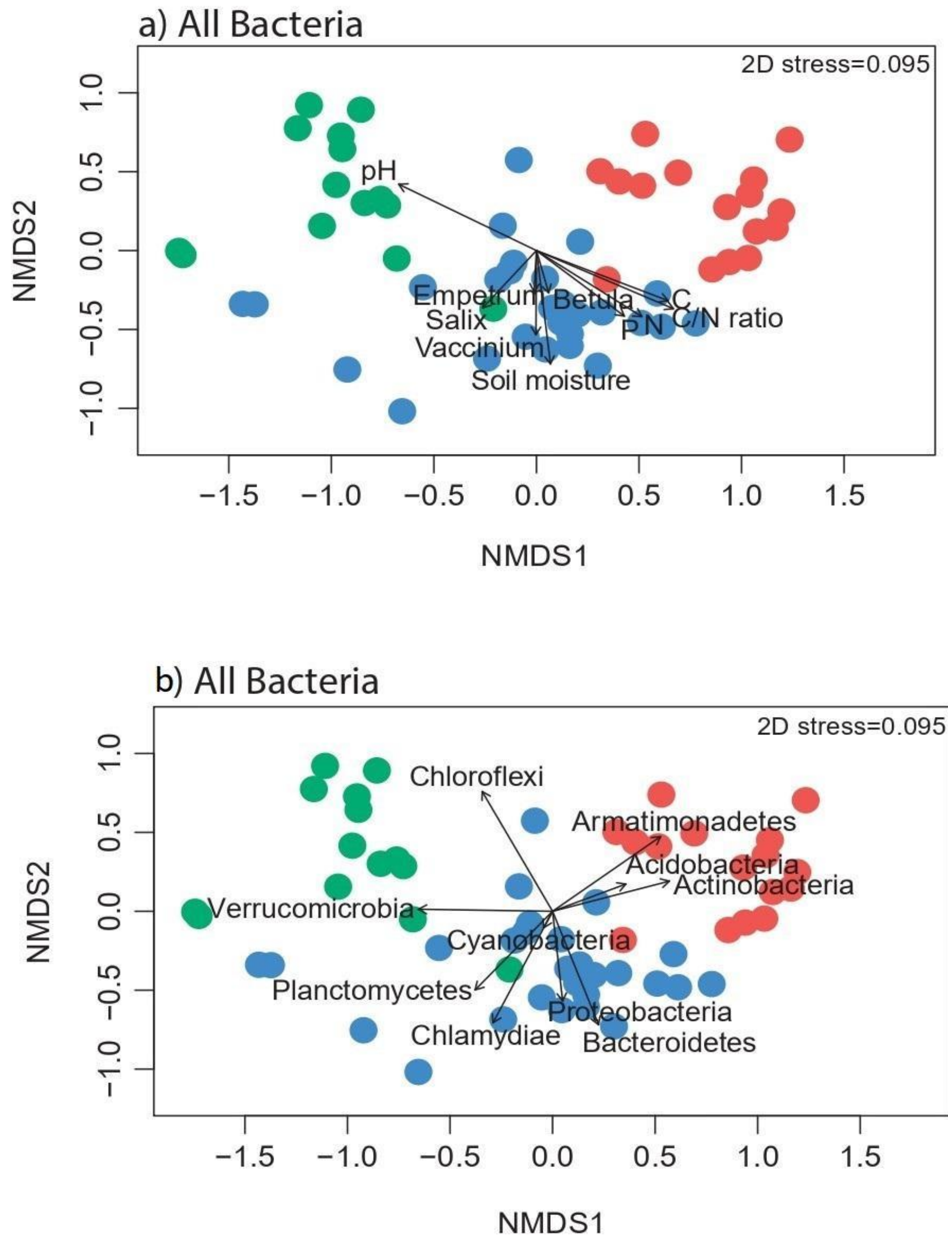


Figure 4. Nonmetric multidimensional scaling (NMDS) ordinations of the differences (Bray–Curtis distance) in composition of bacterial communities (Hellinger transformed ASVs abundances) in the habitats studied (green, Bare Ground plots; red, Biological Soil Crusts plots; blue, Vascular Vegetation plots) for the total bacteria communities. Arrows represent projections of a) edaphic variables (pH, soil moisture, carbon, nitrogen and phosphorus content, and C/N ratio) and the relative abundance values of the shrub genera (*Betula*, *Empetrum*, *Salix*, and *Vaccinium*) ; and b) relative abundances of dominant phyla.



4.3.4 Effect of shrub coverage on bacterial community composition

PerMANOVA analysis was used to test for the effect of shrub community composition on soil communities. When the abundance of different shrubs and the soil parameters were tested

independently, *Salix* coverage explained the highest variance in community composition for many groups (Supplementary Table 5), and *Betula* coverage, never significant, was the main determinant only for Cyanobacteria community composition (Table 2). pH was the second strongest predictor, explaining the highest variance in community composition for many groups (Table 2). Finally, C, N and soil moisture, and *Empetrum* abundance had a significant independent effect on the variance of total community and, to a different extent, on the phyla studied (Table 2).

4.4 Discussion

Our study reports the differences in richness and community composition along a gradient of vegetational complexity in Western Greenland. Our findings show that the structure of soil bacterial communities is strongly influenced by vegetation complexity and by the identity of dominant shrub genera. Our results partly confirm previously observed patterns, but also offers new insights. For example, Wallenstein *et al.* (2007) found that bacterial communities in Alaskan acidic tundra soils were dominated by Acidobacteria in tussock tundra, while Proteobacteria dominated shrub tundra. In our study, Acidobacteria clearly preferred BSC soils that had the lowest pH, but they were much less diverse in vascular vegetation plots with similar pH that were dominated by dwarf shrubs (Supplementary Figure 1).

Table 2. **Proportion of variation in bacterial community composition in the vascular vegetation plots explained by soil variables and relative abundance of shrub genera, based on permutational multivariate analyses of variance.** Variables with significant results in individual analyses (Supplementary Table 6) were added sequentially in reverse order on explained variance to a combined model. Variables that remained significant in the combined model are in bold.

All Bacteria			**Acidobacteria**			**Actinobacteria**			**Armatimonadetes**		
Variable	Variance (%)	*p*	Variable	Variance (%)	*p*	Variable	Variance (%)	*p*	Variable	Variance (%)	*p*
Salix	**16.467**	**0.0001**	*Salix*	**21.028**	**0.0001**	*Salix*	**20.615**	**0.0001**	*Salix*	**9.416**	**0.0001**
pH	**7.193**	**0.0013**	pH	**7.184**	**0.0039**	pH	**7.667**	**0.0005**	pH	4.565	0.2019
C	**6.068**	**0.0073**	C	**6.422**	**0.0103**	*Vaccinium*	**9.157**	**0.0006**	Residuals	86.019	
Empetrum	**4.867**	**0.0307**	N	3.369	0.2002	*Empetrum*	**4.512**	**0.0399**			
C/N ratio	3.305	0.2331	C/N ratio	3.901	0.1180	C	3.258	0.1665			
N	**4.606**	**0.0442**	P	1.958	0.7156	N	1.904	0.6643			
Vaccinium	2.490	0.5848	*Vaccinium*	3.192	0.2379	Soil moisture	**7.241**	**0.0009**			
Soil moisture	**4.644**	**0.0426**	*Empetrum*	3.366	0.1984	C/N ratio	2.202	0.5044			
P	2.839	0.4011	Soil moisture	**4.891**	**0.0428**	P	2.332	0.4502			
Residuals	47.521		Residuals	44.689		*Betula*	2.561	0.3713			
						Residuals	38.551				
Bacteroidetes			**Chlamydiae**			**Chloroflexi**			**Cyanobacteria**		
Variable	Variance (%)	*p*	Variable	Variance (%)	*p*	Variable	Variance (%)	*p*	Variable	Variance (%)	*p*

Salix	**17.137**	**0.0001**	*Empetrum*	**7.805**	**0.0001**	*Salix*	**12.335**	**0.0001**	*Betula*	**7.118**	**0.0091**
pH	5.227	0.0566	*Vaccinium*	**5.855**	**0.0034**	*Vaccinium*	**9.338**	**0.0003**	*Salix*	**6.257**	**0.154**
Empetrum	4.988	0.0713	*Salix*	**5.608**	**0.0045**	*Empetrum*	**5.505**	**0.0167**	Residuals	86.626	
C	4.594	0.1047	Soil moisture	4.516	0.0635	pH	4.155	0.1127			
Residuals	68.053		N	3.531	0.4173	C	4.113	0.1166			
			Residuals	72.684		Soil moisture	**6.741**	**0.0020**			
						N	1.893	0.9105			
						P	3.270	0.3373			
						C/N ratio	1.946	0.9000			
						Residuals	50.704				

Planctomycetes			**Proteobacteria**			**Verrucomicrobia**		
Variable	Variance (%)	p	Variable	Variance (%)	p	Variable	Variance (%)	p
Salix	**12.656**	**0.0001**	*Salix*	**15.488**	**0.0001**	pH	**15.560**	**0.0001**
pH	**6.477**	**0.0046**	pH	**7.236**	**0.0007**	*Salix*	**8.908**	**0.0002**
Vaccinium	**7.521**	**0.0019**	C	**5.573**	**0.0113**	C	**7.659**	**0.0005**
C	2.953	0.5087	*Empetrum*	**5.308**	**0.0148**	N	2.675	0.4706

C/N ratio	3.843	0.1747	C/N ratio	3.681	0.1367	Soil moisture	**6.714**	**0.0029**
Empetrum	3.938	0.1549	*Vaccinium*	3.729	0.1319	P	3.104	0.2894
N	3.536	0.2572	Soil moisture	**5.806**	**0.0082**	C/N ratio	2.549	0.5301
P	3.024	0.4711	N	2.584	0.5444	*Empetrum*	4.447	0.0503
Residuals	56.051		P	2.799	0.4284	*Vaccinium*	1.774	0.8979
			Residuals	47.797		Residuals	46.610	

For Proteobacteria, our data showed that phylum-level patterns can mask potentially important class-level differences. Out of the four Proteobacteria classes, three showed highest richness in shrub-dominated plots, while Alphaproteobacteria showed highest richness in BSC soils (Figure 1), a pattern identical to that of Acidobacteria. Acidobacteria are generally considered k-strategists, with lower growth rates, but high efficiency in converting nutrients to biomass and high tolerance to toxic compounds. This results in a greater ability to compete in oligotrophic environments, which accords with their preference for BSC plots (Kielak *et al.* 2016). Instead, Proteobacteria are generally considered copiotrophic organisms. In the Arctic tundra, organisms of this phylum have been reported to be more abundant after fertilization experiments (Koyama *et al*. 2014), likely due to increased organic matter input by vascular plants (Ramirez *et al.* 2010).

The highest number of indicator ASVs recorded in BG plots suggests that these habitats harbour a unique pool of bacteria, adapted to thrive in these conditions. Similar patterns have been reported for fungi in Eastern Greenland (Grau *et al.* 2017). With the metabarcoding approach it is not possible to estimate the proportion of sequences derived from relic DNA, often considered to be the majority of the total DNA in low biomass soils (Carini *et al.* 2016). Although we cannot rule out the possibility of some of the observed patterns to be due to such relict sequences, the sequences found exclusively in BG plots in the dataset often belonged to stress-tolerant taxa, such as those of the phylum Chloroflexi. If these taxa are typical of bare-ground habitats, the recent expansion of shrubs into these habitats would likely result in the local extinction of these stress-tolerant microbes, since they could be expected to be outcompeted in fully vegetated habitats.

The highest richness observed for BG and VV plots relative to BSC (Figure 1) differs from results found on a primary successional gradient of an Arctic glacier foreland, where richness positively correlated with vegetation complexity (Kwon *et al.* 2015). Our results also differs from previous comparisons of vegetated and non-vegetated soils, where lower diversity was observed in the former (Tam *et al.* 2001; Kumar *et al.* 2016). In this study, plant coverage is among the most crucial environmental factors influencing bacterial community composition.

