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Supraglacial ecosystems: ecological role and anthropogenic impact

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1. GENERAL INTRODUCTION

The cryosphere is the portion of the Earth that presents water in the solid phase for one or more months of the year. It covers up to one fifth of the Earth's surface and includes snow, ice caps, ice sheets, sea ice, permafrost, ice clouds and glaciers (Vincent et al. 2004; Fountain et al. 2012; Boetius et al. 2015; Barry 2011). The cryosphere is important for many reasons: it is a water and nutrients reservoir and source, and represents a habitat for many organisms (Fountain et al. 2012). The increase of the temperature due to global warming is dramatically affecting this environment in all its components and these effects are going to be more and more evident (Templer et al. 2017; Hotaling et al. 2017). Therefore, it is important to fully understand the dynamics concerning this environment.

One of the less investigated aspect in the past, that now is of increasing interest, concerns the microbiology of these environments, especially on glaciers that together with ice sheets have been recently considered as a biome (Anesio et al. 2012). Glaciers are mass of moving snow or ice and present three typical ecosystems: the supraglacial, the englacial and the subglacial ecosystems. The supraglacial environment is the most studied because it is the more accessible and has a higher interaction with the atmosphere and the surrounding environment (Hodson et al. 2008). The supraglacial environment presents two distinct areas: the accumulation zone where there is a net gain of mass along the year and the ablation zone where there is a net loss of mass, these two zones are separated by the so called equilibrium line (Bakke et al. 2011). The ablation area is the one that presents most of the psychrophilic microorganisms because of the largest presence of sediment that decreases the albedo effect (the ability of the glacial ice to reflect the light instead of adsorbing it) increasing the melting rate and consequently liquid water availability, and especially because of the presence of cryoconite holes (Cook et al. 2016a,b).

1.1. Cryoconite holes

Cryoconite (*krýos* = icy + *kónis* = dust) is a fine-grained dust of atmospheric origin that once deposited on the glaciers' surface absorbs the heat from the sun and melts the underlying ice forming cryoconite holes: pits full of melting water with the sediment on the bottom (Nordenskiöld 1872; Wharton et al. 1985). These microhabitats can widely vary in dimensions, indeed their diameter ranges between few centimetres up to more than one metre, while their depth can reach more than 50 cm (Fountain et al. 2004a) (Fig. 1). The presence of the sediment on the bottom adsorbs the heat from the sunlight and allows the maintenance of liquid water, indeed when the temperature goes below zero it can form a superficial lid that can be also very thick (up to 20 cm) and isolates the cryoconite hole, but still can be penetrated by the sunlight that warms up the

sediment and keeps liquid the melting water (Fountain et al. 2008) (Fig. 1c-d). Albeit the sunlight can penetrate quite thick layers of snow and ice, when the temperature gets very cold the whole cryoconite hole will completely freeze (Fountain et al. 2004a). Their size depends on many factors, the most important are the dynamics they have to face, like supraglacial streams, strong wind and precipitations and the movements of the glacier itself, that together can wash away and destroy these structures that will form again once the sediment will find a suitable spot (McIntyre 1984). Another important factor for their formation is the sediment thickness, indeed, if too thick (> 5 mm), it isolates the ice from the sun instead of warming it up, showing an opposite effect (Fountain et al. 2004a) and forming peculiar deposits of sediments (i.e. dirt cones) (Betterton 2001). At first there were many hypotheses about cryoconite origin (cosmic origin, long or short atmospheric-transport, clay and bird's guano) (Cook et al. 2016b; Zarsky et al. 2013; Franzetti et al. 2017a; Nordenskiöld 1883). Nowadays it is common thought that this sediment is mostly deposited by the wind from local sources present in the areas close to the glacier, while a smaller fraction is deposited after a long-range atmospheric transport (Franzetti et al. 2017a; Dong et al. 2016b), indeed also anthropic products (i.e. pollutants) were proven to reach glaciers, even if released in the environment more than 100 km far away (Ferrario et al. 2017; Ferrario et al. 2017a).

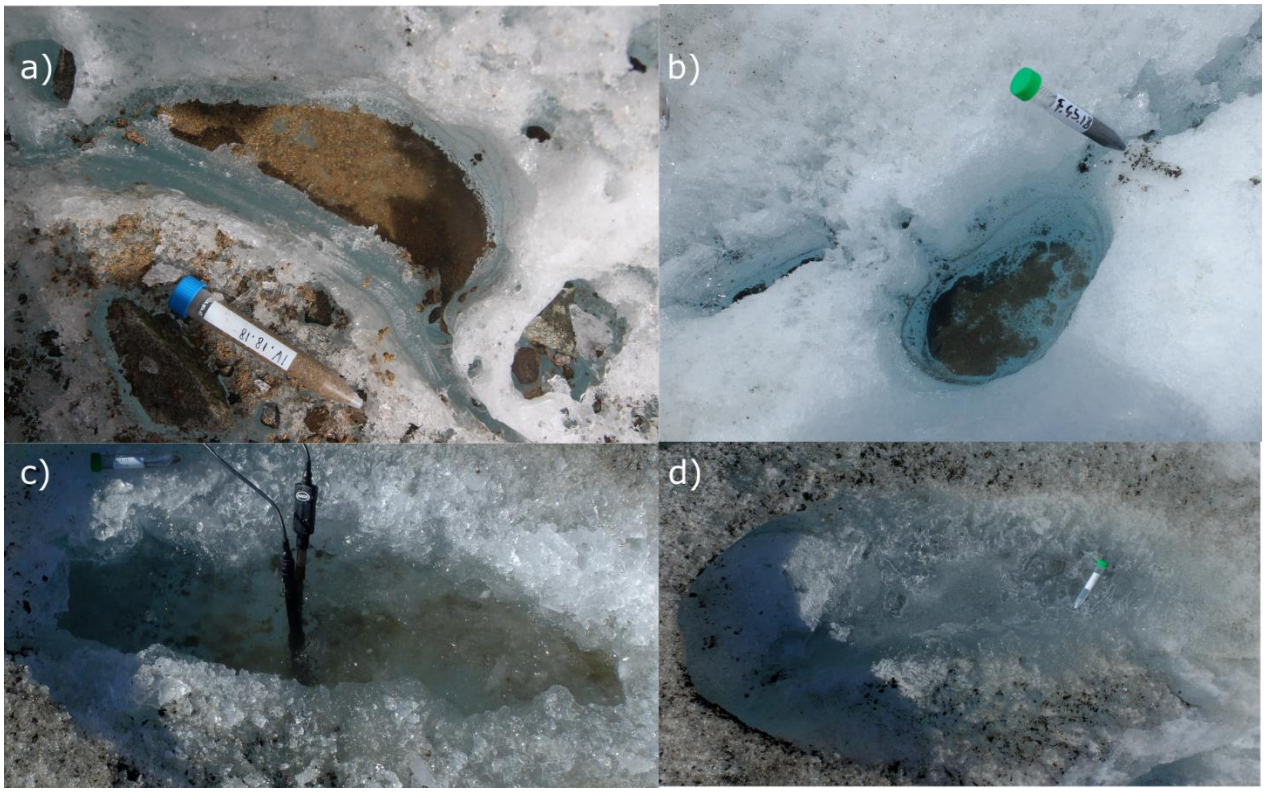

Figure 1. Examples of cryoconite holes. a) Cryoconite hole of Glaciar Iver (Chilean Andes), b) Cryoconite hole of Forni glacier (Italian Alps), c-d) Cryoconite hole of Forni glacier without and with ice lid respectively.

The inorganic fraction of cryoconite is mostly dominated by quartz minerals, but also phyllosilicates and tectosilicates can be present (Dong et al. 2016b; Langford et al. 2010; Hodson et al. 2010). Cryoconites can appear with different aspects, sometimes is just loose sediment and sometimes it can form real granules (maximum diameter 3,5 mm), which are the consequence of the biological activity of filamentous Cyanobacteria and that during quite stable conditions may persist over more than one year; it is even possible to recognize layers that show the seasonal activity of Cyanobacteria (Takeuchi et al. 2010). Also extracellular polymeric substances (EPS) production contributes to granules formation since after they accumulation they can act as a sticky body that entraps the particulates that float in the melting water (Langford et al. 2010). Even if algae and Cyanobacteria have been reported in cryoconite communities and have similar ecological roles, so fare there are no evidences that algae contribute to granules formation (Hodson et al. 2010; Takeuchi etl. 2010).

Cryoconite holes host diverse microorganisms like bacteria, microalgae, fungi, viruses and also other phyla like Rotifera, Annelida, Tardigrada, Nematoda and Arthropoda (Zawierucha et al. 2015). The reason why they have such a biodiversity is that they provide protection from the high UV radiation, relatively higher temperature and nutrients availability and also protection from the harsh wind that can blow in these extreme environments (Musilova et al. 2015; Zawierucha et al. 2015). There are many strategies that microorganisms adopt to survive in this environment: EPS production can provide protection from desiccation, allow biofilm formation, provide protection from the high UV radiation or also cryoprotection (Langford et al. 2014; Chrismas et al. 2016). An adaptation to increased survival to the high UV radiation is pigment production that is a strategy adopted for example by bacteria, algae and tardigrades (Singh et al. 2011; Zawierucha et al. 2015; Yallop et al. 2012; Jagannadham et al. 2000). So far, most of the biological studies focused on bacteria papulation inhabiting cryoconite sediment mostly through Next Generation Sequencing (NGS) techniques and snap-shot studies (Edwards et al. 2016; Franzetti et al. 2013). Cryoconite holes' biota started to be studied at the beginning of the 21 century and immediately gained attention because of its potentialities since cryoconite holes were recognized to have an important nutrient budget (Säwström et al. 2002; Hodson et al. 2005). A few studies investigated the metabolisms and the carbon cycle in cryoconite holes, and results showed that there is a significantly higher carbon cycling by heterotrophic bacteria than on ice, and comparing community respiration with the net primary production, results proved that the community is mainly autotrophic (Nicholes et al. 2019; Anesio et al. 2009). On the Alps the predominant metabolism seems to be oxygenic photosynthesis, with a contribute also from aerobic anoxygenic phototrophs (AAPs) in the energy input in cryoconite

system. AAPs are heterotrophic bacteria that store energy thanks to phototrophic processes and are typical of oligotrophic environments (Fenchel 2001; Franzetti et al. 2016). Also nitrification and denitrification were reported in cryoconite on a Asian glacier (Segawa et al. 2014). However, nitrification and denitrification were not predominant on an Italian glacier (Franzetti et al. 2016). Therefore, cryoconite bacterial metabolisms need further investigation to understand the dynamics in these microhabitats. But this purpose is not easy, since we have to take into account that even if mostly oxic, they have anoxic niches and they host a bacterial community that can adapt to and tolerate different conditions (Poniecka et al. 2018, 2020). Furthermore, they can evolve and change their composition along the ablation season. It was reported on Italian Alps that bacterial communities at the beginning of the ablation season are composed mostly by autotrophic bacteria that can produce the organic matter necessary for heterotrophs' growth that predominate in the end of the ablation season (Franzetti et al. 2017b). Nevertheless, the same trend was not observed on the GrIS (Greenland Ice Sheet), probably because of the less accentuated differences in temperatures and atmospheric conditions along the ablation season in this area. Therefore, underlying the contest in which bacterial communities in cryoconite holes are studied is fundamental to better understand the dynamics concerning these structures.

1.2. Glaciers retreat

Glaciers are important water reservoir, they have an essential role for many reasons: they provide water (Fig. 2) and regulate down-valley water temperature and quantity, hydropower and agriculture can sometimes depend on them (Carey et al. 2016). The awareness of the effects of global warming is increasing more and more, and glaciers retreat is one of the most evident consequences (Roe et al. 2017; Solomon et al. 2007). Glacial ice loss is therefore an inevitable effect that cannot be underestimated, especially for what concerns the future scenario that is going to show up. For this reason, it is important also to understand how the habitat is going to change and if these changes are going somehow to influence the surrounding ecosystem. An important aspect that is now under investigation is the colonization of deglaciated areas that are increasing as the consequence of glaciers retreating (Zumsteg et al. 2012; Rime et al. 2016).

Figure 2. Deglaciated area downvalley of the San Lorenzo Glacier (Chilean Patagonia).

Going into details of these dynamics, it looks like that, immediately after glacier retreat, barren soil is poor in organic matter and nitrogen content, which increase in less recently deglaciated area where also vegetation has developed. Nutrients input have three main sources: i) deposition of allochthonous material, ii) primary production of autotrophic microorganisms and iii) the material previously present in the subglacial environment that is now ice-free (Bradley et al. 2014). Cyanobacteria are quite abundant in those kind of soils, where pH is also higher than in cryoconite, probably because of the lower organic matter content (Kastovská et al. 2005). In particular Cyanobacteria belonging to the order of Nostocales were reported to be representative of communities of recently deglaciated soils, this aspect is important since these bacteria are responsible for nitrogen fixation, and consequently, for soil enrichment in nitrogen that is given also by the atmospheric input (like for other nutrients; *e.g.,* carbon) (Kastovská et al. 2005; Schmidt et al. 2008; Rime et al. 2016). However, nitrogen content increases also with vegetation, because of plant litter and nitrogen-fixing bacteria that are typical of the rhizosphere (Bradley et al. 2014). Furthermore, Cyanobacteria orders seem to change along the chronosequence from orders typical of glacial environments to orders typical of soil crusts (Schmidt et al. 2008). Alpha diversity of this

kind of environment is similar to that of other glacial environments for both fungi and bacteria, but different colonization strategies are believed to happen for the two kingdoms. Indeed, while fungi look more dispersed by the wind, bacterial communities of recently deglaciated areas share more similarities with those of glacial habitats (Rime et al. 2016). Another important role of Cyanobacteria (and probably other phyla) is to avoid soil loss through their exopolysaccharides production that can promote soil aggregation and stability, furthermore they provide organic matter for heterotrophic taxa in the early stages of the succession, while in the late stages there is also plant organic matter (Schmidt et al. 2008). A few studies conducted so far demonstrated that bacterial communities of recently deglaciated barren soils seem to come from supra- and subglacial environments and community composition may depend on the order of arrival (priority effect) (Rime et al. 2016; Symons et al. 2014), while fungal communities do not seem at the same way endogenous and proved to be different from those of supra- and subglacial environments (Rime et al. 2016). Both of these communities dynamically change along the chronosequence also in relation to plants communities (Brown et al. 2014). To conclude, more studies are still necessary to better understand the dynamics involving bacteria, fungi and plants colonization of deglaciated areas, indeed there are still open questions regarding which is the most important source of these communities (autochthonous or allochthonous) and how important are the interactions between different communities (Brown et al. 2014; Bradleyet al. 2014).

1.3. Bioalbedo

Pigments production, the survival strategy of microorganisms in environments with high UV radiation already mentioned above, can be very strong and affect the supraglacial environment. Indeed, there are a few strains of microalgae that form algal blooms that can develop on both ice and snow and can last also several weeks (Hoham et al. 2020). They affect the upper surface (~ 2 cm) of ice and are subjected to many environmental stressors (*e.g.*, high UV radiation, freeze-thaw cycles, low nutrients concentration) Williamson et al. 2019) (Fig. 3a-b). The organisms involved in this phenomenon are different species of microalgae, such as *Chlamydomonas nivalis*, *Ancylonema nordenskioeldii, Chloromonas sp., Sangiuna nivaloides* and *Mesotaenium berggrenii* (Williamson et al. 2018; Gradinger et al. 1996; Takeuchi et al. 2004; Hoham et al. 2020), which optimum temperature range mostly between 0 °C and 5 °C (Hoham et al. 2020). Their growth seems to decrease with the altitude and their biomass seems to increase close to the margin of glaciers

(Williamson et al. 2019).