In our study, a significant proportion of bacterial community variance was also explained by edaphic parameters (e.g. pH, C/N ratio), that were different among habitat types. Of these, soil pH was the best predictor of community composition and had a key role in predicting the richness and relative abundance of many taxonomic groups (Table 1). In agreement with other studies on arctic communities (Chu *et al.* 2010; 2011; Männistö *et al.* 2007), the effect of pH was significant in all habitats and its influence on community composition remained significant even when habitat type was accounted for. Indeed, the important role of pH on bacterial communities has been observed on a global scale (Lauber *et al.* 2009), even if the direct mechanism by which it regulates microbial communities composition and functionality remains largely unknown (Malard and Pearce 2018). Lauber *et al.* (2009) suggested an indirect pH effect on the availability of different cations fundamental for life. A strong correlation between the relative abundance of genes encoding several metabolic and transport pathways and pH increase has been documented, suggesting a possible greater metabolic activity of bacterial cells in higher-nutrient and alkaline conditions (Bahram *et al.* 2018).

The C/N ratio, an indicator of substrate quality, was also an important parameter in determining changes in microbial communities (Table 1). N availability is an important determining factor for soil life forms (Chen *et al.* 2014; Leff *et al.* 2015). In general, oligotrophic species (k-strategists) dominate under N-limiting conditions, such as polar regions, while under abundant N concentrations copiotrophic species (r-strategists), able to utilize more labile C sources, prevail (Fontaine *et al.* 2003; Chen *et al.* 2014). We found a higher richness and abundance of Actinobacteria and Armatimonadetes, and a lower richness and abundance of Planctomycetes in BSC plots than in BG and VV ones, with a higher C/N ratio (Supplementary figure 1). Among these phyla, Armatimonadetes, although poorly studied, are generally considered oligotrophic (Lee *et al*. 2014) and Actinobacteria, usually associated with plant roots, have been found in many desert soils (Anandan *et al*. 2016), while Planctomycetes are usually more abundant in bulk soils than in the rhizosphere (Derakshani *et al*. 2001). A higher relative abundance of Bacteroidetes and partially

Proteobacteria, generally copiotrophic, were found in VV plots in respect to BG and BSC ones, with a more balanced C/N ratio (Supplementary Figure 3 and Figure 2).

Across the three habitats, the phylum Chloroflexi showed a clear preference for the bare ground habitat (Figure 4b; Supplementary Figures 1 and 3). This phylum mostly includes oligotrophic organisms, apparently with greater stress-tolerance and/or lesser competitive capabilities than most other phyla (Costello and Schmidt 2006). In particular, the dominant Ktedonobacteria class showed a higher richness and abundance in the BG plots (Figure 1 and Supplementary Figure 2) and was the class level grouping most influenced by edaphic parameters (Supplementary Table 2). This group is known to include organisms well adapted to extremely oligotrophic conditions and has been found dominant in volcanic soils of the Atacama Desert (Lynch *et al.* 2012) and in cinder deposits of the Kilauea volcano in Hawaii (King and King 2014) as well. Additionally, it has been recently shown that organisms belonging to this class are optimal CO and H_2 oxidizers and therefore considered pioneer organisms, allowing to fix atmospheric gasses in nutrient limiting environments (Islam *et al.* 2019).

Members of Verrucomicrobia, although still poorly studied, have been recorded in soils from many different biomes, even in Antarctica (Bergmann *et al.* 2011), and reported to be more abundant in plant rhizospheres compared to bulk soils in temperate environments (Jesus *et al.* 2010; Rocha *et al.* 2010). Their close relationship with plants is also indicated by the fact that plant extracts must be added to culturing media in order to successfully isolate members of the Spartobacteria class (Sangwan *et al.* 2004). Although the richness of Verrucomicrobia did not differ significantly among the habitats studied, members of this phylum, including Spartobacteria, were significantly more abundant in BG plots (Supplementary figure 2), possibly due to their greater tolerance to low nutrient conditions. In fact, this phylum includes many slow-growing organisms that are highly sensitive to changes in soil properties and, therefore, good indicators for changes in chemical factors linked to fertility (Navarrete *et al.* 2015).

Members of the phylum Cyanobacteria, despite the lack of a clear abundance pattern, had a higher richness in BG plots (Supplementary Figure 1). Filamentous cyanobacteria, in particular, have proved to be key organisms in early stages of soil development (Budel *et al.* 2016), due to their involvement in N fixation, moisture retention, soil surface stabilization and accumulation of organic matter in nutrient-limited environments, such as Antarctic ice-free regions (Cary *et al.* 2010). For this reason, we propose that BSC samples, characterized by abundant mosses and lichens, had a lower diversity of Cyanobacteria than BG plots because the latter reflects the earlier stages of microbial colonization.

4.5 Conclusions

This study provides a detailed picture of the landscape-level compositional dynamics of soil bacterial communities in Western Greenland, where information about soil microbiota is limited. It represents, to our knowledge, one of the first metabarcoding assessments of Arctic soil bacterial communities underlying different vegetation types. Bacterial richness did not correlate with increasing vegetation complexity, but there is evidence suggestive of a possible local loss of species connected to the expansion of shrubs at the expense of other soil habitats. Additionally, we found that community composition was strongly differentiated between the habitats and was strongly shaped by the vegetation composition in well vegetated plots.

The present report provides a status of the bacterial community composition, serving as a baseline for long-term monitoring, close to the vegetation transect 'NERO line' established to monitor future changes in the species composition of the plant communities.

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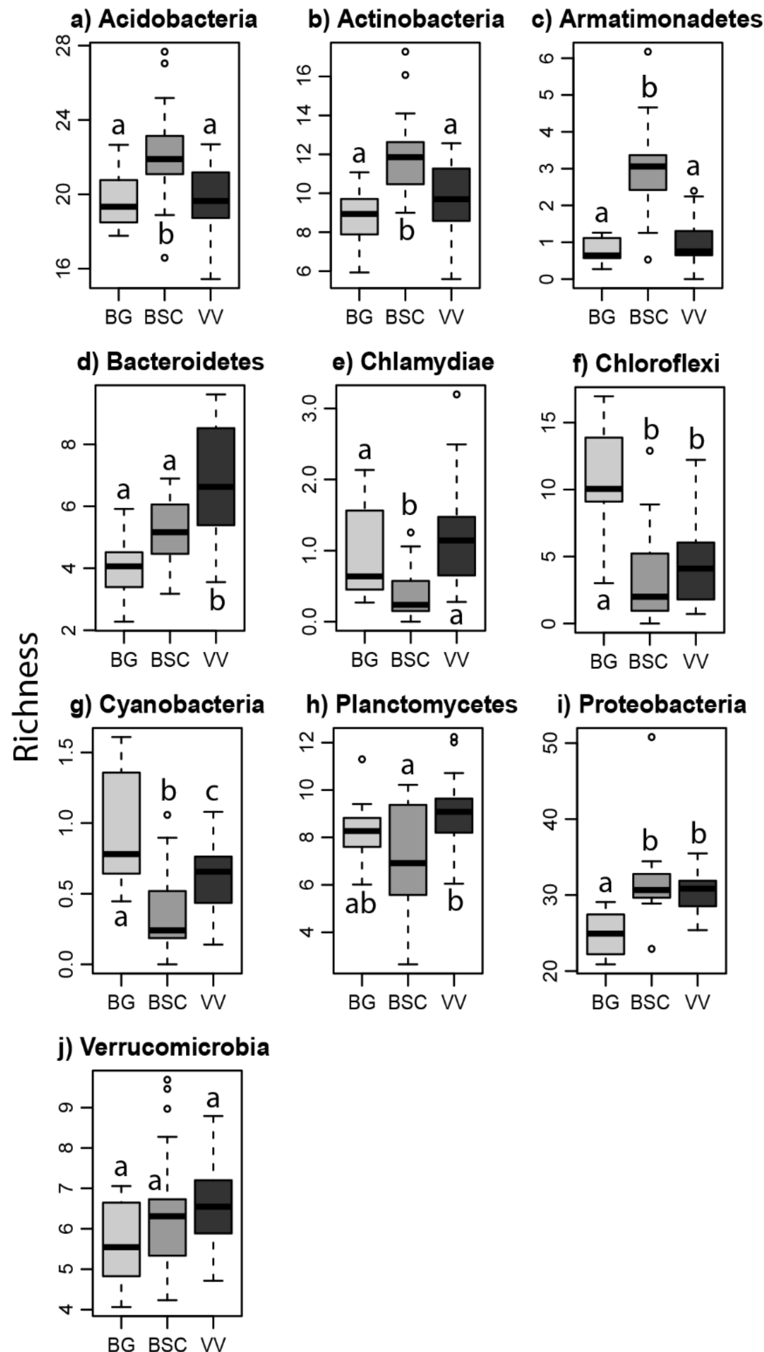
Author Contributions

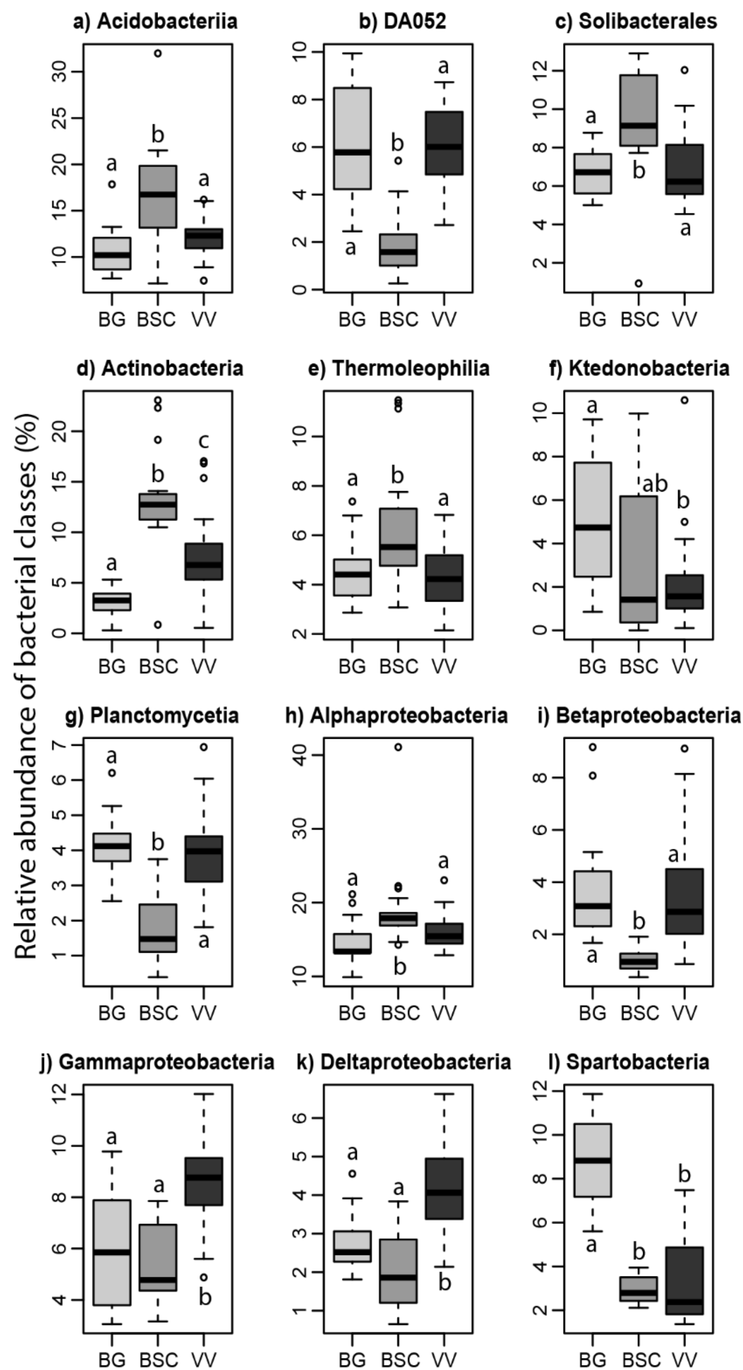
FC, LZ and JG planned and designed the experiment. CC collected the samples. FC performed DNA extraction. FC and JG performed data processing and analyses. FC, LZ and JG wrote the paper with inputs from SO and CC. FD actively participated in the revision of the manuscript.

Supplementary Material

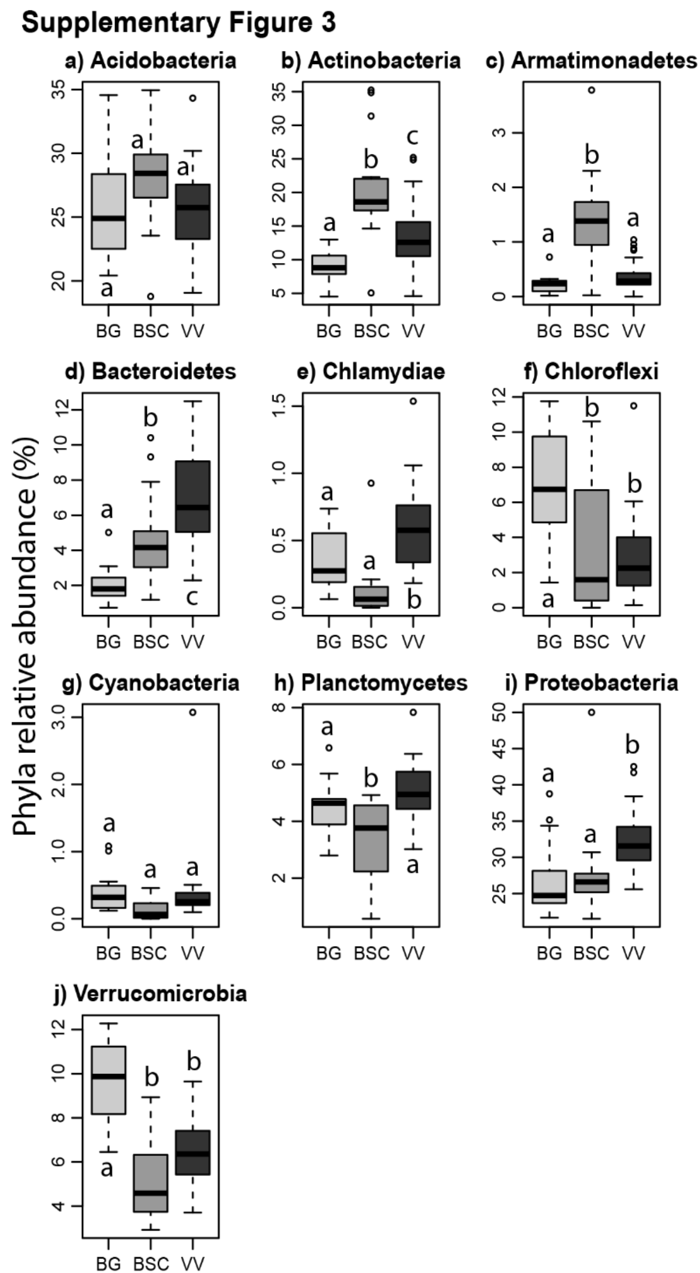
Supplementary Figure 1. Relative richness of the 10 dominant bacterial phyla in each habitat (light grey, Bare Grounds; dark grey, Biological Soil Crusts; black, Vascular Vegetation). Letters indicate significant differences in one-way ANOVA post-hoc Tukey's HSD test (significant for $p<0.05$).



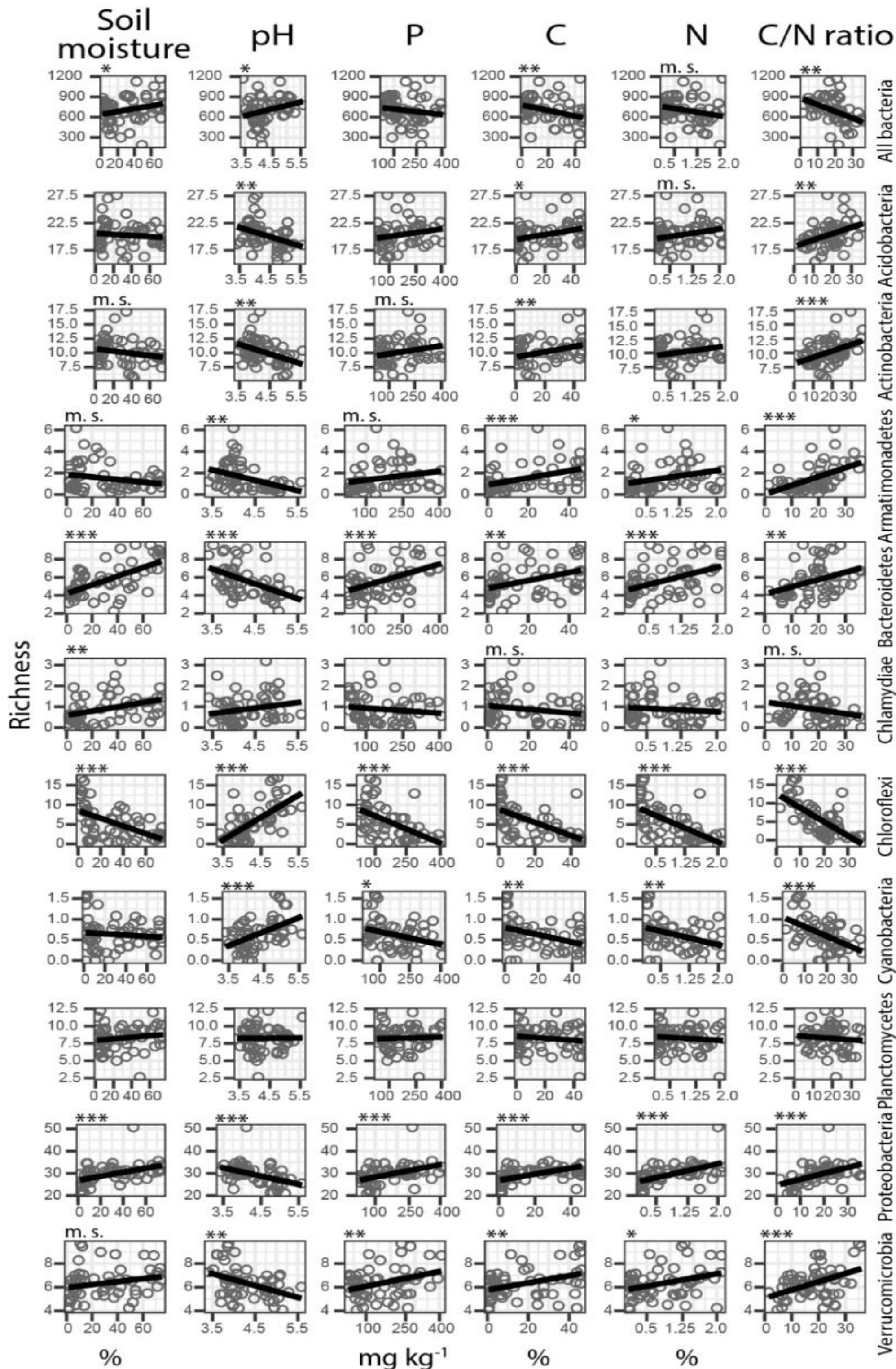
Supplementary Figure 2. **Relative abundance of the 12 dominant bacterial classes in each habitat (light grey, Bare Grounds; dark grey, Biological Soil Crusts; black, Vascular Vegetation).** Letters indicate significant differences in one-way ANOVA post-hoc Tukey's HSD test (significant for p<0.05).



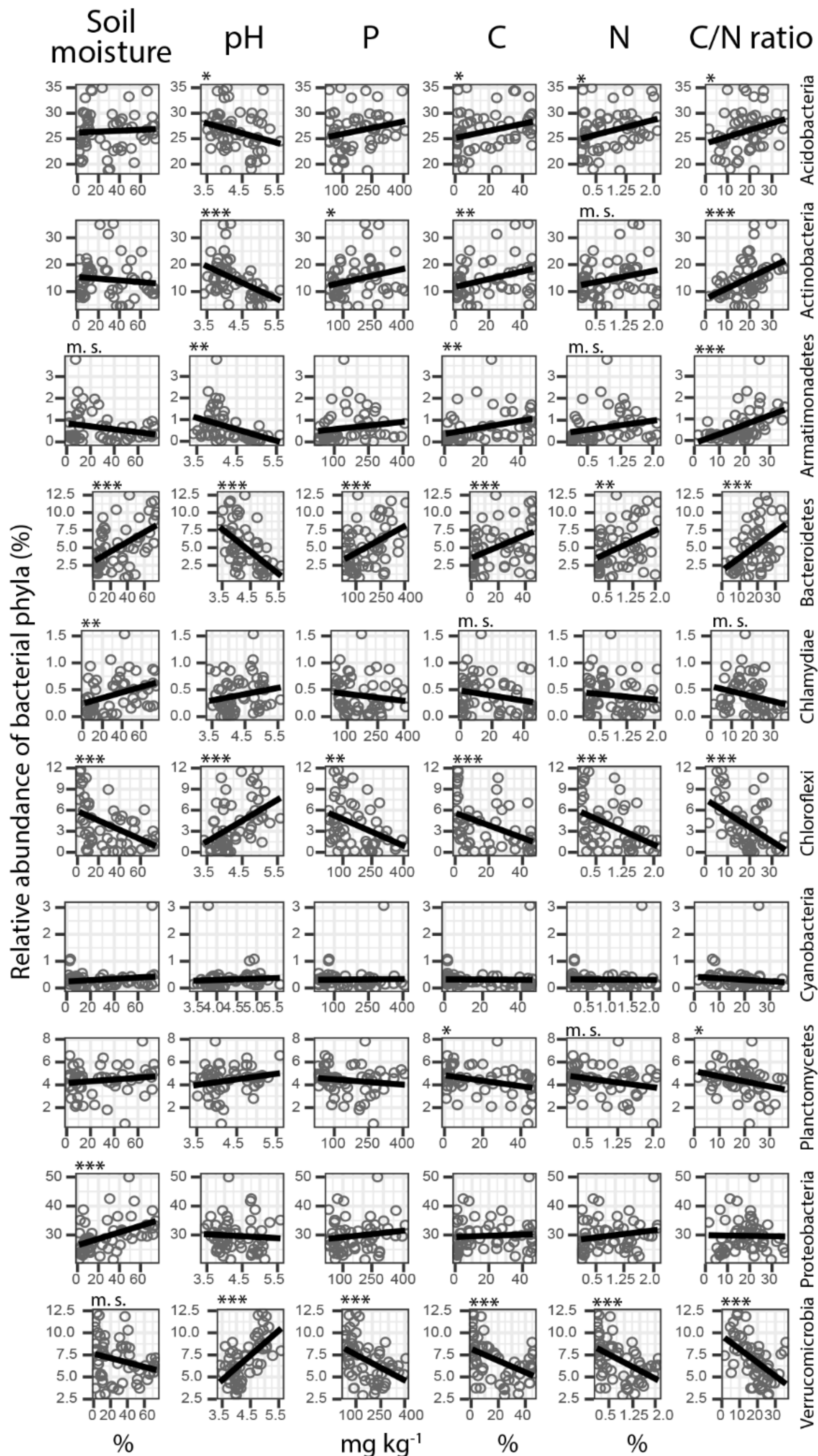
Supplementary Figure 3. **Relative abundance of the 10 dominant bacterial phyla in each habitat (light grey, Bare Grounds; dark grey, Biological Soil Crusts; black, Vascular Vegetation).** Letters indicate significant differences in one-way ANOVA post-hoc Tukey's HSD test (significant for p<0.05).