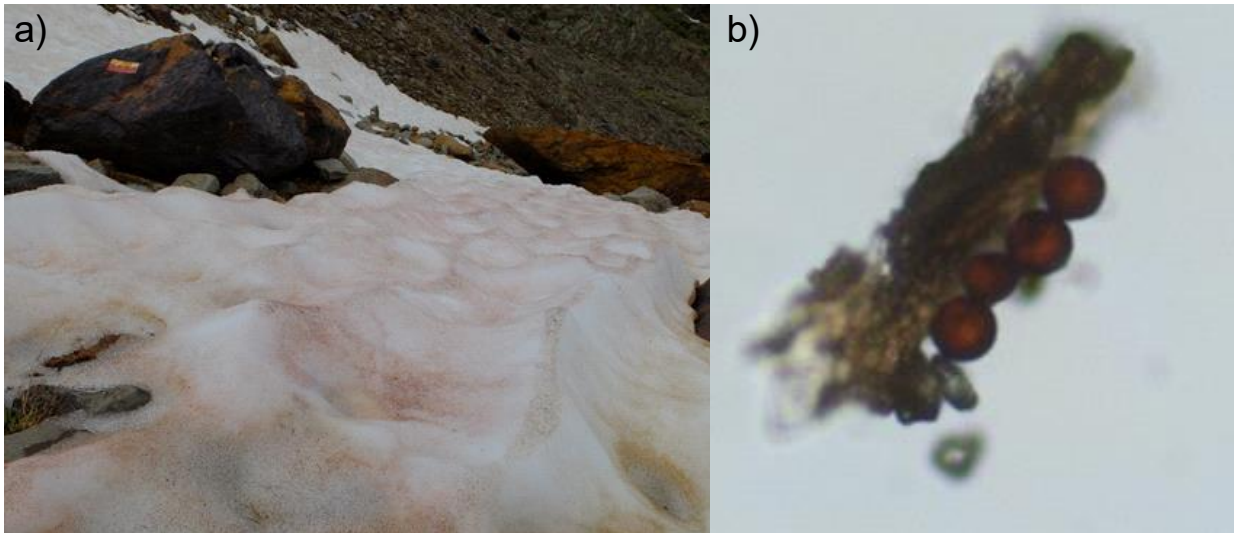

Figure 3. Algal bloom on snow cover. a) Example of an algal bloom on a snow cover, b) Chlamydomonas nivalis captured with an optic microscope.

Snow and ice algae can be divided according to the main strains that inhabit them and according to their life cycles. Snow algae are mostly Chlamydomonadales and have a three-stage life cycle: when cells are buried by winter snow-pack and there is no water and light availability they are dormant cysts (no photosynthesis and no motility), when snow starts melting and water availability increases they germinate as green-colored "swarmers" (active motility and active photosynthesis), and in the end they became red-colored snow-algal "hypnoblasts" (active motility and active photosynthesis) (Dial et al. 2018). Ice algae instead, Zygnematophyceae show a single-stage life cycle, with no motility and dark pigments (Dial et al. 2018).

Pigmented algae and bacteria, fine-grained debris and organic matter of both biotic and abiotic origin contribute to the decrease of the albedo, indeed they adsorb the heat from the sunlight warming up and melting the underlying ice (Cook et al. 2017; Stibal et al. 2017; Takeuchi et al. 2001b). The effect of the biological matter only on the albedo is called bioalbedo; indeed optical properties of cells can drastically affect the albedo (Cook et al. 2017). This effect has a positive feedback, since ice, while melting, lowers its albedo more and more and provides liquid water availability that promotes algal bloom, which are enhanced by liquid water, nutrient availability and light (Yallop et al. 2012; Lutz et al. 2016; Stibal et al. 2017), and can also be stronger than the effect of other abiotic impurities (*e.g.*, black carbon) (Stibal et al. 2017). This phenomenon is so strong that has been proposed to be included in climate models (Lutz et al. 2016). Therefore, it is important to investigate its dynamics and how to include it in albedo estimating models. Differently from bacteria

communities in snow, microalgae forming algal blooms seem more cosmopolitan regardless geochemical and mineralogical composition of glaciers (Lutz et al. 2016). Therefore, including this phenomenon in climate change models will be easier, since there are no changes in its behaviour according to the area where it is located, the only factor that seems to affect differently the albedo is the type of pigment, indeed phenolic pigments look like the major responsible of albedo decrease (Williamson et al. 2020). In particular, they shade the photosystem from the sunlight, adsorb it and finally release it as heat (Hoham et al. 2020). Another important aspect concerning the role of phenolic pigments in microalgae is that they can avoid photoinibition, a possible effect due to extremely high UV radiation already observed in bacteria and algae (Figueroa et al. 1997; Sinha et al. 2002), allowing an improved algae development (Williamson et al. 2020). Furthermore, looks like dark (red, purple) pigments are the "hottest" particles, while green pigments are not such responsible of heat adsorption (Dial et al. 2018). The study of these microalgae should be performed adopting both microscopic and molecular approaches, indeed microscopy does not allow sometimes to identify some species that apparently look very similar, *e.g., Ancylonema nordenskioeldii* and *Mesotaenium berggrenii* (Hoham et al. 2020). Furthermore, to distinguish these two strains is neither easy with a traditional molecular approach, it is therefore necessary to process the sequences adopting the olygotyping pipeline (Eren et al. 2013), which uses only hypervariable oligos of the amplified genes and allows to discern between different, but at the same time very similar algal species (Lutz et al. 2018). Furthermore, not only microscopy and the molecular approach are useful to study this phenomenon, also satellite imagery allows to have a more complete comprehension of the entity of these blooms including their effect on albedo (Stibal et al. 2017). This is important since algal blooms can reach very big dimensions and have a potential effect of great entity considering that they have been proposed as one of the causes of the decrease of the GrIS, and the consequent increase of sea level (Williamson et al. 2019). Indeed, the combination of both microbiological and remote-sensing approaches will allow to obtain more suitable models to understand the characteristics of these strains, and consequently to include them in models that will describe their occurrence and extension (Williamson et al. 2019). These aspects still need further improvements, for example the resolution of remote sensing images is very variable and the main difficulty is to standardize this method; for example the degree of anisotropy has high spatio-temporal variability, that means that both homogeneity of snow/ice surface and time of images capture is important to compare images, for example they should be taken with a constant angle with the sun (Cook et al. 2017). It is also important to account for both direct (pigments) and indirect

(meltwater increase) effects of algal blooms in models and furthermore pigments can also affect the spectral signature according to what type of pigment they are (Cook et al. 2017). It is therefore important to carry on the research in this direction since more data are still necessary to improve the understanding of algal blooms that can decrease albedo of 13 % more than without this phenomenon (Lutz et al. 2016).

Table 1 List of the main algae responsible of both ice and snow algal blooms.

Algal species	Matrix	Ref.
Ancylonema nordenskiöldii	Ice	(Lutz et al. 2018)
Mesotaenium berggrenii	Ice	(Lutz et al. 2018)
Microglena sp.	Snow	(Lutz et al. 2015)
Chloromonas reticulata	Snow	(Novakovskaya et al. 2018)
Hydrurus sp.	Snow	(Remias et al. 2013)
Chloromonas brevispina	Snow	(Rezanka et al. 2008)
Sanguina aurantia	Snow	(Procházková et al. 2019)
Chlainomonas kolii	Snow	(Novis et al. 2008)
Chlainomonas rubra	Snow	(Novis et al. 2008)
Sanguina nivaloides	Snow	(Procházková et al. 2019)
Cylindrocystis brébissonii	Ice	(Takeuchi et al. 2004)
Closterium sp.	Ice	(Takeuchi et al. 2004)
Chlamydomonas nivalis	Snow	(Takeuchi et al. 2006)
Chlorella	Snow	(Davey et al. 2019)

Chloromonas hindakii	Snow	(Procházková et al. 2019)
Chloromonas chenangoensis	Snow	(Hoham et al. 2007)
Chloromonas hohamii	Snow	(Matsuzaki et al. 2014)
Chloromonas pichinchae	Snow	(Matsuzaki et al. 2014)
Chloromonas polyptera	Snow	(Hoham et al. 1983)
Chloromonas rosae	Snow	(Remias et al. 2018)
Scotiella cryiophila	Snow	(Remias et al. 2018)
Chloromonas rubroleosa	Snow	(Ling et al. 1993)l
Chlorosarcina antarctica	Snow	(Ling et al. 2002)
Desmotetra antactica	Snow	(Ling et al. 2001)
Desmotetra aureospora	Snow	(Ling et al. 2001)

1.4. Contamination

Glaciers can be negatively affected by biological contamination (i.e. algal blooms), but this problem is not the only one concerning these environments. Indeed, glaciers can act as condensers that can receive pollutants from the atmosphere and accumulate them especially with big snow events (Calamari et al. 1991; Ferrario et al. 2017a), avoiding a further revolatilization: "mountain-cold trapping" (Miner et al. 2017). For this reason, glaciers are not only water and nutrients, but also pollutants reservoirs (Miner et al. 2017). An important aspect is that not only currently used contaminants, but also those from the past (*e.g.*, DDT, PCBs) have been stored in the ice pack of glaciers, until the increased melting rate because of global warming allowed their release in melting water, and consequently in the down-valley ecosystems (Ferrario et al. 2017a; Miner et al. 2017). The atmospheric transport of these pollutants can cover very long distances, reaching also the most remote areas (Antarctic, Arctic), and this phenomenon has been observed for different compounds,

like organochlorine pollutants (OCPs), heavy metals and polycyclic aromatic hydrocarbons (PAHs) (Li et al. 2017; Miner et al. 2017; Jiao et al. 2021). This aspect gained a big interest when persistent organic pollutants (POPs) whose use was forbidden, were unexpectedly found in proglacial lakes (*e.g.*, PCBs and DDT) and glaciers were proposed as secondary sources of legacy pollutants (Bogdal et al. 2009). Indeed, it is also possible to reconstruct the history looking at pollutants in ice cores, even lead from the Roman Republican and Imperial periods has been found in an ice core from Greenland (McConnell et al. 2018). Therefore, we can only wonder what the World War I brought on the Alps for example. It is therefore important to fully investigate both qualitatively and quantitatively which pollutants are stored in glaciers. Once deposited on glaciers, depending on their physicochemical characteristics, pollutants can accumulate in the sediment (i.e. cryoconite) or remain in the ice, but not much is known about the dynamics that interest them on glaciers. Furthermore, a big issue is their release into melting water and the possibility that they accumulate in the downvalley trophic chains. On glaciers, pollutants degradation can occur through photolysis or hydrolysis (Ferrario et al. 2017; Gbeddy et al. 2020; Prosen et al. 2005), but not much is known about biodegradation which should not be underestimated. For this reason, in this thesis, one chapter will be dedicated to a review about pollutants biodegradation in supraglacial ecosystems.

2. Aim and structure of the thesis

In the first part of this thesis the temporal variability of supraglacial and periglacial microbial communities has been addressed. At first, we investigated the evolution of bacterial communities along the ablation season and during different years (**Chapter 3**). This study was conducted on Forni Glacier (Italy). We sampled cryoconite hole sediments in different years and in different times along the ablation seasons and we characterized the structure of the bacterial communities with the aim of determining their temporal dynamics. After addressing the temporal trend of the bacterial community composition, we test whether, at shorter time scale, daily temporal dynamics of the metabolism expression patterns occurs in cryoconite holes. To this end, we sampled the same cryoconite hole on Forni glacier in different times during one sunny day in July 2018 (**Chapter 4**). Samples were analysed by shotgun metatranscriptomics in order to quantify the expression level of the main genes involved in the main energy and carbon metabolism.

Bacterial communities of the supraglacial ecosystem are connected with periglacial habitats through melted water and aeolic transport and are partly responsible of the colonization of recently deglaciated areas. For this reason, we also investigated, at long time scale, the chronosequence in a glacier forefield to appreciate changes in function of the deglaciation time. In 2015 sediment

samples of Forni glacier forefield were sampled along three 18-year chronosequence transects from the glacier terminus (**Chapter 5**). Bacterial and fungal community were analysed by 16S rRNA and ITS1 sequencing, respectively. Plant communities were also characterised in the surrounding of sampling location.

A further aspect that is investigated is the spatial variation of supraglacial bacterial communities at both local and global scale (**Chapter 6** and **7**). This is an important aspect that allow to understand to which extent all the data reported in literature so far about supraglacial bacterial communities are useful to draw conclusions at a more general scale, not only in the context of the area/glacier where these results have been obtained.

Cryoconite holes are inhabited not only by bacteria, indeed many other taxa can be found in these microhabitats, and all of them interact. This aspect is extremely overlooked, but it is at the same time extremely important, since not only spatio-temporal features may drive bacterial community composition, but also the presence/absence of other taxa. It is therefore essential to assess who does and who does not live in cryoconite holes to fully understand the biological dynamics in supraglacial ecosystems (**Chapter 8**).

The last aspect that is reported in this thesis concerns both the direct and indirect anthropic impact on supraglacial ecosystems. Indeed, human behaviour is having an important impact on all our ecosystems, temperatures are increasing more and more, and contaminants are still released in the environment and all these aspects are affecting glacier ecosystems.

Two factors have been investigated and are reported in this thesis: algal blooms and microplastic contamination.

Global warming is favouring the so called albedo feedback, that is the blooming of rich-pigments algae on both snow and ice that are decreasing the albedo. It is extremely important to study these phenomena, because they are strictly correlated with the melting rate and the decreasing albedo of glaciers and can reach a significant extent (**Chapter 9**).

Glaciers contamination is a quite assessed aspect, since they can act as condensers and store pollutants deposited by precipitations or after atmospheric transport. Unfortunately, not much is known about pollutants biodegradation in supraglacial ecosystems (**Chapter 10**). We therefore investigated first the literature reporting all the available data about this particular aspect and reported them in a small review. Then we looked for and found microplastics on glaciers (**Chapter 11**), and the main aim of this part of the research is to know which contaminants are present on glaciers and which is their fate in these environments. It is an important piece to know about

microplastics on glaciers, since their adsorption capacity of other pollutants will allow to avoid underestimating the presence of these dangerous compounds in glacier environments. All these aspects will allow to better set up experiments to investigate biodegradation on glacier ecosystems.

3. BACTERIAL COMMUNITIES OF CRYOCONITE HOLES OF A TEMPERATE ALPINE GLACIER SHOW BOTH SEASONAL TRENDS AND YEAR-TO-YEAR VARIABILITY.

The content of this chapter has been published in the following paper:
Francesca Pittino, Maurizio Maglio, Isabella Gandolfi, Roberto Sergio Azzoni, Guglielmina Diolaiuti, Roberto Ambrosini, and Andrea Franzetti. 2018. "*Bacterial Communities of Cryoconite Holes of a Temperate Alpine Glacier Show Both Seasonal Trends and Year-to-Year Variability*" 59 (77): 1–9. https://doi.org/10.1017/aog.2018.16.

ABSTRACT. Cryoconite holes are small depressions of the glacier surface filled with melting water and with a wind-blown debris on the bottom. These environments are considered hot spots of biodiversity and biological activities on glaciers and host communities dominated by bacteria. Most of the studies on cryoconite holes assume that their communities are stable. However, evidence of seasonal variation in cryoconite hole ecological communities exists. We investigated the variation of the bacterial communities of cryoconite holes of Forni Glacier (Central Italian Alps) during the melting seasons (July– September) 2013 and 2016, for which samples at three and five time-points, respectively were available. Bacterial communities were characterized by high-throughput Illumina sequencing of the hypervariableV5−V6 regions of 16S rRNA gene, while meteorological data were obtained by an automatic weather station. We found consistent trends in bacterial communities, which shifted from cyanobacteria-dominated communities in July to communities dominated by heterotrophic orders in late August and September. Temperature seems also to affect seasonal dynamics of communities. We also compared bacterial communities at the beginning of the melting season across 4 years (2012, 2013, 2015 and 2016) and found significant year-to-year variability. Cryoconite hole communities on temperate glaciers are therefore not temporally stable.