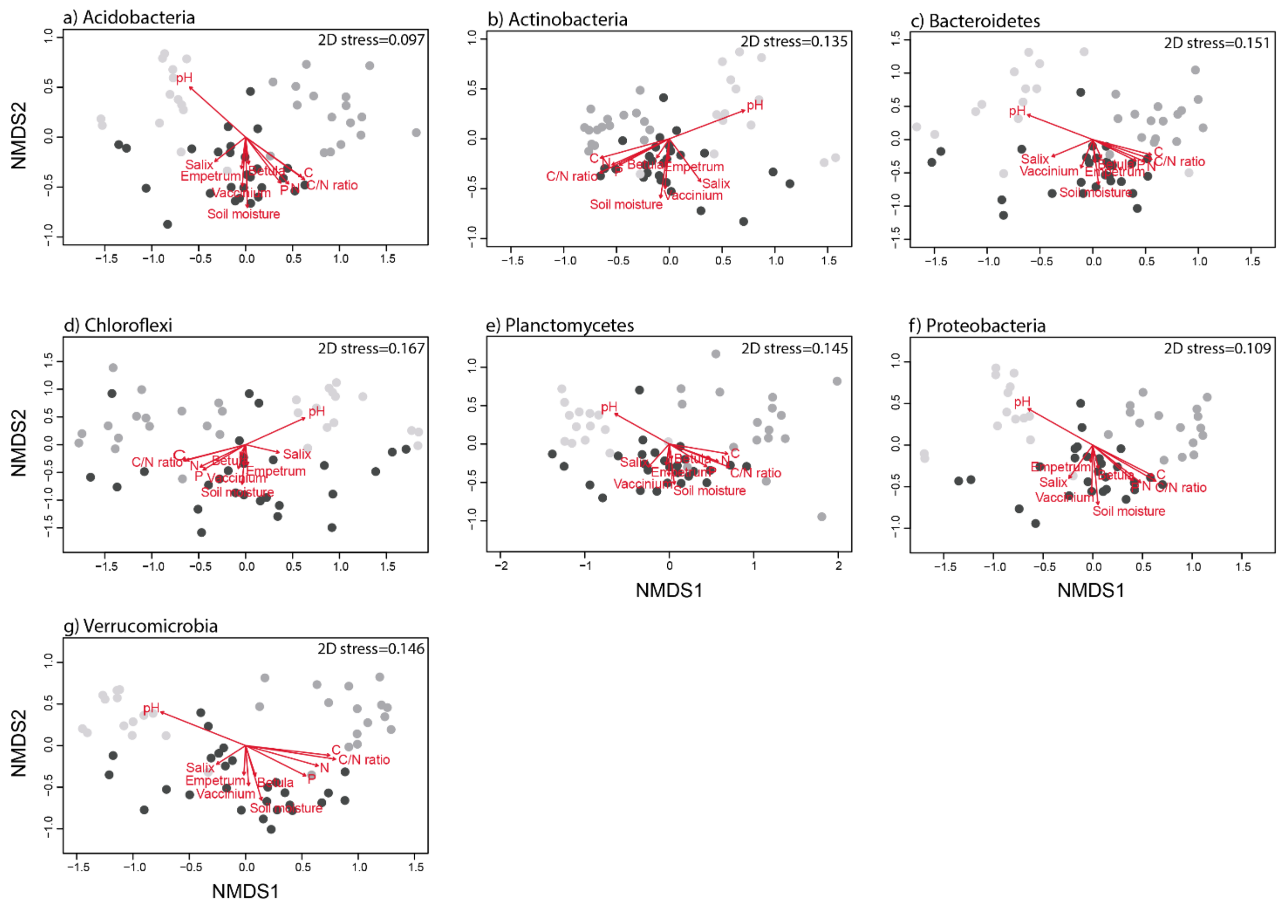
Supplementary Figure 4: **Scatter plots for the variation of richness (y-axis) of the total bacterial community and of relative richness of 10 dominant phyla in response to different soil parameters (soil moisture, pH, P, C and N content and C/N ratio; x-axis).** The significance of the regressions is indicated as *** p<0.001, ** p<0.01, * p<0.05, m.s. (marginally significant) p<0.1.



Supplementary Figure 5: Scatter plots for the variation of relative abundance (y-axis) of 10 dominant phyla in response to different soil parameters (soil moisture, pH, P, C and N content and C/N ratio; x-axis). The significance of the regressions is indicated as *** p<0.001, ** p<0.01, * p<0.05, m.s. (marginally significant) p<0.1.



Supplementary Figure 6. Nonmetric multidimensional scaling (NMDS) ordinations of the differences (Bray–Curtis distance) in composition of 7 bacterial phyla (Hellinger transformed ASVs abundances) in the habitats studied (light grey, Bare Ground; dark grey, Biological Soil Crusts; black, Vascular Vegetation) for the total bacteria communities. Edaphic variables (pH, soil moisture, C, N and P content, and C/N ratio) and the relative abundance values of the shrub genera (*Betula*, *Empetrum*, *Salix*, and *Vaccinium*) have been fitted in the ordinations.



Supplementary Table 1: Characteristics of the 20 sampling plots (altitude, coordinates and pH in single samples). For plots of vascular vegetation covered soils the percentage of the most abundant shrub genera are reported.**Supplementary Table 1**: Characteristics of the 20 sampling plots (altitude, coordinates and pH in single samples). For plots of vascular vegetation covered soils the percentage of the most abundant shrub genera are reported.

Plots	Altitude (m a.s.l.)	Geographic coordinates	pH			*Salix*	*Betula*	*Vaccinium*	*Empetrum*
Vascular Vegetation plot 1	49	64°07'53"N 51°23'10"W	4.30	4.10	4.04	1	12	5	25
Vascular Vegetation plot 2	23	64°07'55"N 51°23'04"W	3.69	3.96	3.70	0	0	10	60
Vascular Vegetation plot 3	43	64°07'56"N 51°23'02"W	3.41	3.61	3.79	0	90	5	0
Vascular Vegetation plot 4	41	64°07'58"N 51°22'58"W	3.52	3.75	3.79	0	20	35	2
Vascular Vegetation plot 5	34	64°08'04"N 51°22'48"W	4.07	3.99	3.61	0	0	0	60
Vascular Vegetation plot 6	0	64°08'12"N 51°22'36"W	4.07	4.44	4.48	0	62	0	23
Vascular Vegetation plot 7	29	64°08'05"N 51°22'48"W	4.76	3.94	4.74	2	0	15	5
Vascular Vegetation plot 8	68	64°08'18"N 51°22'27"W	4.31	4.43	4.67	0	0	0	90
Vascular Vegetation plot 9	109	64°08'25"N 51°22'18"W	4.73	5.05	4.75	30	0	15	5
Biological Soil Crust plot 1	0	64°08'12"N 51°22'36"W	3.86	4.02	3.87				
Biological Soil Crust plot 2	23	64°67'56"N 51°23'05"W	3.96	4.17	4.28				
Biological Soil Crust plot 3	43	64°07'56"N 51°23'02"W	4.19	3.81	4.11				
Biological Soil Crust plot 4	66	64°07'53"N 51°23'12"W	4.16	3.86	3.79				
Biological Soil Crust plot 5	83	64°07'51"N 51°23'21"W	4.03	3.97	4.07				
Biological Soil Crust plot 6	107	64°07'53"N 51°23'11"W	3.77	3.91	3.99				
Bare Ground plot 1	15	64°14'48"N 51°17'45"W	4.95	4.76	5.14				
Bare Ground plot 2	25	64°10'44"N 51°22'31"W	4.82	4.94	5.29				
Bare Ground plot 3	45	64°07'59"N 51°22'43"W	4.87	5.59	5.28				
Bare Ground plot 4	77	64°07'53"N 51°23'20"W	5.09	4.92	4.78				
Bare Ground plot 5	109	64°08'25"N 51°22'17"W	4.95	5.06	5.05				

Supplementary Table 2. Regression slopes and r^2 for the variation of relative richness and abundance of 13 dominant classes in response to soil physicochemical characteristics (significant for $p<0,05$; §marginally significant, $p<0.1$; n. s. non-significant).

		Soil moisture (%)		pH		P content (mg kg^{-1})		C content (%)		N content (%)		C/N ratio	
		slope	r^2	slope	r^2	slope	r^2	slope	r^2	slope	r^2	slope	r^2
Acidobacteria	Richness	n. s.		-3.391	0.3227	0.0114	0.1369	0.1031	0.299	2.3898	0.2105	0.2282	0.3524
	Abundance	n. s.		-3.261	0.1385	0.0129	0.0813	0.1159	0.1809	2.8395	0.1421	0.2297	0.1683
DA052	Richness	0.0149	0.0971	n. s.		n. s.		-0.0164§	0.0491	n. s.		-0.0391	0.0730
	Abundance	0.0428	0.1402	n. s.		n. s.		-0.0318	0.0245	n. s.		-0.0990	0.1368
Solibacteres	Richness	-0.0249	0.1249	-0.7141§	0.0386	n. s.		n. s.		n. s.		n. s.	
	Abundance	-0.0350	0.1084	-1.2498	0.0594	n. s.		n. s.		n. s.		0.0787	0.0557
Actinobacteria	Richness	-0.0173§	0.0427	-1.5948	0.2293	0.0044	0.0594	0.0434	0.1667	0.7915	0.0643	0.1023	0.2263
	Abundance	n. s.		-5.762	0.307	0.0176	0.1043	0.1433	0.1844	3.070	0.1066	0.3705	0.3045
Thermoleophilia	Richness	n. s.		-0.3770§	0.04111	n. s.		0.0118§	0.0403	n. s.		0.0287	0.0642
	Abundance	-0.0295	0.1121	n. s.		n. s.		n. s.		n. s.		n. s.	
Ktedonobacteria	Richness	-0.0869	0.3038	3.8960	0.2927	-0.0173	0.2274	-0.1245	0.3012	-3.4805	0.3166	-0.2815	0.3714
	Abundance	-0.0586	0.229	1.6522	0.0764	-0.0087	0.0873	-0.0589	0.1029	-1.7415	0.1236	-0.1306	0.1236
Planctomycetia	Richness	n. s.		1.4772	0.1463	-0.0058	0.0854	-0.0609	0.2632	-1.4291	0.1895	-0.1196	0.2406
	Abundance	n. s.		1.3011	0.2243	-0.0049	0.1207	-0.0477	0.3093	-1.1072	0.2188	-0.0976	0.3092
Alphaproteobacteria	Richness	n. s.		-3.095	0.1178	0.0105§	0.0452	0.1097	0.1537	2.6498	0.1163	0.2337	0.1679
	Abundance	n. s.		n. s.		n. s.		n. s.		n. s.		n. s.	
Betaproteobacteria	Richness	0.0247	0.1562	1.0359	0.1289	n. s.		-0.0228	0.0536	n. s.		-0.0484	0.0592
	Abundance	0.0230§	0.0433	0.2243	0.2606	-0.0061	0.0655	-0.0497	0.1202	-0.9310§	0.0472	-0.1085	0.1393
Gammaproteobacteria	Richness	0.0391	0.2616	-0.9517	0.0617	0.0047	0.0596	0.0253§	0.0386	0.8251	0.0629	n. s.	
	Abundance	0.0507	0.2862	-1.276	0.0748	0.0060	0.0650	n. s.		0.8072§	0.0321	n. s.	
Deltaproteobacteria	Richness	0.0401	0.3819	n. s.		0.0051	0.1062	0.0225§	0.0432	0.8630	0.102	n. s.	
	Abundance	0.0320	0.292	n. s.		n. s.		n. s.		n. s.		n. s.	
Spartobacteria	Richness	-0.0255	0.6181	0.6948	0.2084	-0.0038	0.2501	-0.0191	0.1546	-0.6709	0.2668	-0.0312	0.0915
	Abundance	-0.0654	0.2718	4.5543	0.6567	-0.0176	0.3858	-0.1152	0.4166	-3.2009	0.4318	-0.2549	0.4895

Supplementary Table 3. Regression r^2 and significance of each variable fitted in the NMDS analysis of the total community (Figure 4 a and b). Significant values in bold.

Variables	**Community composition**	
	r^2	p value
pH	**0.6272**	**0.001**
Soil moisture	**0.5208**	**0.001**
P	**0.3557**	**0.001**
C	**0.5137**	**0.001**
N	**0.4399**	**0.001**
C/N ratio	**0.5821**	**0.001**
Salix	**0.2012**	**0.001**
Betula	0.0751	0.127
Vaccinium	**0.2841**	**0.002**
Empetrum	0.0975	0.137
Acidobacteria	**0.1573**	**0.010**
Actinobacteria	**0.3608**	**0.001**
Armatimonadetes	**0.5010**	**0.001**
Bacteroidetes	**0.5678**	**0.001**
Chlamydiae	**0.5837**	**0.001**
Chloroflexi	**0.6936**	**0.001**
Cyanobacteria	0.0128	0.753
Planctomycetes	**0.3907**	**0.001**
Proteobacteria	**0.3244**	**0.001**
Verrucomicrobia	**0.5743**	**0.001**

Supplementary Table 4. Proportion of variation in bacterial community composition, explained by soil variables calculated independently with permutational multivariate analysis of variance, based on Hellinger-transformed community matrices. Significant variables were included in the final model for each group (Table 1).