3.1. Introduction

Glaciers and ice sheets have been recently recognized as a terrestrial biome in their own right (Anesio et al. 2012) because they host different ecosystems dominated by microorganisms (Hodson et al. 2008; Boetius et al. 2015). Among glacial environments, cryoconite holes are considered hot spots of biodiversity (Cook et al. 2016a; Cook et al. 2016b). These peculiar structures are small ponds that form on glacier surface when the cryoconite, a fine-grained wind-borne sediment, accumulates in small depressions and locally decreases the albedo promoting the underlying ice melting. The ponds are then filled by the meltwater and the cryoconite remains at the bottom where it promotes microorganism growth (Wharton et al. 1985). Cryoconite holes range in diameter from few centimetres to more than a metre and host the most metabolically active ecological communities in glacier ecosystems (Laybourn-Parry et al. 2012). These microhabitats host bacteria, tardigrades, rotifers, collembola, algae, viruses and nematodes (Hodson et al. 2008; Cook et al. 2016a; Cook et al. 2016b) and have been studied on glaciers in different geographical areas, such as the Alps (Edwards et al. 2013; Franzetti et al. 2017b), Arctic (Gokul et al. 2016), Greenland (Uetake et al. 2016), Antarctica (Cameron 2012), Himalaya (Takeuchi et al. 2000) and Karakoram (Ambrosini et al. 2017). Bacterial communities of cryoconite holes seem to vary according to ecological conditions of

the holes, particularly with their size and pH (Ambrosini et al. 2017), sediment thickness and organic matter content (Telling et al. 2012) and the hydrology of the glacier surface (Edwards et al. 2011), as well as their location within a glacier (Stibal et al. 2015). Variability among glaciers has also been reported (Franzetti et al. 2016; Liu et al. 2017). Bacterial communities of cryoconite holes seem dominated by Cyanobacteria, Actinobacteria, Proteobacteria and Bacteroidetes on both polar and temperate glaciers (Musilova et al. 2015; Ambrosini et al. 2017; Gokul et al. 2016; Franzetti et al. 2017b). However, most of the studies conducted so far investigated cryoconite bacterial populations by snapshot sampling, probably because of logistic limitations to the sampling design, and almost neglected the temporal variability of the ecological communities of cryoconite holes (e.g. Takeuchi et al. 2010; Singh et al. 2014; Ambrosini et al. 2017). Indeed, to the best of our knowledge, only a few studies investigated the temporal variability of bacterial communities of cryoconite holes. For instance, Takeuchi and others (2010) showed that cryoconite grains form as a consequence of biological activity, especially of filamentous Cyanobacteria. Similarly, Franzetti and others (2017b) indicated that Cyanobacteria are one of the prevalent taxa in cryoconite holes at the beginning of the ablation season on a temperate glacier, but their relative abundance declines in later stages of the melting season, when heterotrophic taxa such as Actinobacteria, Proteobacteria and Bacteroidetes dominate. Hence, cryoconite bacterial communities seem to show temporal variability during one ablation season, shifting from a condition with a prevalence of phototrophs to a prevalence of heterotrophs. This process is probably driven by the increase in the organic matter content of the cryoconite. In contrast, Musilova and others (2015) showed that bacterial communities inhabiting cryoconite holes on the Greenland ice sheet seem stable during one ablation season. This result is supported by the study of Cook and others (Cook et al. 2016a; Cook et al. 2016b), which showed that a perturbation of Greenland cryoconite holes did not change the status of net autotrophy. Different studies thus provided contrasting results on the temporal dynamics of cryoconite hole bacterial communities, the main difference being the evidence, on the one side, of a community stability in Arctic glaciers and, on the other side, of temporal dynamics in cryoconite holes on temperate glaciers. This difference may be due to the fact that, on polar glaciers, holes can persist for years (Porazinska et al. 2004), while they are rather ephemeral on temperate glaciers. Indeed, the intense solar radiation melts them away and releases the cryoconite, which can eventually determine the formation of a new hole during the same ablation season (Cook et al. 2016a; Cook et al. 2016b). Interestingly, one of the few studies on the temporal variability of bacterial communities of cryoconite holes suggested that they may follow the steps of an ecological

succession independently of the age of the hole (Franzetti et al. 2017b). Indeed, communities observed in cryoconite holes on the Forni Glacier (Italian Alps) for example in August, were similar to one another, regardless of the fact that the holes formed in July (i.e. they were 1-month old) or they were newly formed ones. This suggested that communities in new holes can be seeded by those present in previous holes melted away by ablation and that ecological succession of cryoconite bacterial communities continues throughout the ablation season, independently of the time when a hole forms (Franzetti et al. 2017b). Cryoconite communities, however, are seeded also by inputs from near glacier environments (Telling et al. 2012; Stibal et al. 2015; Franzetti et al. 2017a) and possibly by bacteria subjected to long-range transport (Cook et al. 2016a; Cook, et al. 2016b) or deposited by precipitations (Azzoni, personal communication). Inputs from external environments may thus affect the bacterial communities of cryoconite holes and determine year-to-year variability in the observed communities. This variability may be particularly large at the beginning of the melting season, when cryoconite communities may be seeded by bacteria from the melting snow cover, whose composition may vary from year to year for example because of stochastic variability of the geographical origin of the air masses that determines dust-rich precipitations (Azzoni, personal communication). Whether these differences persist along the ablation season or communities quickly tend to a similar composition because of strong selection toward the populations most adapted to the conditions of the cryoconite holes is, however, unknown. This study aims at filling this gap of knowledge by investigating both inter- and intra-annual variability of bacterial communities of cryoconite holes from the Forni Glacier (Italian Alps). In particular, we used Illumina sequencing of the 16S rRNA gene to compare the structure of bacterial communities of cryoconite holes collected at the beginning of the ablation season (i.e. in July) in four different years (2012, 2013, 2015 and 2016). In addition, we investigated the variation of bacterial communities along the ablation seasons of 2013 and 2016 to assess whether recurrent patterns could be observed. Part of the data presented here (i.e. those collected in 2013) has already been published in a previous paper on the temporal variability of cryoconite bacterial communities along one ablation season (Franzetti et al. 2017b). However, the other data are entirely new and allowed investigating different research questions (i.e. interannual variability, repeatability of within-year trends) from those addressed in the previous paper.

3.2. Materials and methods

3.2.1. Filed sampling and meteorological data

Forni Glacier is an Italian valley glacier belonging to Ortles- Cevedale Group (46°24'00"N, 10°35'30"E; Fig. 3.1). Its elevation ranges between 2600 and 3670 m a.s.l (Smiraglia et al. 2015). Samples were collected during the ablation seasons (July–September) of the years 2012 (21 samples), 2013 (60 samples), 2015 (21 samples) and 2016 (60 samples) with a laboratory spoon sterilized with alcohol. Samples were collected in 50 mL Falcon™ tubes kept at 4 °C during the transport to the laboratory, which occurred within 8 h, and then at −20 °C before the analyses. In 2012 and 2015, we collected samples in July only, while in 2013 and 2016 samples were collected at respectively three and five-time points along the ablation seasons (see Supplementary table S1 for details on sampling dates and sample sizes). Detailed meteorological data were recorded by an automatic weather station (AWS) located on the surface of the Forni Glacier at 2660 m. a.s.l., 400 m apart from the area where we collected cryoconite. We defined the beginning of the melting season at a site on the glacier as the date when the snow is completely melted and the bare ice is exposed. This can be precisely defined at the AWS site as the day when the mean daily albedo is lower than 0.30 (Azzoni et al. 2016). However, images from a high resolution webcam installed in the proximity of the glacier revealed that bare ice is exposed at the site where we collected cryoconite up to 7 days earlier than at the AWS site. We thus defined the beginning of the melting season as the 7th day before the date when the mean daily albedo at the AWS was lower than 0.30. The end of the melting season was defined as the date when a snowfall covered the ice and mean daily albedo rised above 0.30 (reaching values of 0.85–0.90, typical of the fresh snow) for the following months. We then calculated the positive daily degree-day (DD) as the cumulated mean daily temperatures above 0 °C and the cumulative incoming shortwave radiation (c-SWIN) as the cumulated daily SWIN since the beginning of the melting season of each year (Fig. S3.1).

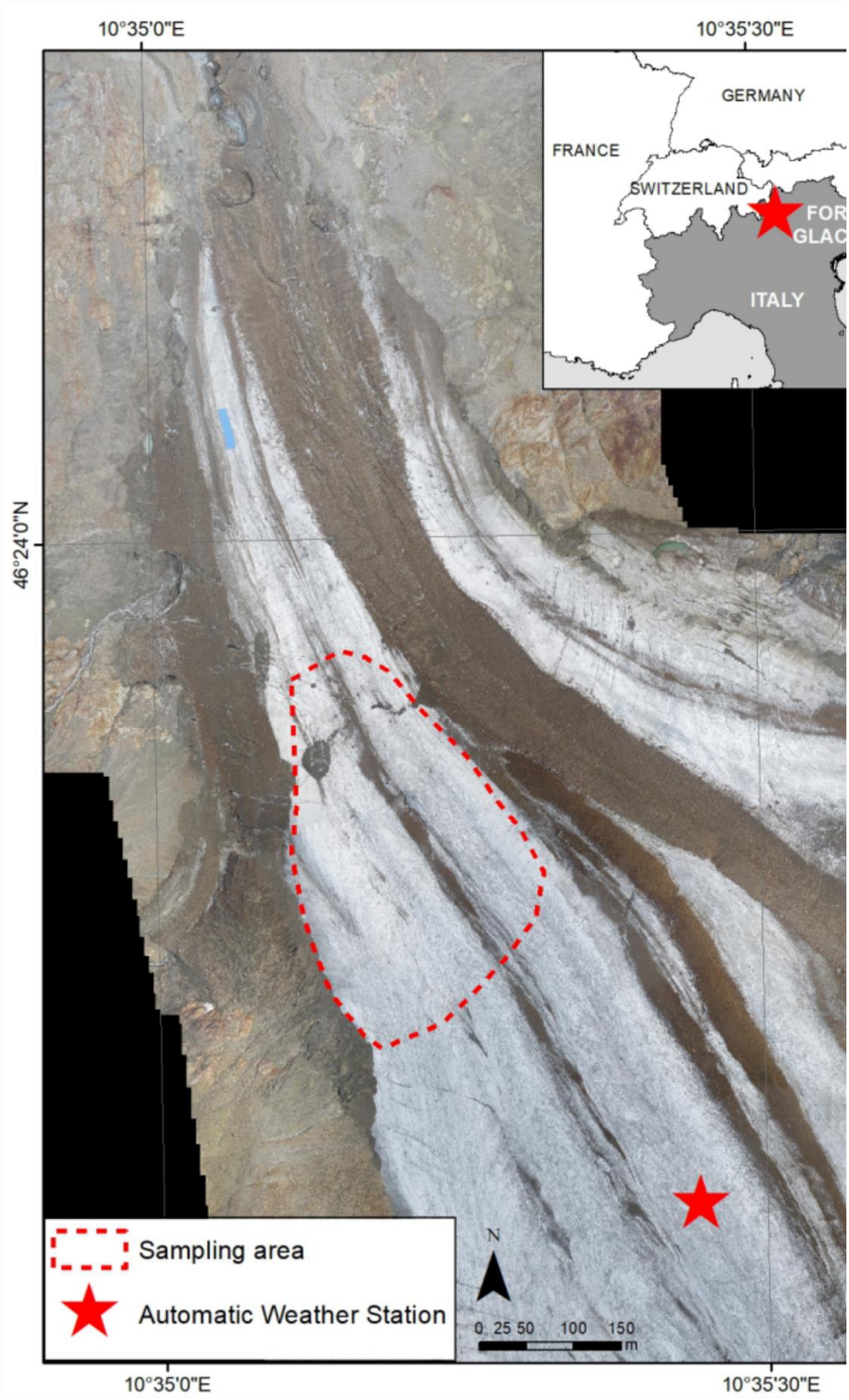

Figure 3.1 Location of the sampling area on the ablation tongue of Forni Glacier (Stelvio Park, Central Italian Alps). The sampling area is reported with a red-dashed line, the AWS is represented by a red star. The base map is produced from an Unmanned Aerial Vehicle flight in 2014.

3.2.2. DNA extraction and amplification

Total DNA was extracted from 0.5 g of each cryoconite sample with the FastDNA® Spin for Soil kit (MP Biomedicals, Solon, OH, USA) according to the manufacturer's instructions. A first PCR

amplification was performed on the V5-V6 hypervariable regions of 16S rRNA gene for each sample to evaluate its quality on the original and on the 1:10 dilution to identify inhibition or insufficient samples. A second PCR was then performed with GoTaq® Green Master Mix (Promega Corporation, Madison, WI, USA) and 1 µM of each primer, for a final volume of 2 × 50 µL for each sample. Illumina adapters (6 bp) were added at the 5' end. 783F and 1046R primers were used (Huber et al. 2007; Wang and Qian 2009) and the cycling conditions were: initial denaturation at 94 °C for 4 min; 28 cycles at 94 °C for 50 s, 47 °C for 30 s, and 72 °C for 45 s and a final extension at 72 °C for 5 min. Amplicons were then purified with Wizard® SV Gel and PCR Clean-up System (Promega Corporation, Madison, WI) and quantified with Qubit® (Life Technologies, Carlsbad, CA). Libraries were prepared with nine samples each, identifiable thanks to different barcode pairs. Library preparation with the addition of standard Nextera indexes (Illumina, Inc., San Diego, CA) and sequencing with MiSeq Illumina platform (Illumina, Inc., San Diego, CA), using a 2 × 250 bp paired-end protocol, were performed at Parco Tecnologico Padano (Lodi, Italy).

3.2.3. DNA sequence processing and statistical analyses

The obtained reads were demultiplexed according to the indexes. The Uparse pipeline was used for the following elaborations (Edgar 2013). Forward and reverse reads were merged only if with zero mismatches and quality filtered with default parameters. Operational taxonomic units (OTUs) were defined with an aggregative clustering of sequences with 97% of sequence identity. Suspected chimeras and singleton sequences (i.e. sequences appearing only once in the whole dataset) were removed. Singletons (OTUs present once in one sample only) were removed from the analyses because their inclusion could inflate variance explained by multivariate analyses (Legendre et al. 2003). OTU classification at order level was inferred with RDP classifier (Wang et al. 2007) with the only exception of Cyanobacteria because the RDP classifier does not report the order level for this taxon (Garrity et al. 2007; Wilmotte et al. 2015). To compare diversity among samples that largely differed in the number of sequences, 20 000 sequences were randomly selected from each sample for which more than 20 000 sequences were available. For the other samples, OTU abundance was normalized to 20 000 sequences by resampling with repetition. Multivariate analyses were based on Hellinger distance, which depends on the differences in OTU proportion between samples, decreases the importance of OTU abundance over their occurrence and avoids the double-zero problem when comparing OTU composition between samples (Legendre et al. 2001; De Cáceres et al. 2010). Principal Component Analysis (PCA) was performed on all samples to visualize data distribution. Redundancy analysis (RDA) and variation partitioning were used to quantify the

variation of community structures according to the meteorological conditions (i.e. DD and c-SWIN), the day of melting season, month and year. Post-hoc tests were also performed to assess pairwise differences between years and months. For RDAs, post-hoc tests were performed by running separate analyses for each pair of years or months and correcting P-values for multiple testing according to the false discovery rate (FDR) procedure (Benjamini et al. 2001). Day of melting season, DD and c-SWIN were highly collinear ($r \geq 0.991$; Fig. S3.1), so they could not be entered simultaneously in RDA models. We thus performed a principal component analysis on DD and c-SWIN by considering all the days in the melting seasons of all years. The first extracted component was strongly collinear with the day of melting season ($r= 0.989$), thus it accounted for the seasonal trend of increasing DD and c-SWIN. This variable was discarded from subsequent analyses because its effect was already accounted for by the day of melting season. In contrast, the second component was almost independent from the day of melting season and c-SWIN ($r< 0.006$) and was slightly but positively correlated with DD ($r = 0.133$). It was therefore used as an index of the deviation of the DD in a particular day from the mean seasonal trend of increasing DD. From here on, we will refer to this variable as temperature index (TI). Positive values of TI thus indicate warmer days than expected for that period of the year. Variation in the abundance of Cyanobacteria and the most abundant orders (see below) according to the variables that significantly affected the structure of bacterial communities identified by the RDAs was investigated by generalized linear models (GLMs) assuming a Poisson distribution and corrected for overdispersion. Also in this case, P-values were corrected using the FDR procedure. Analyses were performed with R 3.4.2 (Team 2014) with the VEGAN, BIODIVERSITYR, MULTTEST, MULTCOMP packages.