Variable	**All Bacteria**		**Acidobacteria**		**Actinobacteria**		**Armatimonadetes**		**Bacteroidetes**		**Chlamydiae**	
	Variance (%)	p	Variance (%)	p	Variance (%)	p	Variance (%)	p	Variance (%)	p	Variance (%)	p
habitat	26.73	0.001	33.18	0.001	25.25	0.001	14.18	0.001	21.73	0.001	6.75	0.001
Soil moisture	8.52	0.001	9.30	0.001	8.53	0.001	4.93	0.001	8.66	0.001	4.09	0.001
pH	15.46	0.001	18.07	0.001	15.72	0.001	6.82	0.001	11.45	0.001	2.58	0.009
P	8.81	0.001	10.12	0.001	8.47	0.001	3.62	0.002	6.05	0.001	2.55	0.014
C	12.29	0.001	15.09	0.001	12.45	0.001	4.65	0.001	7.56	0.001	2.55	0.019
N	10.07	0.001	11.93	0.001	10.06	0.001	3.81	0.001	6.47	0.001	2.75	0.002
C/N ratio	15.02	0.001	18.07	0.001	14.96	0.001	6.05	0.001	9.62	0.001	2.42	0.022

Variable	**Chloroflexi**		**Cyanobacteria**		**Planctomycetes**		**Proteobacteria**		**Verrucomicrobia**	
	Variance (%)	p	Variance (%)	p	Variance (%)	p	Variance (%)	p	Variance (%)	p
habitat	20.07	0.001	9.27	0.001	20.57	0.001	25.65	0.001	29.16	0.001
Soil moisture	7.81	0.001	3.45	0.004	5.58	0.001	8.66	0.001	9.79	0.001
pH	11.00	0.001	4.57	0.001	12.82	0.001	14.63	0.001	17.96	0.001
P	7.29	0.001	2.77	0.042	7.52	0.001	8.49	0.001	11.46	0.001
C	9.21	0.001	4.25	0.001	10.97	0.001	11.68	0.001	15.25	0.001
N	7.56	0.001	3.33	0.005	8.69	0.001	9.80	0.001	13.11	0.001
C/N ratio	10.69	0.001	5.52	0.001	13.02	0.001	14.62	0.001	17.75	0.001

Supplementary Table 5. Proportion of variation in bacterial community composition of VV plots explained by soil variables and shrub genera relative abundance calculated independently with permutational multivariate analysis of variance, based on Hellinger-transformed community matrices. Significant variables (in bold) were included in the final model for each group (Table 2).

Variable	**All Bacteria**		**Acidobacteria**		**Actinobacteria**		**Armatimonadetes**		**Bacteroidetes**		**Chlamydiae**	
	Variance (%)	p	Variance (%)	p	Variance (%)	p	Variance (%)	p	Variance (%)	p	Variance (%)	p
Soil moisture	**7.73**	**0.013**	**7.59**	**0.028**	**8.37**	**0.009**	3.69	0.598	6.14	0.071	**5.60**	**0.023**
pH	**13.54**	**0.001**	**16.89**	**0.001**	**13.75**	**0.001**	**8.12**	**0.001**	**12.91**	**0.002**	4.06	0.350
P	**7.66**	**0.011**	**8.75**	**0.009**	**7.31**	**0.015**	4.00	0.462	5.83	0.069	4.53	0.153
C	**9.27**	**0.002**	**10.92**	**0.004**	**9.87**	**0.002**	4.40	0.310	**6.50**	**0.049**	5.09	0.050
N	**8.05**	**0.005**	**9.13**	**0.010**	**8.48**	**0.014**	4.17	0.386	5.84	0.079	**5.29**	**0.023**
C/N ratio	**8.15**	**0.006**	**8.78**	**0.012**	**7.77**	**0.021**	4.68	0.251	5.68	0.105	4.13	0.310
Salix	**16.47**	**0.001**	**21.03**	**0.001**	**20.61**	**0.001**	**9.42**	**0.001**	**17.14**	**0.001**	**6.34**	**0.003**
Betula	5.74	0.061	6.01	0.069	**6.86**	**0.048**	5.55	0.081	5.13	0.148	4.38	0.213
Vaccinium	**7.97**	**0.004**	**8.34**	**0.017**	**11.77**	**0.003**	3.12	0.796	5.41	0.108	**6.96**	**0.002**
Empetrum	**8.19**	**0.006**	**7.64**	**0.028**	**11.12**	**0.004**	3.86	0.474	**6.88**	**0.032**	**7.80**	**0.002**

Variable	**Chloroflexi**		**Cyanobacteria**		**Planctomycetes**		**Proteobacteria**		**Verrucomicrobia**	
	Variance (%)	p	Variance (%)	p	Variance (%)	p	Variance (%)	p	Variance (%)	p
Soil moisture	**7.47**	**0.006**	4.40	0.247	5.48	0.063	**7.85**	**0.004**	**11.31**	**0.001**

pH	**8.13**	**0.004**	4.49	0.255	**11.66**	**0.001**	**12.84**	**0.001**	**15.56**	**0.001**
P	**6.93**	**0.018**	3.69	0.476	**5.96**	**0.039**	**7.44**	**0.007**	**10.86**	**0.001**
C	**7.70**	**0.006**	4.56	0.219	**7.22**	**0.011**	**9.00**	**0.003**	**12.45**	**0.001**
N	**6.99**	**0.017**	4.31	0.282	**6.13**	**0.029**	**7.82**	**0.004**	**11.32**	**0.001**
C/N ratio	**6.37**	**0.030**	3.65	0.540	**6.92**	**0.020**	**8.49**	**0.005**	**10.15**	**0.001**
Salix	**12.33**	**0.001**	**5.93**	**0.025**	**12.66**	**0.001**	**15.49**	**0.001**	**14.06**	**0.001**
Betula	4.68	0.184	**7.12**	**0.017**	5.17	0.096	5.68	0.054	5.59	0.090
Vaccinium	**9.39**	**0.001**	5.56	0.066	**7.83**	**0.004**	**8.02**	**0.007**	**7.25**	**0.020**
Empetrum	**8.70**	**0.003**	4.19	0.323	**6.29**	**0.034**	**8.64**	**0.003**	**8.74**	**0.002**

Chapter 5

GlobalFungi, a global database of fungal occurrences from high-throughput-sequencing metabarcoding studies

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Abstract

Fungi are key players in vital ecosystem services, spanning carbon cycling, decomposition, symbiotic associations with cultivated and wild plants and pathogenicity. The high importance of fungi in ecosystem processes contrasts with the incompleteness of our understanding of the patterns of fungal biogeography and the environmental factors that drive those patterns. To reduce this gap of knowledge, we collected and validated data published on the composition of soil fungal communities in terrestrial environments including soil and plant-associated habitats and made them publicly accessible through a user interface at http://globalfungi.com. The GlobalFungi database contains over 600 million observations of fungal sequences across >17 000 samples with geographical locations and additional metadata contained in 178 original studies with millions of unique nucleotide sequences (sequence variants) of the fungal internal transcribed spacers (ITS) 1 and 2 representing fungal species and genera. The study represents the most comprehensive atlas of global fungal distribution, and it is framed in such a way that third-party data addition is possible.

Table 1. List of metadata contained in the GlobalFungi database. The table lists identifiers, units and sources of metadata contained in the database with the description of their content. The data source "original paper" may also represent additional metadata provided by the authors of the paper.

Metadata identifier	Unit	Description of content	Source
Sample ID		unique identifier	generated
Longitude	degrees	Geographical longitude	original paper
Latitude	degrees	Geographical latitude	original paper
Continent		One of the following: Africa/Antarctica/Asia/Australia/Europe/North America/South America	original paper
Sample type		One of the following: soil/rhizosphere soil/litter/litter+humus/deadwood/lichen/shoot/root	original paper
Biome		One of the following: forest biome/woodland biome/shrubland biome/grassland biome/desert biome/tundra biome/mangrove biome/anthropogenic terrestrial biome/marine biome/freshwater biome/polar desert biome	original paper
Sampling year		Year of sample collection	original paper

Primers		Primers used	original paper
pH		pH	original paper
ITS total		Number of full ITS sequences extracted	generated
MAT (°C)	°C	Mean annual temperature from CHELSA database	CHELSA
MAP (mm)	mm	Mean annual precipitation from CHELSA database	CHELSA

5.1 Background & Summary

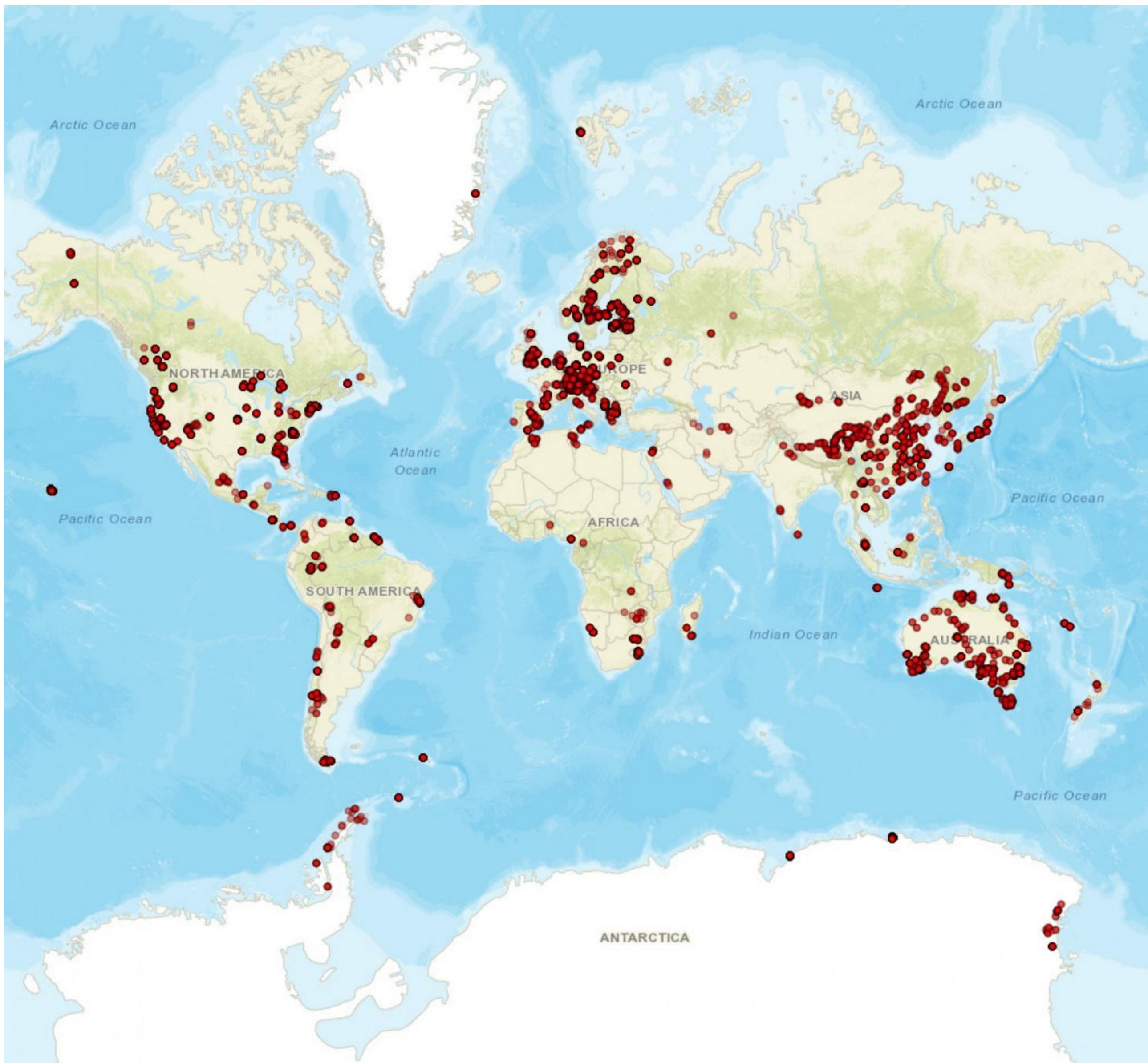
Fungi play fundamental roles in the ecosystem processes across all terrestrial biomes. As plant symbionts, pathogens or major decomposers of organic matter they substantially influence plant primary production, carbon mineralization and sequestration, and act as crucial regulators of the soil carbon balance[1,2]. The activities of fungal communities contribute to the production of clean water, food, and air and the suppression of disease-causing soil organisms. Soil fungal biodiversity is thus increasingly recognized to provide services critical to food safety and human health[3].