3.3. Results

Cyanobacteria and seven orders (Sphingobacteriales, Burkholderiales, Pseudomonadales, Rhodospirillales, Cytophagales, Actinomycetales, Clostridiales) had more than 50 000 sequences in the dataset and were considered abundant taxa (Fig. 3.2).

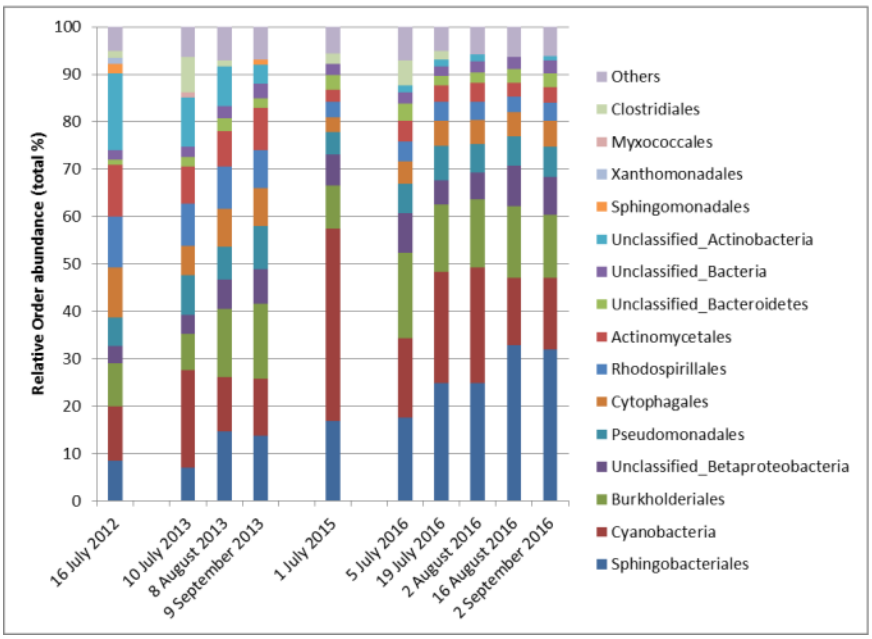

Figure 3.2 Relative abundance of bacterial taxa, mostly at the order level, expressed as the percentage of sequences classified with confidence >90%. Orders whose abundance was <1% were grouped in 'Others'.

Cyanobacteria, Pseudomonadales, Rhodospirillales, Cytophagales and Actinomycetales were present in all years and months. Clostridiales were present in July samples of all years, but they decreased along the ablation season in 2013 and 2016 and were always absent in September samples. We ran a PCA on all samples of all 4 years to visualize data distribution (Fig. 3.3).

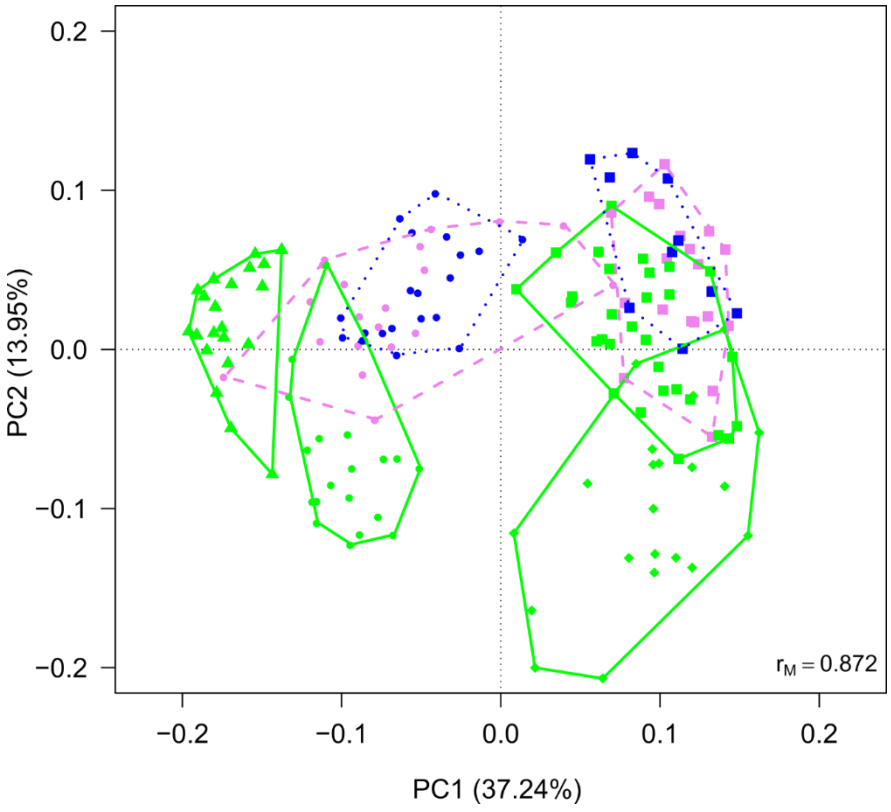

Figure 3.3 PCA plot on Hellinger-transformed abundances of each OUT of all the samples. Line colours and styles denote the month when the samples were collected (green solid line= July, violet dashed line=August, blue dotted line=September), while symbols the year (dots=2012, triangles= 2013, diamonds= 2015, squares=2016).

This analysis showed that each year clustered in a rather distinct group mainly along the first axis. A temporal trend of samples collected in different months also seemed to appear along the second axis. This analysis thus suggested that cryoconite hole bacterial communities showed both between- and within-year variability. To investigate between-year variability, we ran a RDA including only July samples of all years to investigate differences in the structure of bacterial communities at the beginning of the ablation season. This analysis showed significant variations in the structure of bacterial communities between years (Table 3.1; Fig. S3.2) and post-hoc tests highlighted significant differences between all pairs of years ($F_{1,49} \geq 15.099$, $P_{FDR} \leq 0.002$).

Table 3.1 RDA of variation of Hellinger-transformed bacterial OUT abundance of July samples of 2012, 2013, 2015 and 2016 according to year

Predictor	Df	Variance	F	P
Year	1	0.017	22.755	0.001
Day of melting season	1	0.002	2.854	0.018
Temperature Index	1	0.006	8.223	0.001
Residuals	116	0.088		

$F_{3,112} = 22.729$, P = 0.001, Adjusted-$R^2 = 0.354$

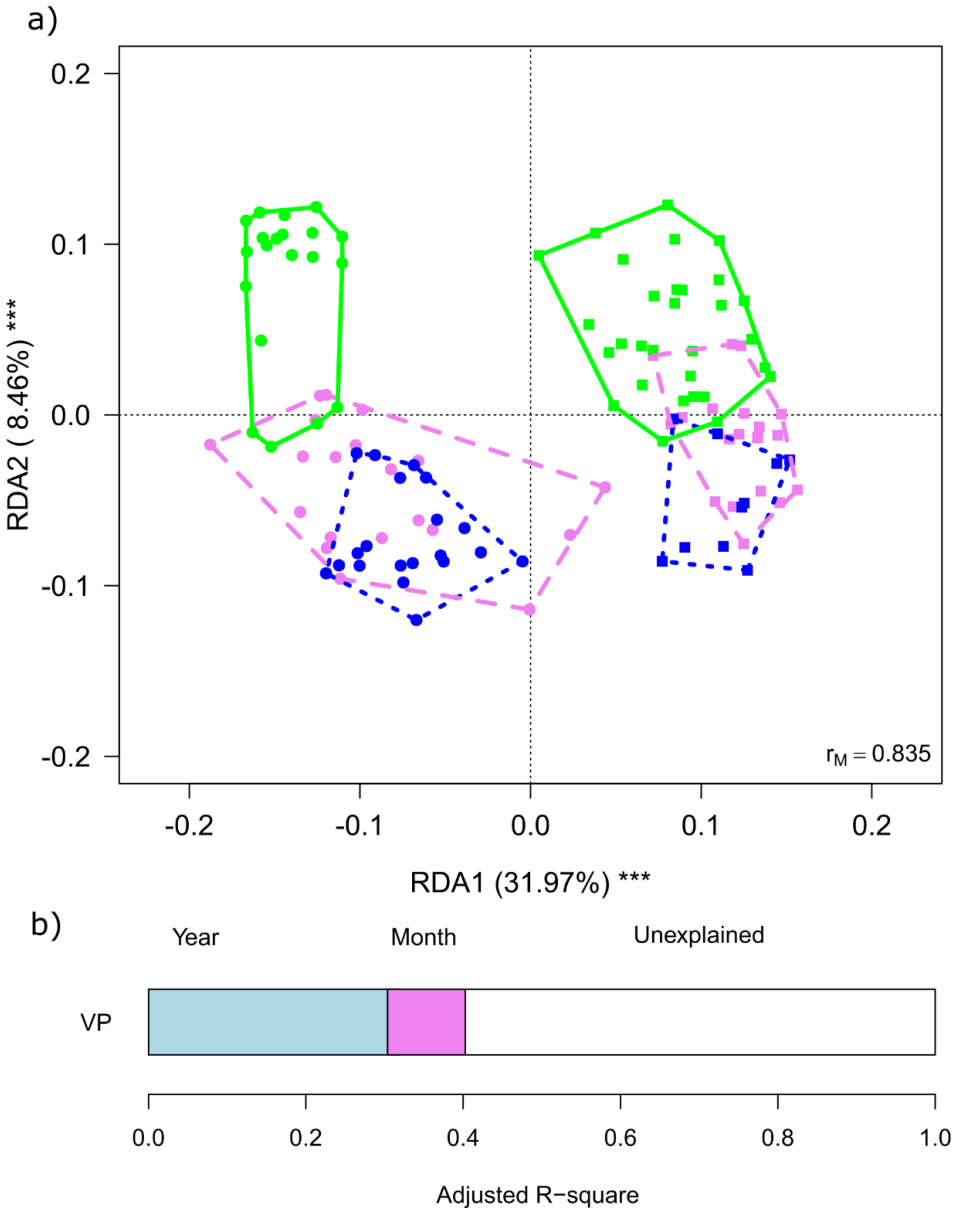

*Figure 3.4 (a) Biplot from RDA on Hellinger-transformed bacterial OUT abundance on year and month. Each point represents one sample. The analysis includes data collected in 2013 (dots) and 2016 (squares) only. Samples collected in different months are indicated by different colours and included in polygons with different line styles (green solid line =July, violet dashed line=August, blue dotted line= September). The percentage of variance explained by each axis and its significance (***: P< 0.001) is reported. rM is the Mantel correlation coefficient between the Hellinger distance between samples and the Euclidean distance between the corresponding symbols in the graph. Values close to one indicate that the graph correctly represents the distance between samples. (b) Results from the variation partitioning showing the amount of variance explained by the independent effects of the predictors entered in the RDA. There was no combined effect of the two variables.*

GLMs indicated that July abundance of all the eight most abundant taxa differed among years ($F_{3,88} \geq 8.891$, $P_{FDR} \leq 0.001$). Post-hoc tests also showed that abundance of Cyanobacteria was lowest in 2012, intermediate in 2013 and 2016 and highest in 2015. Sphingobacteriales were more abundant in 2015 and 2016 than in 2012 and 2013, Burkholderiales were more abundant in 2016 than in all the other years and in 2015 more than in 2013. The abundance of Rhodospirillales and Actinomycetales changed significantly in all years. Pseudomonadales were more abundant in 2013

than in 2012 and 2015 and intermediate in 2016, Cytophagales were more abundant in 2012 than in all the other years, while Clostridiales were more abundant in 2013 than in all the other years and in 2016 more than in 2012 (see Figs S3.3–S3.11 for further details). To gain further insights into within-year variability we focused on data collected in 2013 and 2016 (i.e. the years in which multiple samples were corrected along the ablation season). RDA showed significant variations in the structure of bacterial communities both between years ($F_{1,116}$ = 63.245, P = 0.001) and among months ($F_{2,116}$= 10.722, P = 0.001), with post-hoc tests highlighting significant differences between all pairs of months ($F_{1,116} \geq 12.009$, $P_{FDR} \leq 0.001$). Variation partitioning showed that year per se accounted for 32.01% of variance, while month per se for 9.85%. The amount of variation shared between year and month was null (Fig. 3.4). GLMs showed that abundance of Sphingobacteriales, Cyanobacteria and Clostridiales differed among months ($F_{2,166} \geq 6.284$, $P_{FDR} \leq 0.018$). In particular, abundance of Sphingobacteriales was lower in July than in August and September ($z \geq 6.476$, $P \leq 0.001$), whereas Cyanobacteria were significantly more abundant in July than in the other months ($z \geq 2.351$, $P \leq 0.048$). Clostridiales were more abundant in July, intermediate in August and less abundant in September ($z \geq 2.624$, $P \leq 0.022$). Abundance of Cyanobacteria did not differ significantly between years ($F_{1,116}$= 3.038, P_{FDR}= 0.141), Sphingobacteriales and Burkholderiales were more abundant in 2016 than in 2013($F_{1,116} \geq 10.033$, $P_{FDR} \leq 0.006$), while all the other abundant orders where significantly more abundant in 2013 than in 2016 ($F_{1,116} \geq 10.426$, $P_{FDR} \leq 0.006$). Finally, we aimed at investigating whether meteorological conditions affected the observed within-year trends. To this aim, we ran a RDA including sampling date, expressed as the day of melting season, and TI. This analysis showed that the structure of cryoconite bacterial communities differed between years, changed along the melting season and varied according to TI (Table 3.2, Fig. 3.5a). Variation partitioning also showed that year per se explained 12.11% of the variance, TI 3.93% and their shared effect 18.38%. This probably occurred because TI values differed significantly between years both considering all days during melting seasons ($t_{190,48}$ = 17.209, P < 0.001) and considering sampling days only ($t_{4,27}$ = 2.992, P = 0.037). Day of melting season explained only 1.03% of the variance in the structure of bacterial communities and its contribution was independent of that of all the other variables (Fig. 3.5b). GLMs showed that, after accounting for day of melting season and TI, abundance of Clostridiales, Burkholderiales and Pseudomonadales did not differ significantly between years ($F_{1,116} \leq 5.263$, $P_{FDR} \geq 0.085$), Rhodospirillales, Cytophagales, Pseudomonadales and Actinomycetales were more abundant in 2013 than in 2016 ($F_{1,116} \leq 100.907$, $P_{FDR} \leq 0.001$), while the opposite occurred to Cyanobacteria and Sphingobacteriales ($F_{2,116} \geq 19.999$, $P_{FDR} \leq 0.001$). In

addition, the abundance of no taxon changed with a day of melting season ($F_{1,116} \leq 3.778$, $P_{FDR} \geq 0.594$), while Sphingobacteriales and Burkholderiales increased at increasing values of TI ($F_{2,116} \geq 12.254$, $P_{FDR} \leq 0.004$) and Clostridiales and Cyanobacteria decreased ($F_{2,116} \geq 19.161$, $P_{FDR} \leq 0.001$).

Table 3.2 RDA of Hellinger-transformed bacterial OTU abundance on year, day of melting season and an index of temperatures based on samples collected in 2013 and 2016.