It is of high importance to determine how environmental factors affect the diversity and distribution of fungal communities. So far, only a few studies have focused on fungal distribution and diversity on global scale[4-6]. Importantly, these single survey studies focused either on a limited number of biomes[4,5], fairly narrow groups within the fungal kingdom[6], or were restricted only to fungi inhabiting soil. Although individual studies have the advantage of standardized methodology across their whole dataset, their limitation is in the limited sampling efforts in space and time that do not allow general conclusions on distribution of fungal taxa. On the other hand, since the advent of high-throughput-sequencing methods, large amounts of sequencing data on fungi from terrestrial environments accumulated along with metadata across numerous studies and allow interesting analyses when combined[7]. As an example of this approach, the meta-analysis of 36 papers made it possible to map global diversity of soil fungi collected in >3000 samples and indicated that climate is an important factor for the global distribution of soil fungi[8]. This approach clearly demonstrated the utility of a meta-study approach to address fungal biogeography, ecology and diversity. In addition, the

compilation of these data demonstrated the fact that symbiotic mycorrhizal fungi that aid cultivated and wild plants to access nutrients, are more likely to be affected by rapid changes of climate than other guilds of fungi, including plant pathogens[8] and helped to identify which fungi tend to follow alien plants invading new environments[9].

Here, we have undertaken a comprehensive collection and validation of data published on the composition of fungal communities in terrestrial environments including soil and plant-associated habitats. This approach enabled us to construct the GlobalFungi database containing, on March 16, 2020, over 110 million unique sequence variants[10] (i.e., unique nucleotide sequences) of the fungal nuclear ribosomal internal transcribed spacers (ITS) 1 and 2, covering >17 000 samples contained in 178 original studies (Figure 1). The ITS region has been used as molecular marker because it is a universal barcode for fungi[11].The dataset of sequence variant frequencies across samples, accompanied by metadata retrieved from published papers and in global climate databases is made publicly available at http://globalfungi.com. To achieve the goal to make published data findable, accessible, interoperable and reusable, the user interface at the above address allows the users to search for individual sequences, fungal species hypotheses[12], species or genera, to get a visual representation of their distribution in the environment and to access and download sequence data and metadata. In addition, the user interface also allows authors to submit data from studies not yet covered and in this way to help to build the resource for the community of researchers in systematics, biogeography, and ecology of fungi.

Figure 1. Map of locations of samples contained in the GlobalFungi database. Each point represents one or several samples where fungal community composition was reported using high-throughput-sequencing methods targeting the ITS1 or ITS2 marker of fungi. The map was created using the 'leaflet' package that uses an open-source JavaScript library for mobile-friendly interactive maps (Leaflet 1.6.0, GNU General Public License).



5.2 Methods

5.2.1 Data selection

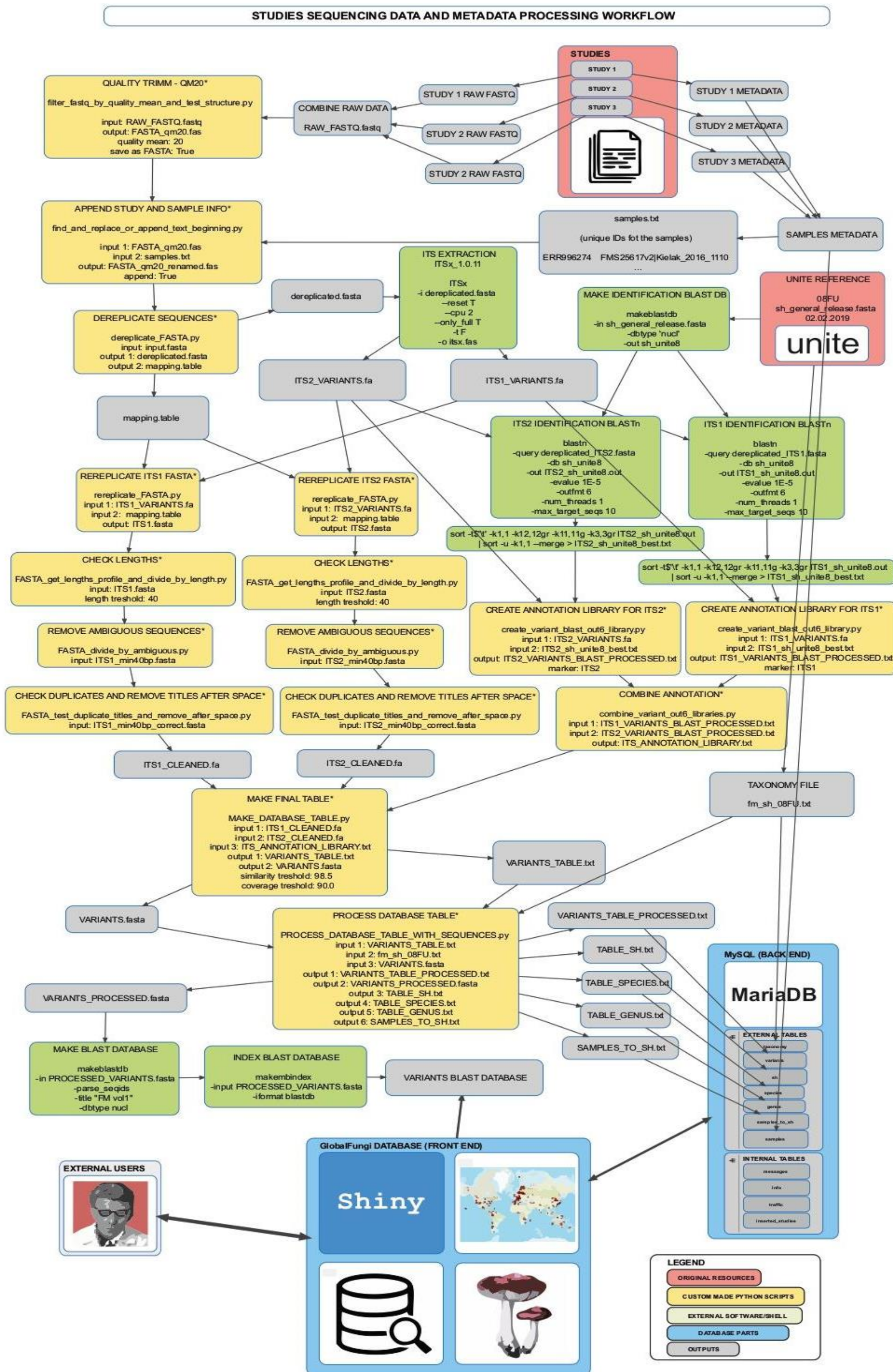
We explored papers fitting with a main criterion, i.e., high-throughput sequencing for the analysis of fungal communities thanks to the ITS region, and that were published up to the beginning of 2019; in total, we explored 843 papers. The following selection criteria were used for the inclusion of samples (and, consequently, studies) into the dataset: (1) samples came from terrestrial biomes of soil, dead or live plant material (e.g., soil, litter, rhizosphere soil, topsoil, lichen, deadwood, root, and

shoot) and were not subject to experimental treatment that artificially modifies the fungal community composition (e.g., temperature or nitrogen increase experiment, greenhouse controlled experiment were excluded); (2) the precise geographic location of each sample was recorded and released using GPS coordinates; (3) the whole fungal community was subject to amplicon sequencing (studies using group-specific primers were excluded); (4) the internal transcribed spacer regions (ITS1, ITS2, or both) were subject to amplification; (5) sequencing data (either in fasta with phred scores reported or fastq format) were publicly available or provided by the authors of the study upon request, and the sequences were unambiguously assigned to samples; (6) the samples could be assigned to biomes according to the Environment Ontology (http://www.ontobee.org/ontology/ENVO)[8]. In total, 178 publications contained samples that matched our criteria (Online-Only Table 1).

5.2.2 Processing of sequencing data

For the processing of data, see Figure 2 and Code Availability section. Raw datasets from 178 studies, covering 17 242 individual samples were quality filtered by removing all sequences with the mean quality phred scores below 20. Each sequence was labelled using the combination of a sample ID and sequence ID, and the full ITS1 or ITS2 fungal region was extracted using Perl script ITSx v1.0.11[13]. ITS extraction resulted in a total of 416 291 533 full ITS1 and 231 278 756 full ITS2 sequences. The extracted ITS sequences were classified according to the representative sequence of the closest UNITE species hypothesis (SH) using BLASTn[14], using the SH created considering a 98.5% similarity threshold (BLASTDBv5, general release 8.1 from 2.2.2019[12]). A sequence was classified to the best best hit SH only when the following thresholds were met: e-value $<10e^{-50}$, sequence similarity >=98.5%. All representative sequences annotated as nonfungal were discarded. All representative sequences classified to any fungal SH and all unclassified sequences were used to build database library of unique nucleotide sequences (sequence variants). The number of sequence variants accessible through the database is 113 423 871.

Figure 2. Processing of raw sequencing data for the GlobalFungi database. Workflow of processing of sequencing data included in the GlobalFungi database.



5.2.3 Sample metadata

Sample metadata were collected from the published papers and/or public repositories where they were submitted by the authors. In some cases, metadata were obtained from the authors of individual studies upon request. The samples were assigned to continents, countries, and specific locations when available, and all sites were categorized into biomes following the classification of Environment Ontology to a maximum achievable depth for each sample. The complete list of metadata included in the database is presented in Table 1.

In addition to the metadata provided by the authors of each study, we also extracted bioclimatic variables from the global CHELSA[15] and WorldClim 2[16] databases for each sample based on its GPS location. Since the results based on CHELSA and WorldClim 2 were comparable, we decided to include those from CHELSA, because precipitation patterns are better captured in the CHELSA dataset, in particular for mountain sites[15].

For each sequence variant that was classified to SH, fungal species name and genus name was retrieved from the UNITE database[12], when available.

5.3 Data Records

The raw sequencing reads used to create the database are available at different locations (see Table 2).

Table 2. List of identifiers and source database of the raw sequencing datasets used.

Database	Accession Identifiers (in superscripts, respectively: dataset reference, study reference(s))

National Center for Biotechnology Information Sequence Read Archive	SRP001058[17,197] SRP001175[18,198], SRP006078[19,199], SRP012868[20,200], SRP013695[21,201], SRP013944[22,202], SRP015735[23,203], SRP016090[24,204], SRP026207[25,205], SRP028404[26,206], SRP033719[27,207], SRP035356[28,208], SRP040314[29,209], SRP040786[30,210], SRP041347[31,211], SRP043106[32,212], SRP043706[33,4,213,214], SRP043982[34,215], SRP044665[35,216], SRP045166[36,217], SRP045587[37,218], SRP045746[38,219], SRP045933[39,220], SRP046049[40,221], SRP048036[41,222,223], SRP048856[42,224], SRP049544[43,225], SRP051033[44,226], SRP052222[45,227], SRP052716[46,228], SRP055957[47,229], SRP057433[48,230], SRP057541[49,231], SRP058508[50,232], SRP058555[51,233], SRP058851[52,234], SRP059280[53,235], SRP060838[54,236], SRP061179[55,237], SRP061305[56,238], SRP061904[57,239], SRP062647[58,240], SRP063711[59,241], SRP064158[60,242], SRP065817[61,243], SRP066030[62,244], SRP066284[63,245], SRP066331[64,246], SRP067301[65,247], SRP067367[66,248], SRP068514[67,249], SRP068608[68,250], SRP068620[69,251], SRP068654[70,252], SRP069065[71,253], SRP069742[72,254], SRP070568[73,255], SRP073070[74,256], SRP073265[75,257], SRP074055[76,258], SRP074496[77,259], SRP075989[78,260], SRP079403[79,261], SRP079521[80,262], SRP080210[81,263], SRP080428[82,264], SRP080680[83,265], SRP082472[84,266], SRP082976[85,267], SRP083394[86,268], SRP083434[87,268], SRP083901[88,269], SRP087715[89,270], SRP090261[90,271], SRP090335[91,272], SRP090490[92,273], SRP090651[93,274], SRP091741[94,275], SRP091855[95,276], SRP091867[96,277], SRP092609[97,278], SRP092777[98,279], SRP093592[99,280], SRP093928[100,281], SRP094708[101,282,283], SRP097883[102,284], SRP101553[103,285], SRP101605[104,286], SRP102378[105,287], SRP102417[106,288], SRP102775[107,289], SRP106137[108,290], SRP106774[109,291], SRP107174[110,292], SRP107743[111,293], SRP109164[112,294], SRP109773[113,295], SRP110522[114,296], SRP110810[115,297], SRP113348[116,298], SRP114697[117,299], SRP114821[118,300], SRP115350[119,301], SRP115464[120,302], SRP115599[121,303], SRP117302[122,304], SRP118875[123,305], SRP118960[124,306], SRP119174[125,307], SRP125864[126,308], SRP132277[127,309], SRP132591[128,310], SRP132598[129,310], SRP136886[130,311], SRP139483[131,312], SRP142723[132,313], SRP148813[133,314], SRP150527[134,315], SRP151262[135,316], SRP153934[136,317], SRP160913[137,318], SRP161632[138,319], SRP195764[139,320]
European Nucleotide Archive Sequence Read Archive	ERP001713[140,321], ERP003251[141,322], ERP003790[142,323], ERP005177[143,324], ERP005905[144,325], ERP009341[145,326], ERP010027[146,327], ERP010084[147,328], ERP010743[148,329], ERP011924[149,330], ERP012017[150,331], ERP013208[151,332], ERP013987[152,333], ERP014227[153,334], ERP017480[154,335], ERP017851[155,336], ERP017915[156,337], ERP019580[157,338], ERP019924[158,339], ERP020657[159,340], ERP022511[160,341], ERP022742[161,342], ERP023275[162,343], ERP023718[163,344], ERP023855[164,345], ERP106131[165,346], ERP107634[166,347], ERP107636[167,347], ERP110188[168,348], ERP112007[169,349]
DNA Data Bank of Japan	DRA000926[170,350], DRA000937[171,351], DRA001737[172,352], DRA002424[173,353], DRA002469[174,354], DRA003024[175,355], DRA003730[176,356], DRA004913[177,357], DRA006519[178,358], DRP002783[179,359], DRP003138[180,360], DRP005365[181,361]
Dryad Digital Repository	https://doi.org/10.5061/dryad.2fc32[182,362], http://dx.doi.org/10.5061/dryad.n82g9[183,363], http://dx.doi.org/10.5061/dryad.2343k[184,364], https://doi.org/10.5061/dryad.gp302[185,365], http://dx.doi.org/10.5061/dryad.cq2rb[186,366], https://doi.org/10.5061/dryad.8fn8j[187,367], https://doi.org/10.5061/dryad.216tp[188,368]
GenBank	KAYV00000000.1[189,369], KAYU00000000.1[190,369], KAYT00000000.1[191,369], SAMN02934078[192,370], SAMN02934079[193,370]
Australian Antarctic Data Center database	http://dx.doi.org/10.4225/15/526f42ada05b1[194,371]
Supplemental Data	Hartmann et al (2012)[Supplementary_Data2,372], Rime et al (2016)[Fungi_SeqsID,373]

The database contains two data types: sequence variants (individual nucleotide sequences) and samples. For each sequence variant, the following information is stored: sequence variant code, identification of samples where sequence variant occurs and the number of observations, the SH of best hit (when available), fungal species name (when available), fungal genus name (when available). For each sample, metadata information is stored (Table 1). Sequence data and metadata are accessible at Figshare195 (GlobalFungi_ITS_variants.zip, GlobalFungi_metadata.xlsx). All database content is accessible using a public graphical user interface at http://globalfungi.com.

5.4 Technical Validation

The technical validation included the screening of the data sources, sequencing data and data reliability. Regarding the data source screening, the data sources (published papers) were screened to fulfil the criteria outlined in the Methods section. The dataset was thoroughly checked for duplicates, and for all records that appeared in multiple publications, only the first original publication of the dataset was considered as a data source. Considering sequence quality, we have only utilized those primer pairs that are generally accepted to target general fungi (see Online-only Table 1)[7,196]. Sequences were quality filtered by removing all sequences with the mean quality phred scores below 20 and sequences that did not represent complete ITS1 or ITS2 after extraction or those that were identified as chimeric by the ITS extraction software[13] were removed. All representative sequences where the BLASTn search against the UNITE database[12] resulted in a nonfungal organism, were discarded.

For data reliability, the geographic location represented by the GPS coordinates was validated first. For each sample set, the geographic location of the sample described in the text of the study was confronted with the location on the map. For those samples where disagreement was recorded (e.g., terrestrial samples positioned in the ocean or where the location of another region than described in the text), the authors of each study were asked for correction. Studies that could not be reconciled in this way were excluded from our database. The quality of sample metadata was checked and if they

were outside the acceptable range (such as content of elements or organic matter >100%), these invalid metadata were removed.

5.5 Usage Notes

The user interface at http://globalfungi.com enables the users to access the database in several ways (Figure 3). In the taxon search, it is possible to search for genera and species of fungi and for the 98.5% SH species hypotheses of UNITE, contained in the general release 8.1 from 2.2.2019.

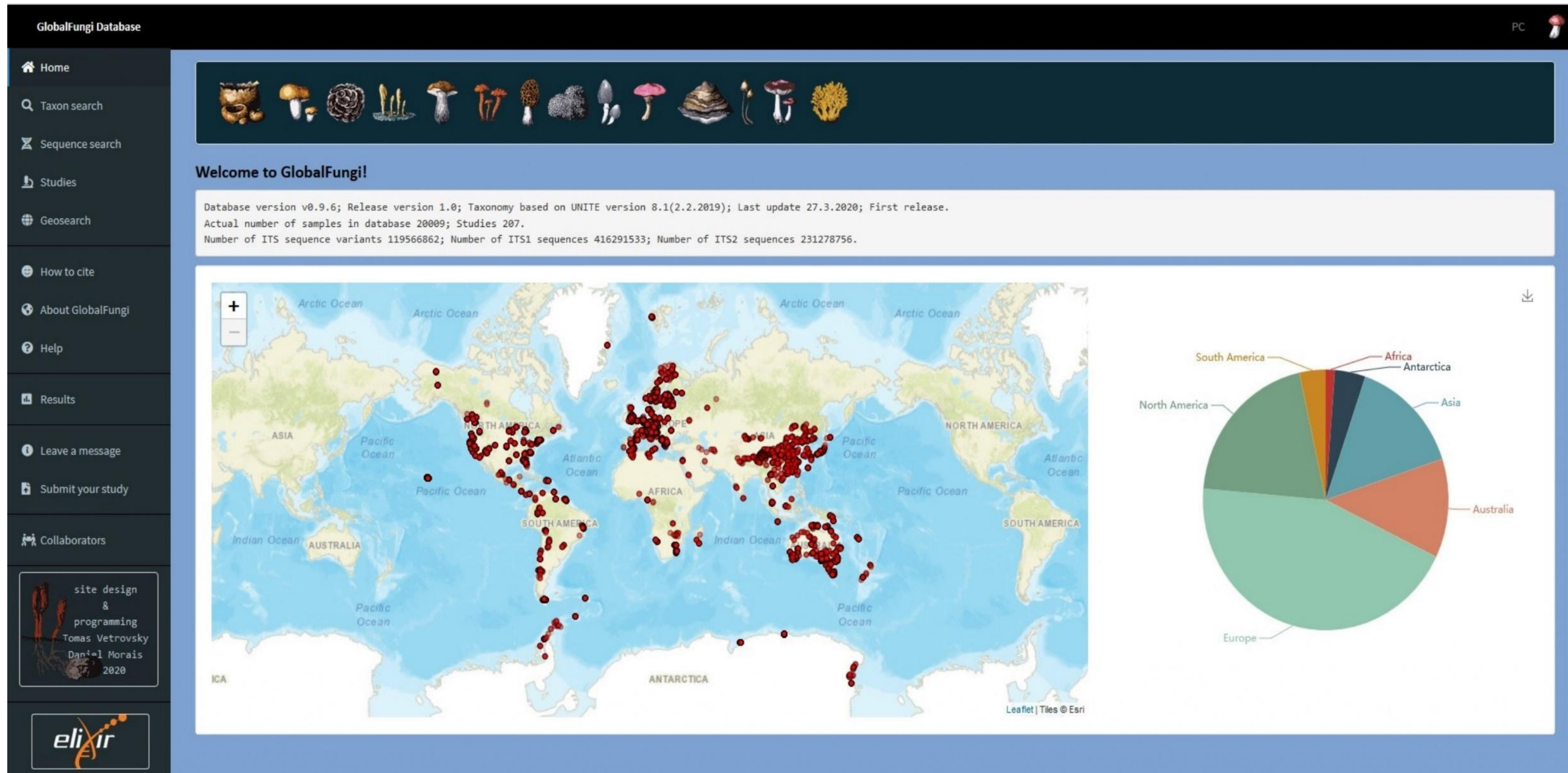
The search results open the options to download the corresponding SH or the corresponding sequence variants. It is also possible to view a breakdown of samples by type, biome, mean annual temperature, mean annual precipitation, pH, and continents. The results also contain an interactive map of the taxon distribution with relative abundances of sequences of the taxon across samples and a list of samples with metadata. Several modes of filtering of results are available as well.

In the sequence search, it is possible to search for multiple nucleotide sequences by choosing if the result will be the exact match or a BLAST result. The BLAST option gives the possibility to retrieve the sequence variant best hit in the database, or, when only one sequence is submitted, it gives the possibility to display multiple ranked high score hits among the sequence variants.

It is also possible to open individual studies and access their content. Finally, in the geosearch, users can select a group of samples on the map, with a range of tools, and retrieve data for these samples (such as the FASTA file with all occurring sequence variants).

Importantly, the database is intended to grow, both by the continuing activity of the authors and by using the help of the scientific community. For that, the “Submit your study” section of the web interface enabling the submission of studies not yet represented is available to users. The submission tool guides the submitting person through the steps where details about the publication, samples, sample metadata and sequences are sequentially collected.

Figure 3. User interface to access the GlobalFungi database.



The submitted data will be added twice a year, each year, to the existing dataset after processing and validation by the authors. Thus, users inserting their data, besides a precious contribution to mycological progress, will benefit several advantages as make their data accessible to the international scientific community in an easily accessible form and get visibility. Users can also maximize their visibility by approving to add their name and affiliation to the online list of collaborators and/or to the GlobalFungi Group Author' list that will be mentioned in future publications describing the database content, its development, or metastudies using the whole database.

Among the possible uses of the GlobalFungi Database, fungal ecologists will be able to link fungal diversity data with the panel of collected metadata, which should allow them to determine the environmental factors driving the fungal diversity. This kind of study can be done at different geographic levels, from country scale up to the entire world, and for all the fungal communities or by focusing on some ecosystem compartments. This should lead to a better understanding of the biogeography of the fungal diversity. Větrovský et al.[8] brought interesting findings by doing this for soil fungal communities at the scale of the globe. The evolutionary biologists could study, for example, the effect of global change on the fungal diversity by comparing the natural versus anthropogenic biomes. In addition to focus on the fungal diversity, some studies could trigger specific fungi. Thus, mycologists could determine the biogeography of one specific fungal species. They could also determine the composition of the fungal communities associated with the focused species and detect some potential recurrent fungal associations. The GlobalFungi Database could also speed up the progress in fungal taxonomy by highlighting the existence of a high number of fungal sequences not currently assigned to species along with environmental metadata promoting thus the interest in their description.

Code Availability

The workflow included several custom made python scripts (labelled by star in the Figure 2) which are accessible here: https://github.com/VetrovskyTomas/GlobalFungi.

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Author contributions

T.V., P.K. and P.B. jointly conceived the study. C.L. and coordinated data acquisition, T.V., D.M., M.K. and P.B. designed the database, T.V. and D.M. developed the database and created the user interface. C.A.G., S.A.H., B.D.B., K.B., V.B., F.D., R.Z.H., M.J., J.K., C.L., S.L., R.L.M., T.Mar., T.Maš., L.Me., L.Mi., T.Mi., S.M., D.N., I.O., S.P.C., M.Š., K.Š., V.T., M.U., L.V., J.V. and L.Ž. identified data sources, processed and analysed sequencing information, collated and analysed metadata. P.B. drafted the manuscript along with T.V., C.L. and P.K. All authors wrote and reviewed the manuscript.

T.V., D.M., P.K. and C.L. contributed equally to this study.

Competing interests

The authors declare no competing interests.