Predictor	Df	Variance	F	P
Year	1	0.017	22.755	0.001
Day of melting season	1	0.002	2.854	0.018
Temperature Index	1	0.006	8.223	0.001
Residuals	116	0.088		

$F_{3,112} = 22.729$, $P = 0.001$, Adjusted-$R^2 = 0.354$

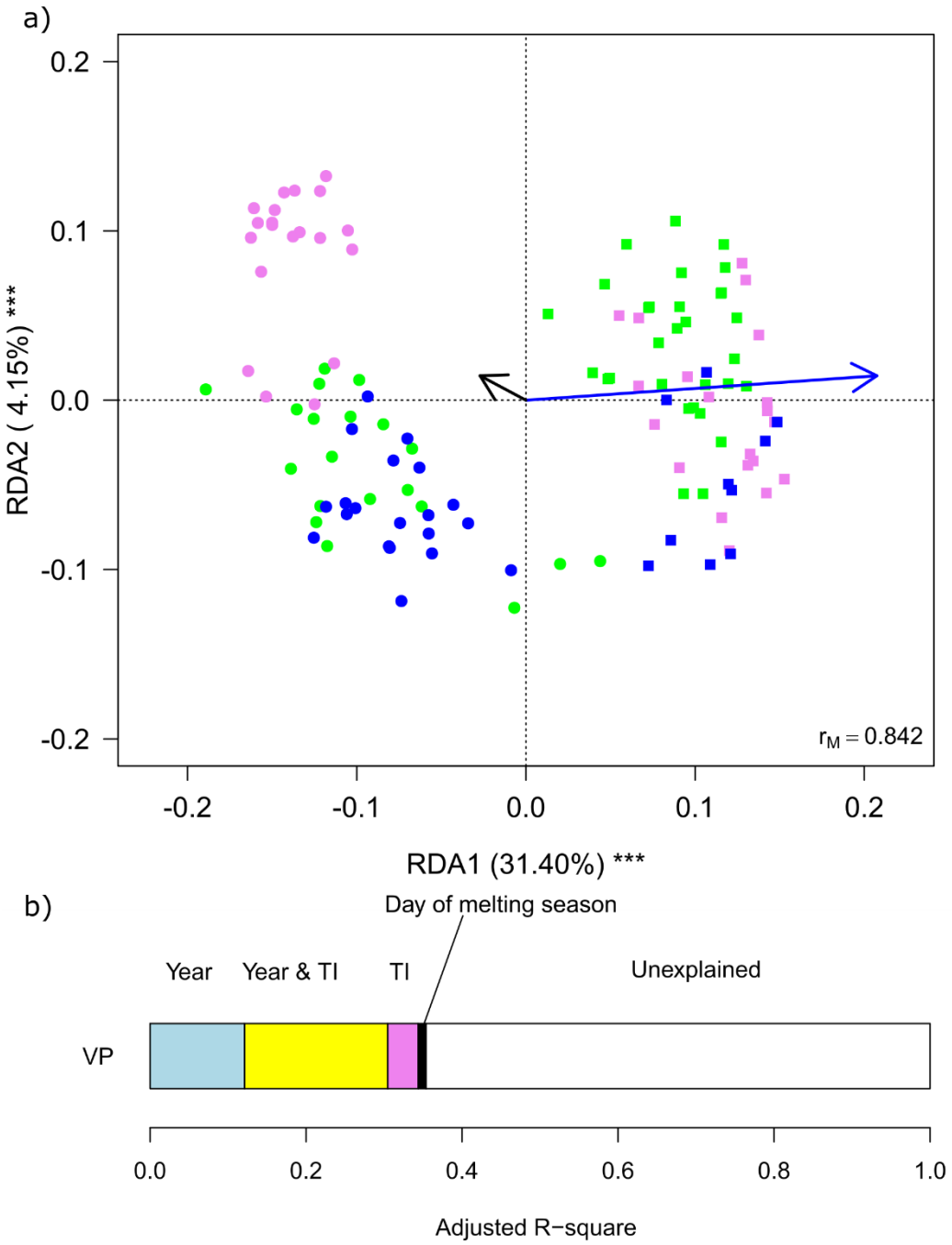

*Figure 3.5 (a) Biplot from RDA of Hellinger-transformed bacterial OUT abundance on the year, day of melting season and temperature index (TI). Each point represents one sample. The analysis includes data collected in 2013 (dots) and 2016 (squares) only. Samples collected in different months are indicated by different colours (green=July, violet=August, blue= September). Arrows represent noncategorical constraining variables (black arrow=day of melting season, blue arrow=TI). The percentage of variance explained by each axis and its significance (***: P<0.001) are reported. rM is the Mantel correlation coefficient between the Hellinger distance between samples and the Euclidean distance between the corresponding symbols in the graph. Values close to one indicate that the graph correctly represents the distance between samples. (b) Results from the variation partitioning showing the amount of variance explained by the independent and combined effects of the predictors entered in the RDA. The amount of variance explained by the shared contribution of TI and day of melting season was null as well as that explained by the shared contribution of year and day of melting season and by all the three variables simultaneously.*

3.4. Discussion

This study investigated the variation in the structure of bacterial communities of cryoconite holes on a temperate mountain glacier. Overall, the most abundant taxa (Cyanobacteria, Sphingobacteriales and Burkholderiales) were the same in all years and have been described as typical of cryoconite holes worldwide (Ambrosini et al. 2017; Gokul et al. 2016; Uetake et al. 2016; Franzetti et al. 2017b). Their relative abundance can markedly vary among geographical areas; for instance, on the Rotmoosferner Glacier (Austria), Proteobacteria were more abundant than on Forni, while Bacteroidetes were more scarce (Edwards et al. 2013). On the Baltoro Glacier (Pakistani Karakoram), the most abundant orders were Burkholderiales, Enterobacteriales and Sphingobacteriales (Ambrosini et al. 2017). Finally, Cameron and others (2012) showed that cryoconite bacterial communities varied according to their geographical location on a global scale. Indeed, they found that communities from individual glaciers clustered together, probably because of similar sources of organisms and/or similar environmental selection pressures. Our results also showed that the structure of bacterial communities can change temporally both within and between ablation seasons. Autotrophs (namely Cyanobacteria), in particular, decreased and heterotrophs (namely Burkholderiales and Sphingomonadales) increased during the melting season in both years. Exceptions to this general pattern occurred, however. For instance, the heterotrophic Clostridiales (phylum Firmicutes) were particularly abundant at the beginning of the ablation season and then disappeared in later samples in both years. Probably, this has occurred because this order is strictly anaerobic (Galperin 2013; Gandolfi et al. 2013; Musilova et al. 2015) and it can be outcompeted by other taxa when the microhabitat becomes richer in organic carbon, more oxygenated and less subjected to extreme conditions. Variation in the structure of bacterial communities along one melting season was already observed in a previous work (Franzetti et al. 2017b) based on a subset of the data included in the present study (i.e. the samples collected in 2013). However, this work adds to previous knowledge not only the information that a similar pattern could be observed also in 2016, but also that the structure of the bacterial communities sampled in the same month differed between years already at the beginning of the melting season, a period for which data on four different years were available. In addition, there was no evidence of an interaction effect between year and month or between year and day of melting season, which indicated that the trend was similar in both years. Importantly, these seasonal trends seem to occur rather quickly on a temperate glacier, as we observed differences even between samples collected 15 days to one another, particularly at the beginning of the melting season. The ecological processes

that affected these trends seemed mainly driven by temperature. In fact, we found that a variable (TI), which indicated periods when DD were higher or lower than what was expected on the basis of the mean seasonal temperature trend, explained a larger amount of variation in community composition than the temporal trend per se (i.e. day of melting season). In particular, abundance of Cyanobacteria decreased at increasing values of TI, while Sphingobacteriales and Burkholderiales increased. In addition, TI, explained a large amount of variation in combination with year because 2016 was significantly warmer than 2013. Taken together, the evidences collected in the present study thus suggested that the structure of the bacterial communities differed among years already at the very beginning of the ablation season and then changed according to 'parallel' seasonal gradients in different years, which, probably, were mainly due to temperature and drove communities toward an increase in heterotrophic taxa and a decrease in autotrophic ones. What we observed in cryoconite holes seemed therefore similar to ecological successions occurring in areas with similar climate conditions but in different biogeographical regions, where successions tend to the same climax (e.g. a tropical forest) but species composition differs. An alternative explanation of the observed data is that cryoconite communities follow exactly the same succession each year, but, by chance, we sampled different stages of the succession in 2013 and 2016. However, we consider this second hypothesis unlikely because in 2016 we collected samples approximately every 2 weeks instead of once per month as we did in 2013. Thus, data collected in 2016 should have revealed if communities had gone through the same (or similar) stages as those observed in 2013. For instance, RDA plots should have shown a large overlap between samples collected in the same month (Figs 3.3 and 3.4), which never occurred. Rather, samples collected in different years were always separated along the first axis. We thus discarded this hypothesis and considered the existence of 'parallel' trends in the variation of community structures as most likely, based on the present results. Large variability in the structure of bacterial communities seems, therefore, to exist already at the beginning of the melting season. Admittedly, we have no information on the processes that can explain such variability. We can speculate that these differences could be due both to the dynamics that occur under the snow cover during the winter season and affect buried cryoconite bacterial communities and to bacteria inputs from the snow cover and from surrounding environments at the very beginning of each melting season. On temperate mountain glaciers, cryoconite holes rarely persist throughout one melting season because the high ablation disperses the cryoconite (Takeuchi et al. 2000; Franzetti et al. 2017b), which can eventually form another hole (Cook et al. 2016a; Cook et al. 2016b). Even more rarely,

they persist for more than one melting season. New holes thus form each year at the beginning of the melting season and an ecological succession, therefore, starts each year. Indeed cryoconite and associated bacteria remains on the glacier surface throughout winter and may form new holes as far as the conditions return favourable. Unfortunately, samples from late September of one year and late June of the following year were not available for the present study. Their collection and analysis would be an important goal for future studies in this field, as it will shed light on the processes driving the onset of new ecological successions on glaciers at the beginning of each ablation season. The present results, however, showed large differences between September and July communities, thus suggesting that large changes occurred during the non-melting season when the glacier surface is covered by snow. Bacterial inputs to cryoconite can also largely differ between years. For instance, a recent study on snow samples collected in very different geographical areas, including Forni Glacier (Azzoni, personal communication), highlighted that snow bacterial communities can include taxa transported over long-range, probably from the area of origin of the air mass that produced the precipitation. Moreover, the relative contribution of distant sources to wind-blown bacteria that reach glacier surface may differ between years (Stibal et al. 2012a) We can, therefore, hypothesize that, at the beginning of the melting season, bacteria in the cryoconite already present on the glacier surface mix both with those in the melting snow and with those transported by wind from near-glacier environments, forming communities that markedly differ from year to year. Another interesting finding of this and a previous study (Franzetti et al. 2017b) was that we observed similar communities in samples collected during a single day, but this pattern could not be explained by an ecological succession occurring within each hole, because the intense radiation determined a continuous dismantle and reforming of holes, so that samples collected during a single day probably came from holes of different 'ages'. The fact that at a time point during the ablation season we observed similar communities in holes of different ages suggests that the ecological succession in a new hole apparently re-started from the same stage that could be observed in the other holes present on the glacier at the same time. Again, the information collected in this study did not allow identifying the ecological processes that generated this pattern. However, some hypotheses can be put forward. In particular, we can hypothesize that ablation and runoff may play an important role in homogenizing bacterial communities. Indeed, at the beginning of each ablation season, the spread of cryoconite on the glacier surface due to the intense runoff and the inputs from snow and surrounding environments, can make the bacteria pool on the glacier surface rather homogeneous, but different from the pool that can be observed in a different year. This

process can explain the differences observed among July samples of different years. Later in the season, the continuous dismantle and reforming of holes due to intense ablation and runoff may determine a continuous flow of bacteria among holes and between old and new holes, thus promoting similarity of communities observed at the same time point. In addition, melting water, on the one side, can leach debris from cryoconite holes, but it is also an important source of organic matter (Takeuchi et al. 2000; Franzetti et al. 2017b; Stibal et al. 2008). Thus, runoff can also affect the shift from communities dominated by autotrophic organisms to communities dominated by heterotrophs.

3.5. Conclusions

Overall, the information collected so far allows proposing a model for the processes driving the temporal trends in bacterial community structure of cryoconite holes of Forni Glacier, which can be schematically summarized in the following steps:

(1) At the beginning of the melting season bacterial populations already present in the cryoconite since the previous year, colonize the newly formed holes together with bacteria transported from near-glacier environments, present in the melting snow thaw or, maybe, transported to the glacier from far-away sources. Communities quickly change and populations more adapted to the hole conditions are selected. Clostridiales rapidly decline.

(2) During the first half of the melting season, Cyanobacteria dominate cryoconite communities, stabilize the sediment by favouring grain formation (Takeuchi et al. 2010) and improve the content of organic matter.

(3) Heterotrophic taxa progressively increase with the increase of organic matter due to Cyanobacteria activity and the inputs due to meltwater runoff. Their increase already starts in the first part of the melting season, but they become dominant in the second part. Temperature seems to affect these patterns, accelerating the shift toward more heterotrophic conditions in warm years.

Clearly, this model is based on data collected on a single glacier and on 2 years of data only. Indeed, as far as we know, studies aiming at investigating the temporal variation in the structure of bacterial communities of cryoconite holes on temperate mountain glaciers have been conducted on Forni Glacier only, so there is no information on whether similar patterns could be observed also on other glaciers. In addition, 2016 was warmer than 2013, so the rather strong effect of TI may be due, at least partly, to the hot weather conditions of this year. Caution is therefore necessary when generalizing these results because we are still far from understanding the ecological processes that drive the seasonal variation of bacterial communities of cryoconite holes on mountain glaciers.

3.6. Acknowledgements

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3.7. Data accessibility

Sequence data are available at European Nucleotide Archive (ENA), study accession number PRJEB8981 (http://www.ebi. ac.uk/ena/data/view/PRJEB8981).

3.8. Supplementary material

Table S3.1 Year, month and day of sampling, day of the melting season in which samples have been collected, positive degree day (DD) and cumulative short wave incoming radiation (SWIN) of the day in which samples have been collected.

YEAR	DAY OF SAMPLING	NUMBER OF SAMPLES	PROGRESSIVE DAY OF MELTING SEASON	DD (°C)	c-SWIN (W/m2)
2012	16-Jul	21	38	249.12	8911.80
2013	10-Jul	20	4	32.83	741.87
	28-Aug	20	53	391.77	11531.82
	25-Sep	20	81	507.48	16107.48
2015	1-Jul	21	18	85.80	4572.58
2016	5-Jul	20	12	93.34	2723.60
	19-Jul	10	26	180.39	6764.47
	2-Aug	10	40	283.66	9623.80
	16-Aug	10	54	362.24	12953.65
	2-Sep	10	71	492.79	16494.86

Table S2 OTU abundance for each sample normalized to 20,000 sequences per sample and the relative classification.