Chapter 6

Discussions

Over the past century, CO_2 levels have steadily increased and global temperature has risen accordingly. As early as 1896, Svante Arrhenius laid the foundation for the modern theory of the greenhouse effect and climate change and even now, more than a hundred years since the first early warning, the possible effects of climate change cannot be predicted with certainty (Grassl and Metz, 2013). As soil microorganisms are largely responsible for cycling of soil organic carbon and other nutrients, they have a key role in climate feedback, including the production and consumption of greenhouse gases such as CO_2, CH_4 and N_2O. Despite the number of studies carried out so far, it is difficult to predict whether soil will become a source or sink of greenhouse gases under future climate scenarios (Friedlingstein et al., 2006; Wang et al., 2017) . Therefore, it will be necessary to understand how climate change will affect the soil community composition and biodiversity, the organic matter decomposition dynamics and the resulting nutrient cycling patterns, before predicting the fate of global C and N cycles, as well as the functioning of natural and managed ecosystems. For example, warming may directly influence the kinetics of microbial enzymes, accelerating the rates of organic matter decomposition, in particular a greater temperature sensitivity for decomposition of recalcitrant carbon pools could occur (Knorr et al., 2005). Moreover, climate-driven changes may indirectly affects plant productivity and diversity, which, in turn, alter soil physicochemical conditions, the supply of carbon to soil and the structure and activity of microbial communities involved in decomposition processes and carbon release from soil (Bardgett et al., 2008)

Given the key role of microorganisms in maintaining the balance of cold environments, they could be viewed both as sentinels and amplifiers of global change, especially if we consider the spatial coverage of the corresponding biomes. In fact, the ecosystems at the coldest margins of life on Earth, including alpine and arctic ecosystems, broadly characterized as the ‘tundra biome’ are among those most vulnerable to rapid climate change (Ernakovich et al., 2014).

This thesis wanted to contribute to this topic, by studying soils from alpine and arctic environments. We firstly investigated the possible interaction between different types of vegetation and below-ground soil microbiome along an alpine climatic gradient. Then, we studied the differences among soil microbial communities under different types of vegetation complexity in Greenland, starting from bare grounds without any apparent coverage, to biological soil crusts dominated by mosses and lichens, and ending with soils covered by vascular vegetation. Both studies expected a shrub expansion at the expense of all other vegetation species.

Higher altitudes are generally associated with increasing environmental harshness, i.e. colder climate, lower nutrient and water availability, increasing acidity and lower plant biomass, which in turn influence microbial activities (Ma et al., 2004; Margesin et al., 2009; Donhauser and Frey, 2018). The same was recorded at the Stelvio Pass, where alpine soils at higher altitude were characterized by lower water, C, H, N-NH_4, and K contents compared to subalpine soils at lower altitude. Using the spectrophotometric assays we obtained the first screening of samples, and a small range of microbial enzymes specific for a few substrates was investigated. It evidenced significantly different enzymatic patterns between alpine and subalpine soils. These preliminary results agree with those obtained by higher resolution approaches, namely metagenomics and metatranscriptomics, which are able to identify a wider range of enzymes. Due to the sequencing of the entirety of microbial genomes and transcripts from a microbiota, metagenomics and metatranscriptomics represent useful tools for globally cataloguing microbial gene and transcript profiles and reflecting overall metabolic functions of microorganisms. The use of these "omic" analyses confirmed the marked differences in microbial community composition of soils below different types of vegetation, suggesting a strong capacity of vegetation to shape the belowground microbiome. Similarly, in Arctic soils, the type of coverage showed to be the main predictor of community composition and the shrub composition a fundamental parameter in vegetated plots.

Although it was assumed that the increase of environmental harshness with altitude should result in a decrease of soil microbiome diversity (Margesin et al., 2009; Donhauser and Frey, 2018), in this

study soils at higher altitude showed higher alpha diversity of the soil microbiome. In accordance with these results, some studies already reported the same opposite trend. A meta-analysis study, where the effects of global change on soils were investigated by C and nutrients additions, highlighted negative effects on microbial richness (Zhou et al., 2020). Bacterial diversity observed in previous comparisons among soils of differing complexity showed to be lower in plots with higher vegetation complexity (Tam et al. 2001; Kumar et al. 2016). As well in Greenland, although bacterial richness did not correlate with increasing vegetation complexity, a high number of species unique of simplest ecosystems was recorded, that are expected to be outcompeted with the expansion of shrubs, suggesting a possible loss of local diversity.

Unlike enzyme assays, metagenomics and metatranscriptomics give us deeper information about microbial community composition and functionality, and exactly who is more abundant or more active under certain conditions. In this study, bacteria and fungi represented the major important groups. Metagenome showed a higher presence of bacteria in all soil communities, while metatranscriptome showed an increase of fungi, although with lower values than bacteria one. Archaea and other non-fungal eukaryota were less abundant. While archaea showed higher abundances in snowbed soils due to their ability to adapt at low temperatures, as already reported by Cavicchioli et al. (2000) for Antarctic soils, other eukaryota were more abundant in shrubland soils. The fungal : bacterial ratio increased significantly in shrubland soils, possibly determined by a greater amount of recalcitrant plant detritus (Lauber et al., 2008; Li et al., 2017) and the higher capabilities of fungi to transform and utilize them (Malik et al., 2016; Lopez-Mondéjar et al., 2018). Looking at the taxonomic composition of the communities, *Proteobacteria* and *Ascomycota* were the most abundant phyla in metagenomic and metatranscriptomic results. Both these phyla have been recorded as dominant in arctic tundra soils in Greenland as well, being able to encompass an enormous level of morphological, physiological and metabolic diversity, and therefore playing a pivotal role in global C and N cycling (Kersters et al., 2006; Egidi et al., 2019). Alpine snowbed soils were also characterised by high activity of *Chloroflexi* and *Acidobacteria*, confirming previous reports that

suggested their association with oligotrophic soils (Costello and Schmidt 2006; Kielak et al. 2016). It agrees with their presence in Greenland's soils beneath lower complexity vegetations. On the other hand, lower altitude soils were characterized by higher activity of *Firmicutes* and *Mucoromycota*. In this light, the GlobalFungi database allows us the possibility to associate fungi to different habitats and soil compartments and follow their distribution across the world, suggesting interesting information about their adaptation to specific environments. As ribosomal proteins are rapidly synthesized by growing cells, in order to ensure sufficient ribosome content in daughter cells, their transcription was used as a proxy of cellular growth (Lladó et al., 2019). In this study, the share of bacterial growth was considerably higher than fungal one, suggesting their faster turnover in terms of biomass.

An increase of arthropods, also called litter transformers, was observed in shrubland soils. Their role will be essential for the shrubs expansion, because they improve the soil fertility. Firstly, arthropods accelerate the breakdown of complex organic molecules into simpler forms, making more easily available to the decomposer microbes, and secondly they affect the physical structure of the soil, improving its porosity, that is essential to provide an adequate aeration and water-holding capacity, and facilitate root penetration.

Exploring the microbial activity, higher enzyme activity was recorded in subalpine soils, associated with higher C, ammonium, H and water contents. Analysing the repertoire of carbohydrate-degrading enzymes, grouped on the base of their participation in the utilization of distinct C sources, CAZymes degrading α-glucans represented the largest share of both the metagenome and metatranscriptome, indicating the importance of the use of reserve C compounds as starch and glycogen. This was true for all soils, except for shrubland soils that showed lower expression of genes targeting α-glucans, along with an increase of genes targeting pectin and, to a lesser extent, cellulose and glucuroxylans. These biopolymers of plant origin are much more recalcitrant compared to α-glucans, suggesting the possible degradation of recalcitrant C sources in subalpine shrubland and, thus, a potential positive feedback to climate warming with the predicted shrubs expansion. Unlike the above results,

spectrophotometric enzyme assays showed that alpine soils compared to subalpine soils were correlated with higher abundance of oxidative enzymes, able to degrade more recalcitrant carbon. The difference of results could be linked to the smaller spectrum of enzymes analyzed with the former approach and to the difficulty to reproduce in laboratory the same environmental conditions of sampling time. Furthermore, abundance of transcripts targeting peptidoglycans showed the same trends as overall bacterial expression. Regarding nitrogen cycling, N is generally a limiting primary nutrient in high altitude ecosystems (Rütting et al., 2015), resulting in a strong competition between plants and microorganisms. High abundances of genes and transcripts involved in microbial ammonia incorporation into glutamine and glutamate were recorded, which are important nitrogen donors in nitrogenous compounds metabolism in microbial cells (Pengpeng and Tan, 2013). These high values suggest a marked ability of microorganisms in ammonium sequestration in all soils. The high share of transporters of organic N containing compounds clearly confirm that organic N recycling is the major pathway in all studied soils. This source is partly supplemented by N_2 fixation. Another essential element to many soil biological processes is P, for nucleic acid synthesis, respiration, and enzymatic activity. Compared to alpine soils, acidic phosphatase and P transporters were more abundant in subalpine soils, suggesting that the higher vegetation productivity characterizing these latter sites, could result in a strong competition for P between plants and microorganisms. For that, microbes enhance their mobilizing and P uptake, contrasting the possible limitation of this nutrient.
KEGG database provides information for understanding high-level functions and utilities of a biological system. As for transporters, the most abundant KEGG metabolisms in both metagenome and metatranscriptome were energy, carbohydrate and amino acids metabolisms in all soil samples, providing the necessary energy and organic sources for microbial growth, mobility and material exchange. Subalpine shrubland soils were associated with a higher abundance of genes and transcripts for environmental adaptation, meaning a great functional performance and an expected more stable and resilient ability to environmental perturbations.

Finally, no significant differences in enzyme activity and communities composition and functioning were recorded, comparing experimental warming plots and relative controls in both alpine vegetation types, despite the increase of temperature recorded within the OTCs. Nevertheless, slight differences in enzyme activity patterns were only observed between OTCs and controls in snowbed soils compared to grassland soils, suggesting that warming may more rapidly affect colder and more vulnerable soils, according to previous studies (Tscherko et al., 2001; Meng et al. 2020; Matteodo et al., 2016). The ongoing studies of warming effects indicate that one or two decades of warming are necessary until pronounced shifts of soil parameters, microbial composition and functioning are recorded (De Angelis et al., 2017, Olivares et al., 2019). We here confirm the need to conduct long-term warming experiments to be able to explore the effect of warming on C and N cycling.

Drawing insights from short-term experimental manipulations and elevation gradient studies, we unravel strong linkages between above- and belowground ecosystems. Results showed that vegetation shift induced by prolonged warming might be substantially more important in size compared to short-term effects in the present vegetation context. If warming did not increase the net primary productivity and accelerate biogeochemical cycling, improving nutrient availability, the space-for-time substitutions may lead to pronounced changes in microbial community structure and function. Indeed, elevational turnover in microbial community composition can be pronounced and varies according to plant type, suggesting that there will be interdependencies among plant and soil microbial responses to climate warming.

Chapter 7

Conclusions and future perspectives

Climate change in cold environments, as arctic and alpine regions, will likely have both short-term effects on ecosystems, by modifying above- and belowground linkages, and long-term impacts, with the expected expansion of shrubs along latitude and altitude, respectively.

In the Arctic, we studied the bacterial communities associated to three different soil habitats in Western Greenland, beneath vegetation increasing coverage. The type of coverage resulted to be the main predictor of community composition, and the shrub composition a fundamental parameter in vegetated plots. Despite bacterial richness did not correlate with increasing vegetation complexity, a high number of unique species were recorded in the simplest ecosystems that are expected to be outcompeted with the expansion of shrubs, suggesting a possible loss of local diversity.

Regarding alpine regions, the upwards subalpine shrubland migration may increase the mean annual soil temperature, decrease the microbial alpha diversity and increase the share of fungi, which are correlated with higher amount of carbohydrate-active enzymes, specific for degrading fungal biomass and recalcitrant plant biopolymers, such as pectin, cellulose, and glucuroxylans.

In this light, shrubs expansion may be connected with a possible loss of species, an increase of more recalcitrant C decomposition, and a decrease of total ecosystem C storage, thereby increasing the efflux of CO_2 to the atmosphere and having a positive feedback to warming. On the other hand, using five years of manipulative warming devices, we demonstrated that increases of mean annual air temperature around 1°C do not provide any evidence of effects, and long-term warming experiments are necessary to detect possible effects on soil parameters and microbial communities.

Although the increasing metagenomic and metatranscriptomic studies represent per se a challenge to our exploration of soil composition and functioning, we should not forget that the complexity of soil as a system itself brings multiple challenges that go beyond basic methodologies. Most importantly, it is the complexity of soil structure with vertical stratification, variation in space and the existence

of specific microhabitats, such as plant root surfaces, rhizosphere, bulk soil which differ largely in properties, nutrient content, rates of biochemical processes and temporal dynamics. Moreover, the size of a soil core is also of limited use when we need to provide answers on the stand level, ecosystem level or biome level. As already done for the metabarcoding data, which were used to create a global fungal database, the increase of the metagenomic and metatranscriptomic studies will allow to have available large amounts of sequencing data on microbial composition and functioning from different terrestrial environments and may give us interesting results when combined. Meta-analysis studies may represent a useful tool to better appreciate how environmental factors may affect the diversity, distribution and functioning of soil microbiota worldwide.

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