Table S2 can be found at https://doi.org/10.1017/aog.2018.16

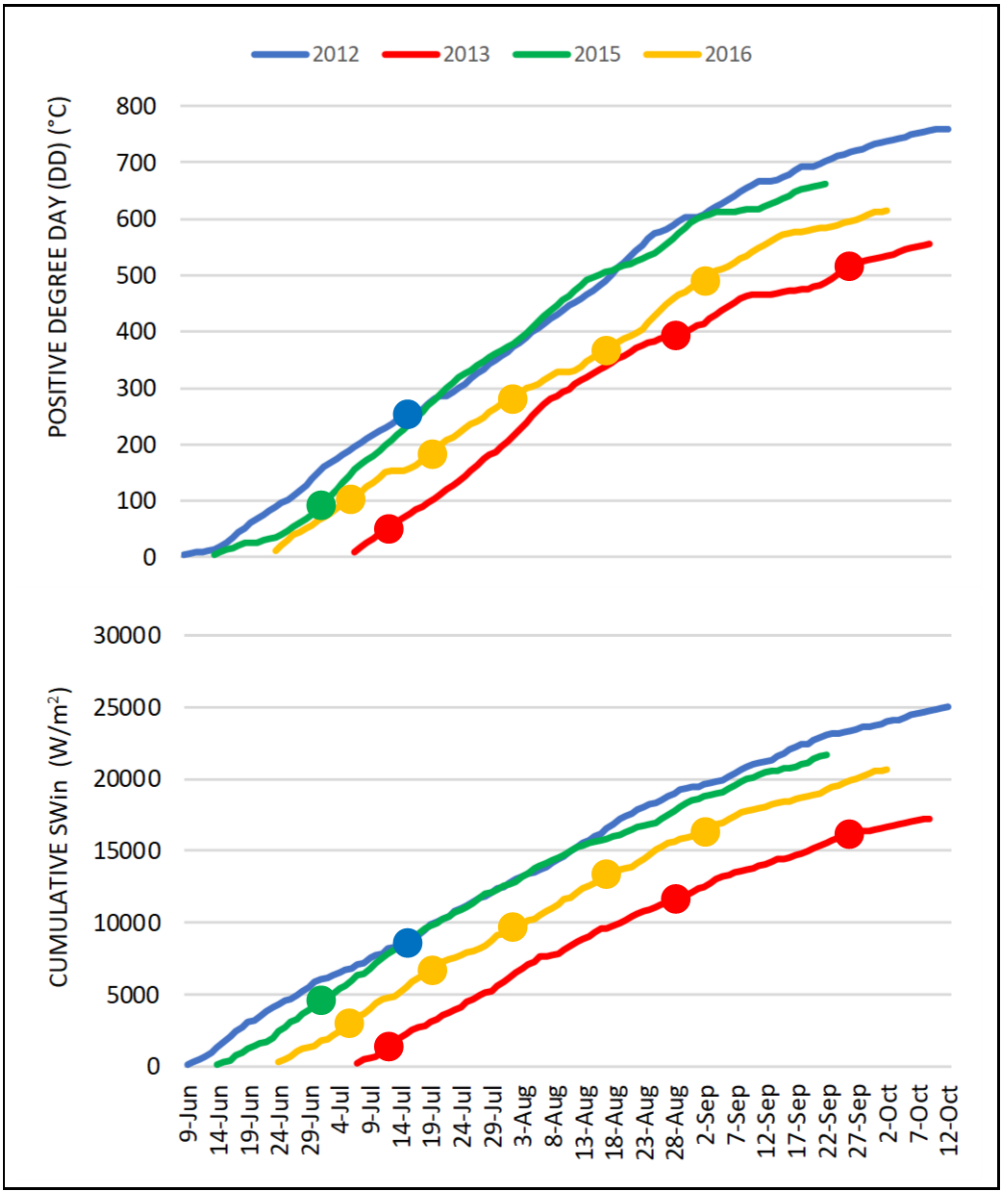

Figure S3.1 Positive degree day factor (DD) and cumulative incoming shortwave radiation (c-SWin) calculated for 2012 (blue), 2013 (red), 2015 (green) and 2016 (yellow) ablation season. The coloured dot indicated days of sampling for each season.

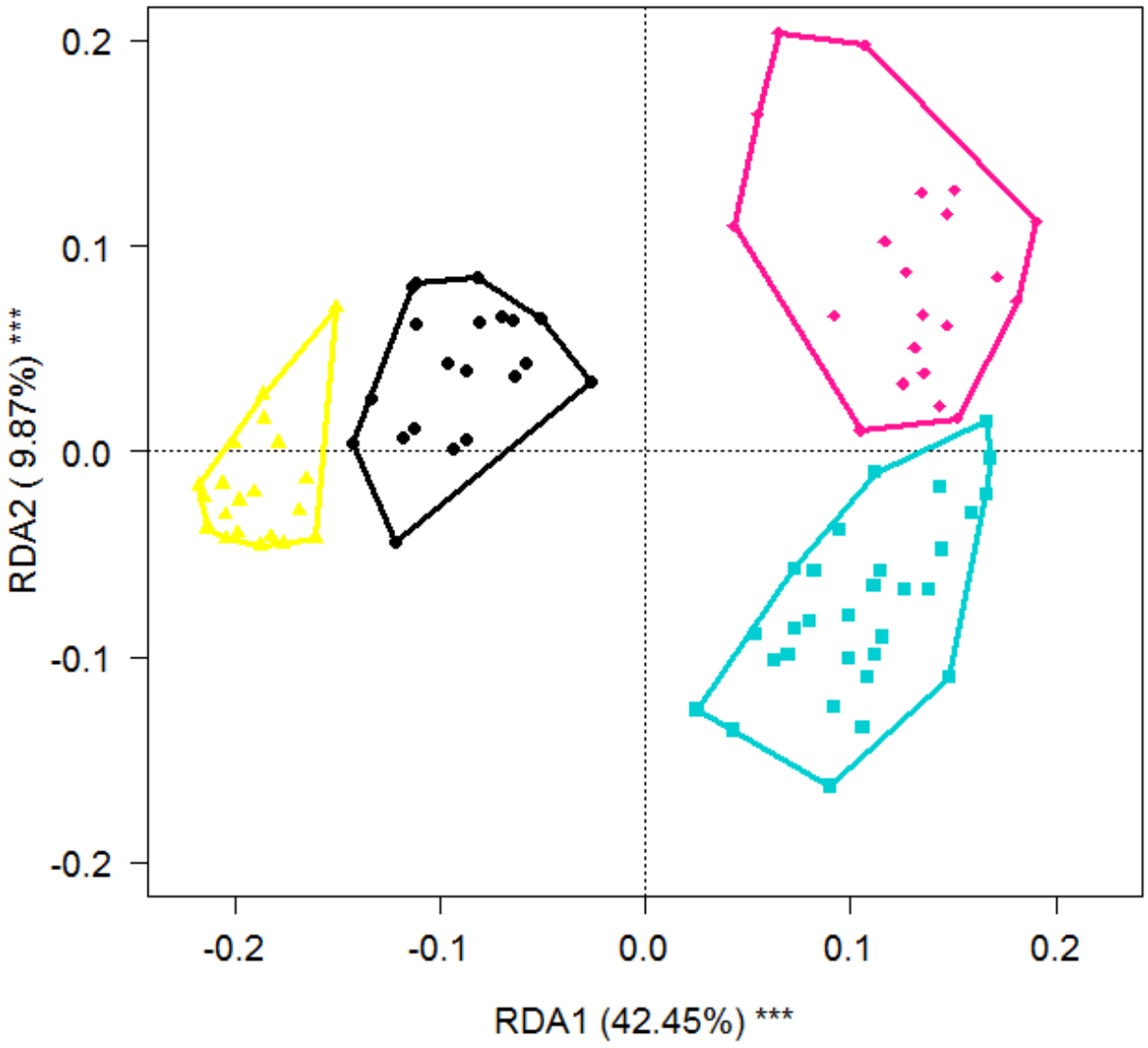

Figure S3.2 RDA on all the samples of July, for every year of sampling. Yellow dots = 2012, black triangles = 2013, pink diamonds = 2015, turquoise squares = 2016.

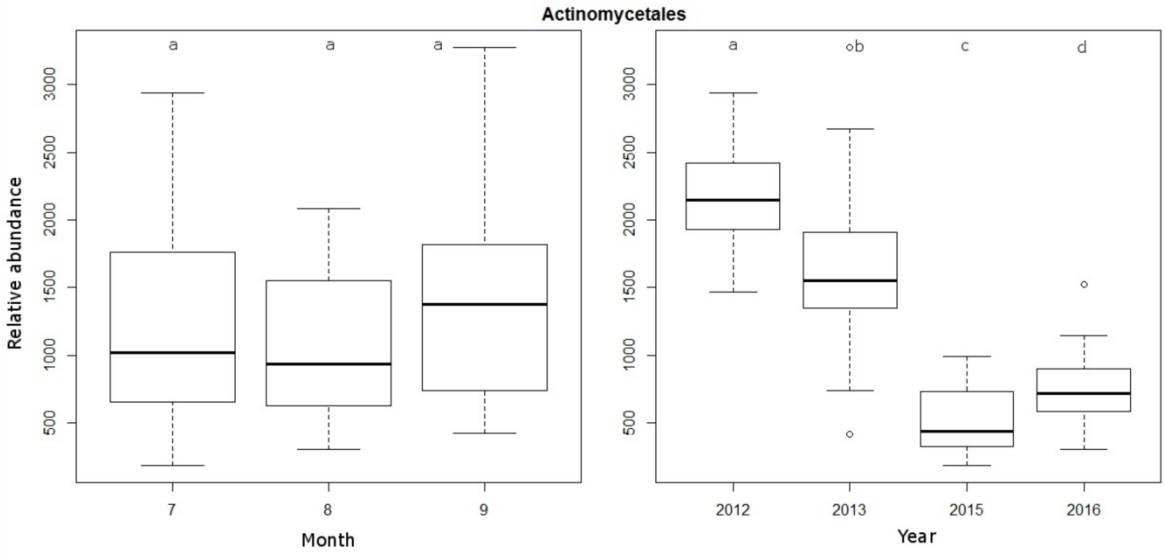

Figure S3.3 Boxplots that represent Actinomycetales variation between years and between months.

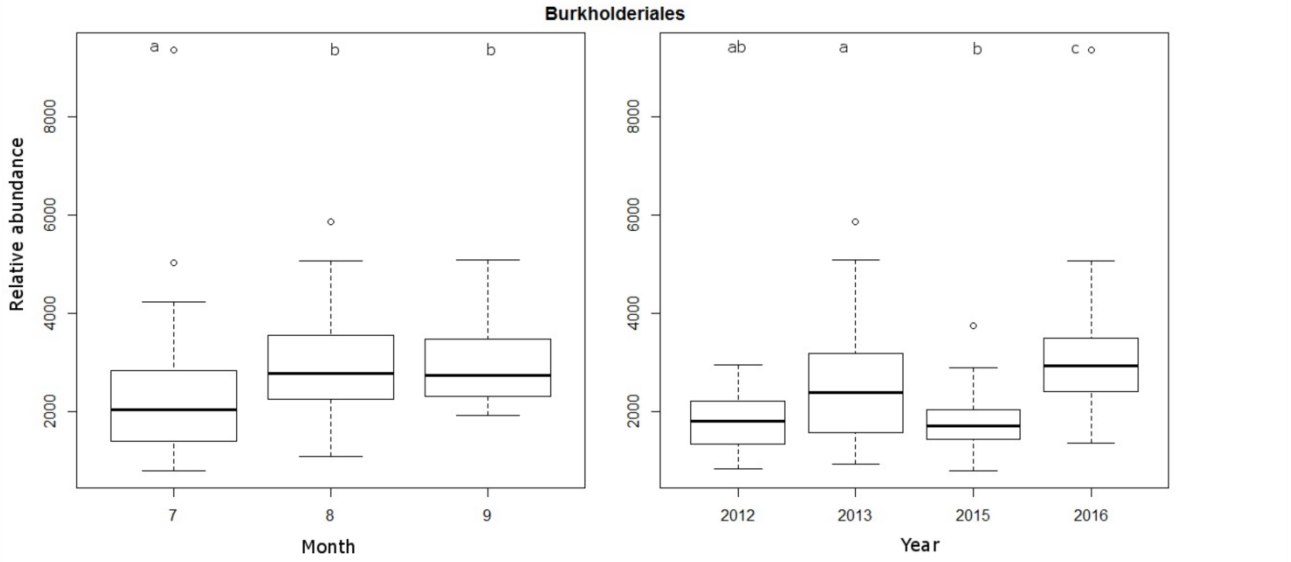

Figure S3.4 Boxplots that represent Burkholderiales variation between years and between months.

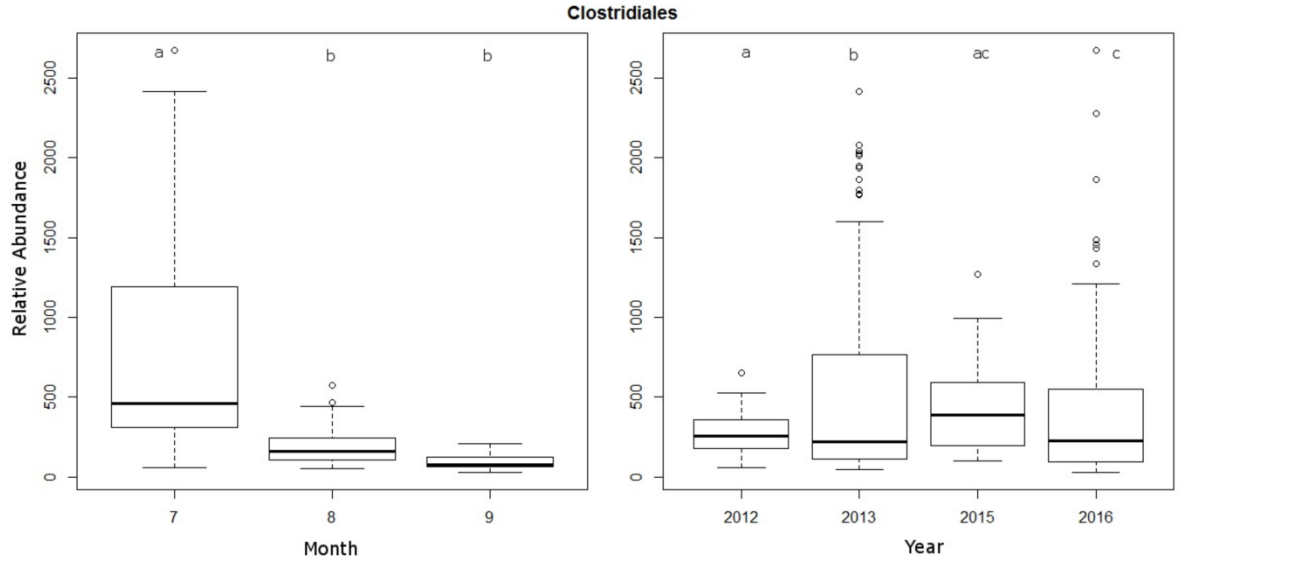

Figure S3.5 Boxplots that represent Clostridiales variation between years and between months.

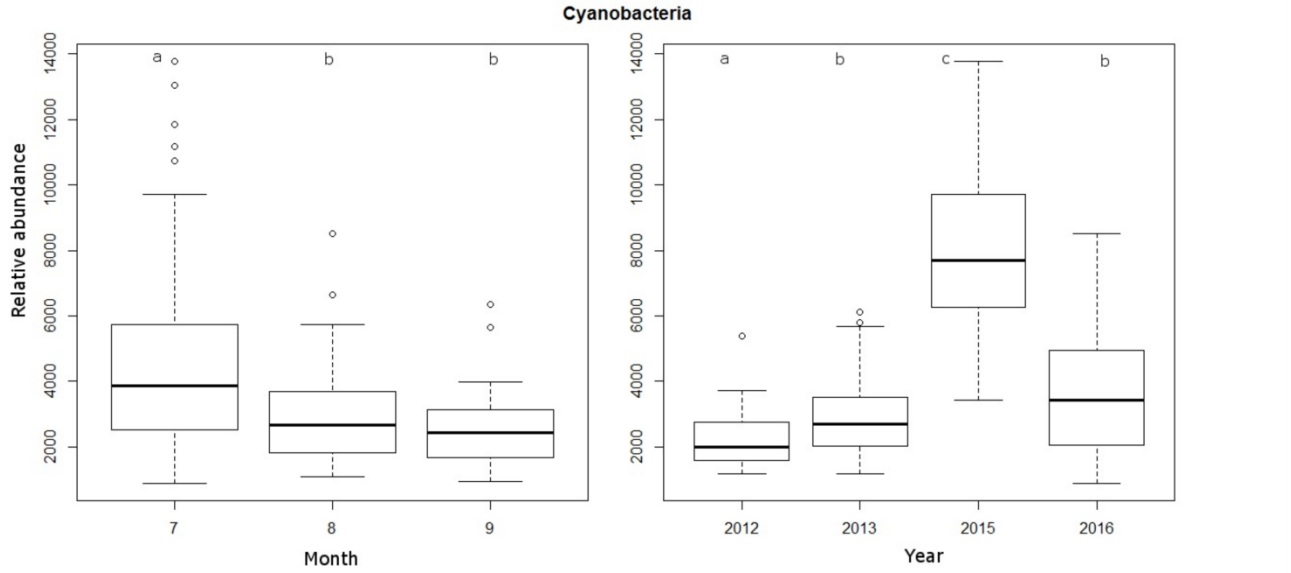

Figure S3.6 Boxplots that represent Cyanobacteria variation between years and between months.

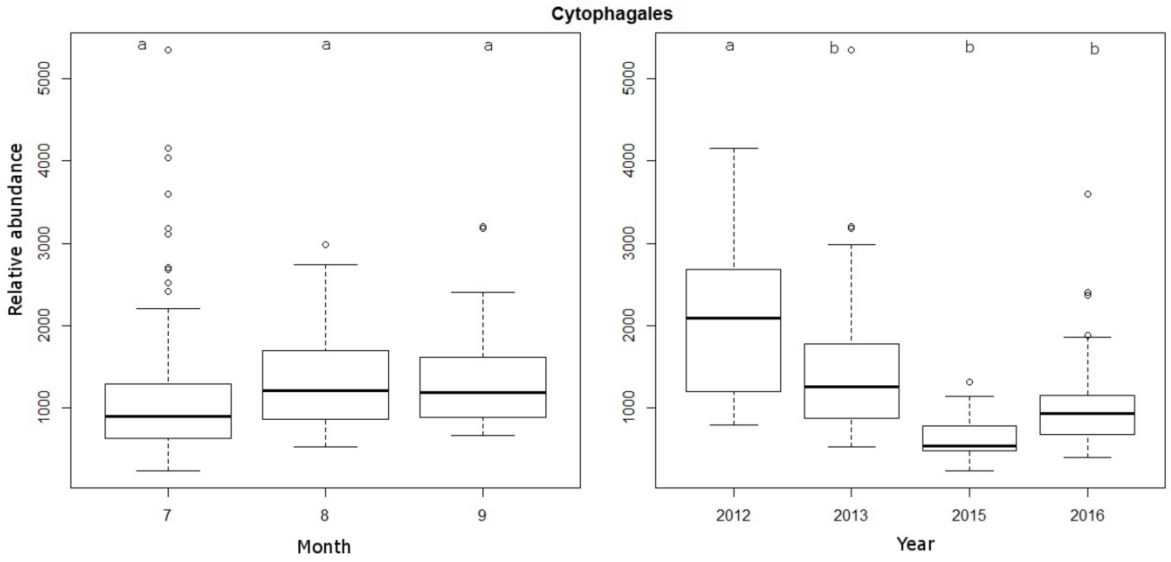

Figure S3.7 Boxplots that represent Cytophagales variation between years and between months.

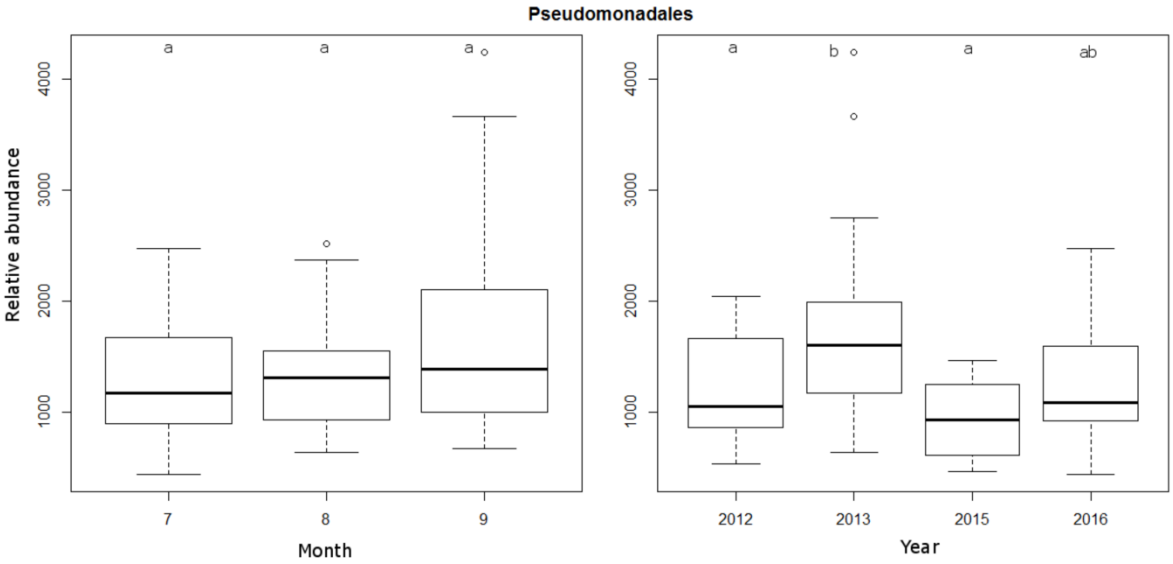

Figure S3.8 Boxplots that represent Pseudomonadales variation between years and between months.

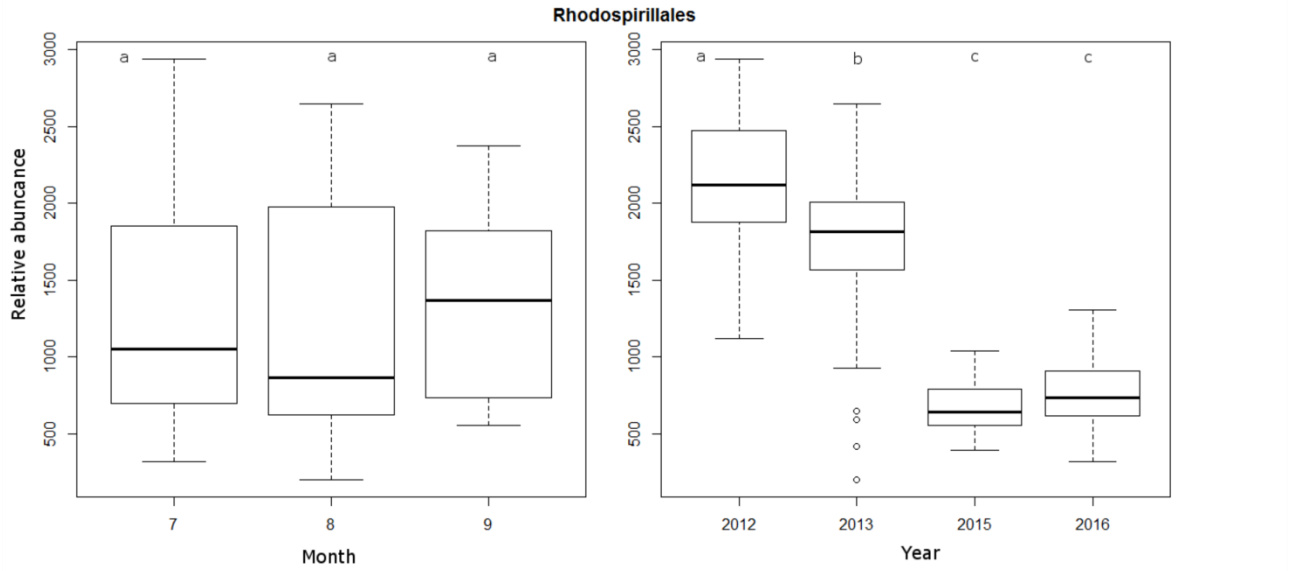

Figure S3.9 Boxplots that represent Rhodospirillales variation between years and between months.

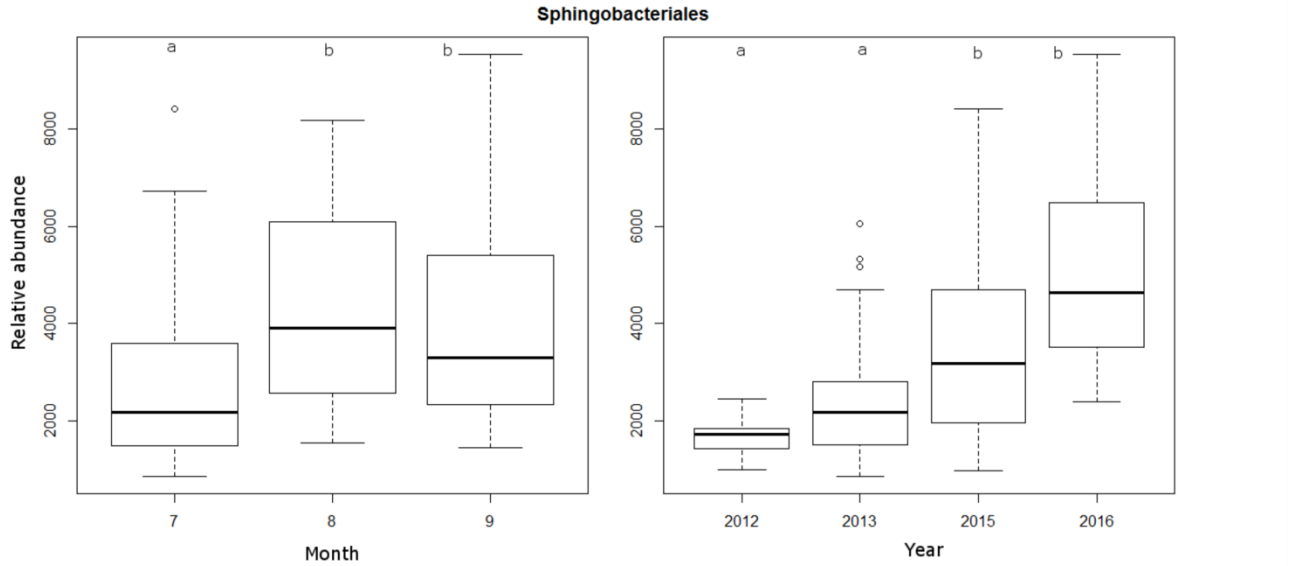

Figure S3.10 Boxplots that represent Sphingobacteriales variation between years and between months.

4. DIEL TRANSCRIPTIONAL PATTERN IN BACTERIAL COMMUNITIES IN CRYOCONITE HOLES

ABSTRACT Despite the harsh environmental conditions, glacier surfaces host metabolically active bacterial communities, especially in cryoconite holes, small ponds filled with melting water and with a fine-grained sediment at the bottom. We investigated the daily changes in transcript profiles of the microbial community of a cryoconite hole on an Alpine glacier. Using a metatranscriptomic shotgun sequencing, we observed different level of expression of the main carbon and energy metabolisms along the day. Oxygenic and anoxygenic photosynthesis peaked their activity at the sunrise and sunset, respectively, and showed an inhibition at midday, in response to high solar radiation. Carbon fixation genes were expressed all day long with the lowest coverage at night. Different microbial populations were responsible for this metabolic function along the day. Cyanobacteria and Algae were the most active primary producers at the sunrise and the sunset, whereas at night and at noon chemosynthetic proteobacteria, likely hydrogen oxidisers, were most active. Furthermore, the observed temporal cascade of transcript peaks of photosynthesis and respiration recalls those occurring in both coastal and open waters in ocean, thus supporting the hypothesis that conserved temporally phased biotic interactions are ubiquitous among aquatic communities worldwide.

4.1. Introduction

Cryoconite holes are small ponds filled with of meltwater with a sediment at the bottom present on the surface of most glaciers. Although they are characterized by extreme conditions such as low temperatures and high solar irradiance, they host bacterial communities with high taxonomic and functional biodiversity (Cook et al. 2016b). It has been supposed that such high functional diversity could be due to the high versatility of some of the most abundant populations (Darcy et al. 2011). In a previous investigation, we demonstrated that these supraglacial communities exhibit high functional biodiversity since they exploit organic matter both as energy and carbon source, and use both oxygenic and anoxygenic photosynthesis with pure autotrophic and mixotrophic lifestyles (Franzetti et al. 2016). Due to the lack of expression studies in these environments, it is currently unknown whether a diel temporal pattern of expression occurs and how it may contribute to the overall functional and taxonomic biodiversity. To fill this gap of knowledge, we collected four cryoconite samples from a single hole along a clean summer day (4.30 am, 7.30 am, 1.30 pm and 7.30 pm) on the Forni Glacier (Italy) on 24 July 2018 and used shotgun metatransctriptomics sequencing to investigate the expression of the main metabolic functions along the day. We focused on carbon and energy metabolisms by comparing the total coverage of marker gene transcripts for photosynthesis, use of inorganic and organic compounds as energy source, respiration and autotrophy/heterotrophy.

4.2. Materials and methods

4.2.1. Sampling and metagenome sequencing

Cryoconite was collected from a single cryoconite hole at four different times (4.30 am, 7.30 am, 1.30 pm, 7.30 pm) on the 24th of July 2018. The cryoconite hole was located on the central ablation tongue of the Forni Glacier (Italy, 46.39868 ° N, 10.58664 ° E) at 2670 m a.s.l. To preserve the RNA content, cryoconite was immediately mixed with a RNA preservative solution (1:5 volume ratio) and stored at -80° within 48 hours. RNA preservative solution was prepared with 4 mL of EDTA 0.5 M, 2.5 mL of $Na_3C_6H_5O_7$ 1M, 70 g of $(NH_4)_2SO_4$, DEPC-H_2O for a final volume of 100 mL (Gray, Pratte, and Kellogg 2013). Total RNA was extracted from 1 g of cryoconite with the RNeasy PowerSoil Total RNA Kit (QIAGEN, Hilden, Germany) according to the manufacturer instructions. After the extraction, residual DNA was removed with the RQ1 RNase-free DNase (Promega) from 6 µL of extracted RNA. The total DNA-free RNA was then treated with the MICROBExpress™ Bacterial mRNAEnrichment Kit (Ambion) and 8 µL of the depleted RNA were retrotranscribed with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) using random primers. Shotgun sequencing was applied to cDNA by HiSeq Illumina (Illumina, Inc., San Diego, CA, USA) using a 150 bp x 2 paired-end protocol on one lane. The paired-end reads were quality-trimmed (minimum length: 80 bp; minimum average quality score: 30) using Sickle (https://github.com/najoshi/sickle). Sequence data were submitted to European Nucleotide Archive (ENA), study accession number PRJEB34670 (http://www.ebi.ac.uk/ena/data/view/PRJEB34670).

4.2.2. Bioinformatics procedures

Filtered reads were co-assembled using IDBA-UD (Peng et al. 2012). IDBA-UD iterated the value of k_{mer} from 40 to 99 (with a step of 5). Predicted genes were inferred from contigs with Prodigal (Hyatt et al. 2010). KO numbers were assigned to the predicted proteins using the on line tool GhostKOALA (Kanehisa, Sato, and Morishima 2016). Lowest Common Ancestor (LCA) algorithm was applied to infer taxonomic affiliation of predicted genes using MEGAN default parameters (Huson et al. 2011). Hierarchical taxonomic data were visualised with Krona (Ondov et al. 2011). Average per-base coverage of predicted genes was calculated using filtered reads with bowtie2 (Langmead et al. 2012), SAMtools (Li et al. 2009) and bedtools (Quinlan et al. 2010). To normalize the different sequencing depth across the samples, sum of gene coverages was normalized to 600,000 for each sample.

4.3. Main text

Cryoconite holes are small ponds full of meltwater with a sediment on the bottom present on the surface of most glaciers. Although they are characterized by extreme conditions such as low temperatures and high solar irradiance, they host bacterial communities with high taxonomic and functional biodiversity (Cook et al. 2016b). It has been supposed that such high diversity could be due to the high versatility of some of the most abundant populations (Darcy et al. 2011). In a previous investigation, we demonstrated that these supraglacial communities exhibit high functional biodiversity since they exploit organic matter both as energy and carbon source, and use both oxygenic and anoxygenic photosynthesis with pure autotrophic and mixotrophic lifestyles (Franzetti et al. 2016). Due to the lack of expression studies in these environments, it is currently unknown whether a diel temporal pattern of expression occurs and how it may contribute to the whole functional and taxonomic biodiversity. To investigate this hypothesis, we collected four cryoconite samples from a single hole along a typical summer day (4.30 am, 7.30 am, 1.30 pm and 7.30 pm) on the Forni glacier (Italy) on July 2018 and we used shotgun metatransctriptomic sequencing to investigate the expression of the main metabolic functions along the day. We focused on carbon and energy metabolisms by comparing the total coverage of marker gene transcripts for photosynthesis, use of inorganic and organic compounds as energy source, respiration and autotrophy/heterotrophy. Table S4.1 reports the marker gene transcripts whose coverage (mean number per base of reads mapping the genes) was used to infer the expression of each metabolism and their normalized coverages. We also used transcript sequences for the taxonomic attribution of microorganisms expressing specific metabolic genes. SM3 reports the transcript coverage of all the annotated KO (KEGG Orthology) molecular functions.

Results showed that carbon and energy metabolisms had different patterns of expression along the day (Fig. 4.1, Tab. S4.1). Before sunrise (4.30 am) aerobic respiration and denitrification were the dominant energy metabolisms, while neither anoxygenic nor oxygenic phototrophy were active (Fig. 4.1).

4.4. Results

Table 4.1 reports the marker gene transcripts whose coverage (mean number per base of reads mapping the genes) was used to infer the expression of each metabolism and their normalized coverages. We also used transcript sequences for the taxonomic attribution of microorganisms expressing specific metabolic genes (SM1). Finally, we report the transcript coverage of all the annotated KO (KEGG Orthology) molecular functions (SM2).

Table 4.1 Transcript coverages of the marker genes.

				Coverage			
Metabolism	**Gene**	**Short name**	**KO Orthology**	**4.30 am**	**7.30 am**	**1.30 pm**	**7.30 pm**
Oxygenic photosynthesis	photosystem II P680 reaction center D2 protein	*psb*D	K02706	2.1	317.4	83.8	163.5
Aerobic anoxygenic photosynthesis	photosynthetic reaction center L and M subunits	*puf*M	K08929	3.2	32.4	0.0	69.6
CO_2 fixation, Calvin-Benson cycle	ribulose bisphosphate carboxylase small and large chain (RubisCO)	*rbc*L	K01602, K01601	25.5	96.8	84.2	50.0
Chitin assimilation	chitinase	*chi*A	K01183	68.7	6.5	0.0	35.1
Hydrolysis O- and S-glycosyl compounds	endo-1,4-beta-xylanase	*xyn*A	K01181	N.D	N.D	N.D	N.D
Carbon monoxide oxidation	carbon-monoxide dehydrogenase medium and large subunits	*cox*ML	K03519, K03520	N.D	N.D	N.D	N.D
Aerobic respiration	cytochrome c oxidase subunit I	*cox*A	K02274	908.8	283.4	677.6	735.1
Dissimilatory nitrate reduction (Denitrification/Nitrification)	nitrate reductase alpha subunit	*nar*G	K00370	748.0	145.8	568.0	454.8
Dissimilatory nitrate reduction (Denitrification)	nitrite reductase (NO-forming) [EC:1.7.2.1]	*nir*K	K00368	831.3	129.3	778.2	408.5
Dissimilatory nitrate reduction (Denitrification)	nitric oxide reductase subunit B [EC:1.7.2.5]	*norB*	K04561	815.5	105.8	666.3	409.1
Ammonia oxidation (nitrification)	ammonia monooxygenase A and B subunits	*amo*A	K10944	0.0	1.0	0.1	0.1
Sulfur oxidation	sulfur-oxidizing protein	*sox* A	K17222	0.0	13.3	0.0	40.3

Hydrogen oxidation	hydrogenase large subunit	*hya* B	K06281	19.0	0.0	26.0	12.5
Nitrogen fixation	nitrogenase iron protein	*nif*H	K02588	0.2	0.0	0.3	2.4

Figure 4.1 Marker transcript coverages cryoconite. psbD, photosystem II P680 reaction center D2 protein; pufM, photosynthetic reaction center M subunit; rbcL, ribulose bisphosphate carboxylase large chain (RubisCO); narG; nitrate reductase alpha subunit; hya B: hydrogenase large subunit; coxA: cytochrome c oxidase subunit I

4.5. Discussion

Results showed that carbon and energy metabolisms had different patterns of expression along the day (Fig. 4.1, Tab. 4.1). Before sunrise (4.30 am) aerobic respiration and denitrification were the dominant energy metabolisms, while neither oxygenic nor anoxygenic phototrophy were active (Fig. 4.1).

According to the taxonomic affiliation of *cox*A and *nir*K, Actinobacteria and Proteobacteria were the most active taxa involved in respiration with slightly different abundances along the day (SM1). Cyanobacterial respiration was active after sunrise (7.30 am) (20% of abundance) and before the sunset (7.30 pm) (6% of abundance) (Fig. 4.1).

Anaerobic respiration also actively occured in cryoconite holes, seems restricted to denitrification since neither iron reduction (rusticyanin) nor dissimilatory sulfite reductase gene (*dsr*A) were actively transcribed (SM2). Consistently, the development of an anoxic zone 2 mm deep has been recently described in cryoconite (Poniecka et al. 2018) and the complete denitrification pathway was also active (*nar*G, *nir*K, *nor*B), as already reported in the cryoconite of a Chinese glacier (Segawa et al. 2014).

As expected, Cyanobacteria and algae were the active oxygenic phototrophs. Aerobic anoxygenic phototrophs (APPs) resulted affiliated to alpha and beta-proteobacteria (SM1) and are known to be photoheterotrophic, thus using organic matter as carbon source and complementing their energy demand with light (Koblížek 2015). Both oxygenic (*psb*D) and aerobic anoxygenic (*puf*M) photosynthesis showed reduced activity at the time of the highest solar irradiance (1.30 pm). For oxygenic phototrophs, this temporal pattern is consistent with the described downregulation of PSI and PSII (*psa* and *pdb* genes) of cyanobacteria after the shift from low to high light intensity (Hihara et al. 2001; Ogawa et al. 2018) as a response to light damages of the photosynthetic apparatus. Expression of oxygenic (*psb*D) and aerobic anoxygenic (*puf*M) photosynthesis peaked after sunrise (7.30 am) and before sunset (7.30 pm), respectively (Fig. 4.1). Interestingly, this pattern of the peak of activity of oxygenic photoautotrophs followed by anoxygenic ones and by the oxidative phosphorylation transcript maxima, recalls those occurring in both coastal and open waters in the Pacific ocean (Aylward et al. 2015).

A relevant expression of the CO_2 fixation genes (*rblc*L) was observed in all the sampling times with the maximum expression after sunrise and the lowest at night. Interestingly, the taxa expressing this metabolic function are extremely variable along the day. Indeed, Cyanobacteria and algae were the most active CO_2 fixators at 7.30 am and 7.30 pm. Cyanobacteria were not expressing RubisCO gene at 1.30 pm, in disagreement with previous reports showing an up-regulation of this gene under high light intensity (Hihara et al. 2001; Ogawa et al. 2018). At this time of the day, Proteobacteria and algae were the active primary producers. The fact that at night and at 1.30 pm phototrophs were not the most active primary producers, suggests that chemolithoautotrophic microorganisms could be active. Ammonia seems not among the possible inorganic electron donors, because the

expression of *amo*A (ammonia monooxygenase A subunit) was negligible at any time, while the oxidation of nitrite to nitrate may be the active chemolitoautrophic pathway because nitrate reductase alpha subunit (*nar*G) was active. However, NarG might catalyse both the oxidation of nitrite and the dissimilatory reduction of nitrate. Moreover, we observed a negligible expression of nitrogenaes (*nif*H), which suggests that nitrogen was not fixed. Therefore, a short nitrogen cycle comprising of dissimilatory nitrate reduction to nitrite and lithotrophic oxidation of nitrite to nitrate could be put forward as a mechanism sustaining the chemosynthesis in these environments. However, the genes for the complete denitrification pathway (*nar*G, *nir*K, *nor*B) showed similar expression levels at all times, suggesting that NarG had reductive catalytic activity rather than oxidative. Carbon monoxide oxidation to CO_2 was previously hypothesized as a possible lithotrophic metabolism in cryoconite due to the high abundance of carbon-monoxide dehydrogenase (*cox*ML) (Franzetti et al. 2016). However, the transcripts of this gene were not retrieved in this study, thus suggesting, as previously reported (Haan et al. 2001), that carbon monoxide is produced by photochemical reactions in presence of snow. Among the different key genes for lithotrophic sulfur oxidation (*apr*A, *dsr*A), only *sox*A was transcribed (SM2). However, *sox* genes were likely harboured by anoxygenic phototrophs (Dahl 2008), since they were retrieved only at 7.30 am and 7.30 pm when also the *puf*M genes were highly transcribed and when the primary production was mainly due to phototrophs. Therefore, we excluded sulfur oxidation as an active chemosynthetic process.
Interestingly, hydrogenase (*hya*B) genes were actively transcribed (Fig. 4.1, Tab. 4.1) when CO_2 fixation was due to non-phototrophs (4.30 am and 1.30 pm). This might indicate that hydrogen oxidation might provide the reducing power for primary production during the night and when photosynthesis is inhibited. This hypothesis is supported by the fact that most of *rblc*L transcripts at 4.30 am and 1.30 pm were taxonomically assigned to *Paracoccus* spp. and *Hydrogenophilus* spp., respectively (SM2), which are known for their capacity to use molecular hydrogen as an electron donor (Bardischewsky et al. 2001; Hayashi et al. 1999). Hydrogen can have both biotic or abiotic origin. Biological hydrogen production could be of both fermentative and photosynthetic origin and is inhibited by oxygen (Benemann 1997; Das et al. 2001). Therefore, these processes could be active in the anoxic layer of the cryoconite (Poniecka et al. 2018). Abiotic hydrogen production has been proposed as a mechanism sustaining lithoautotrophic life in deep and subglacial environments. Abiogenic H_2 is formed due to a variety of water–rock reactions (Telling et al. 2015). Telling and colleagues reported that sufficient H_2 is produced to support methanogenesis under a Greenland glacier (Telling et al. 2015). Moreover, recent studies demonstrated that cryoconite is among the

most radioactive environmental matrices, with activity concentrations exceeding 10,000 Bq kg^{-1} for single radionuclides (Baccolo et al. 2019; Łokas et al. 2016). This radioactivity might cause radiolytic H_2 production in the cryoconite holes, which, in turn, supports bacterial metabolism as already reported in deep subsurface environments (Lin et al. 2005). Further studies are required to confirm the presence of hydrogen in cryoconite and to assess its origins.

This first report of shotgun metatranscriptomics in cryoconite therefore revealed a circadian trend of the main energy and carbon metabolisms, which may contribute to the overall functional and taxonomic diversity of these environments (Fig. 4.2). Different microbial taxa contribute to the same ecosystem functions (e.g. carbon fixation and respiration) along the day and primary productivity was supported by both light and chemosynthesis. This functional redundancy likely enhances the ecosystem stability in such extreme environments. Results from this study also provide pieces of evidence supporting the hypothesis that conserved microbial community transcriptional and biotic interactions, which follow a daily temporal trend, are ubiquitous among aquatic microbial communities worldwide (Ottesen et al. 2014; Aylward et al. 2015).

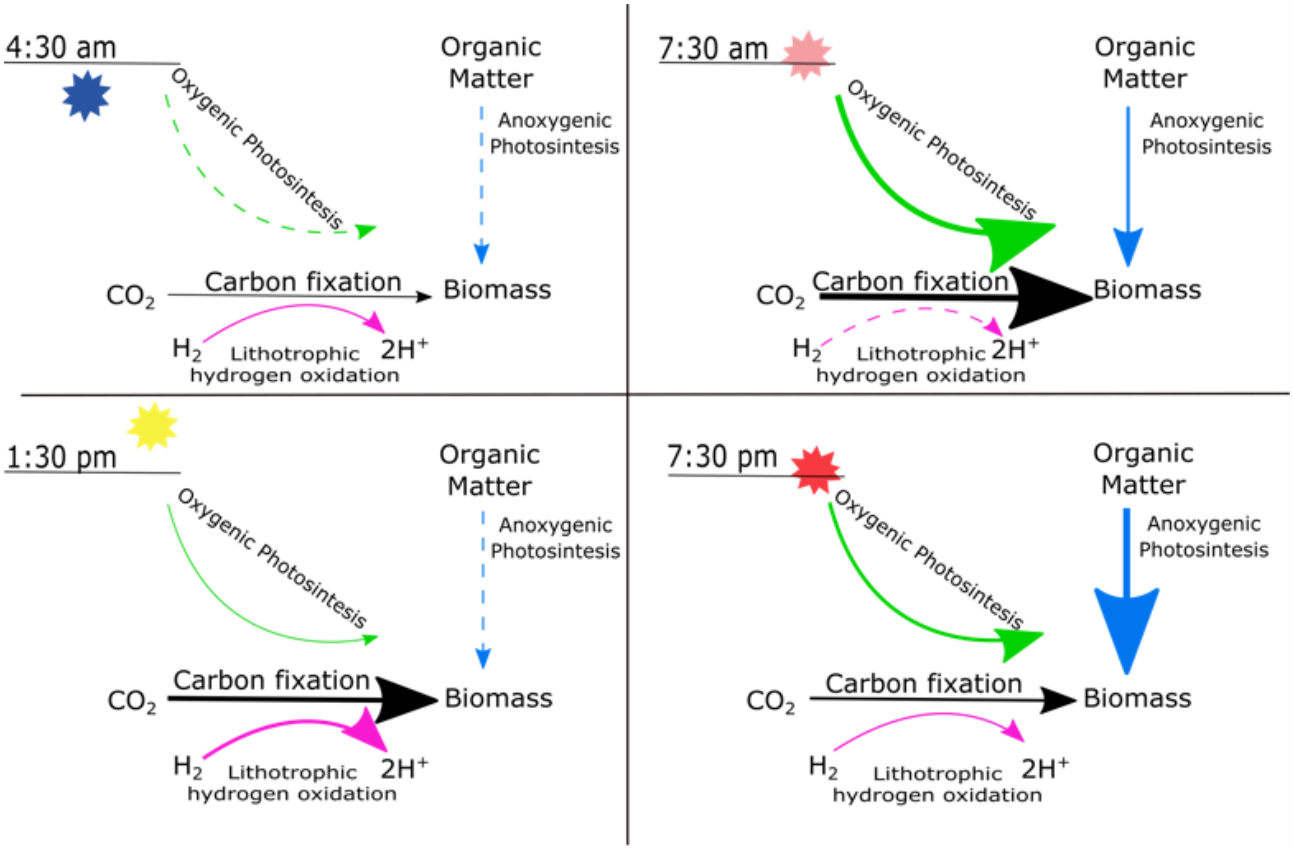

Figure 4.2 Proposed model of a temporal trend of the main energy and carbon metabolisms. Arrows thickness is proportional to transcript abundances.

4.6. Acknowledgments

Authors thank Parco dello Stelvio (Italy) for logistic assistance. This work was partially funded by the University of Milano-Bicocca (PhD fellowship to FP). Bioinformatics analyses have been run on MARCONI server (CINECA, Bologna, Italy).

5. EARLY ECOLOGICAL SUCCESSION PATTERNS OF BACTERIAL, FUNGAL AND PLANT COMMUNITIES ALONG A CHRONOSEQUENCE IN A RECENTLY DEGLACIATED AREA OF THE ITALIAN ALPS

The content of this chapter has been published in the following paper:
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ABSTRACT In this study, the early ecological succession patterns of Forni Glacier (Ortles-Cevedale group, Italian Alps) forefield along an 18-year long chronosequence (with a temporal resolution of 1 year) has been reported. Bacterial and fungal community structures were inferred by high-throughput sequencing of 16S rRNA gene and ITS, respectively. In addition, the occurrence of both herbaceous and arboreous plants was also recorded at each plot. A significant decrease of alpha-diversity in more recently deglaciated areas was observed for both bacteria and plants. Time since deglaciation and pH affected the structure of both fungal and bacterial communities. Pioneer plants could be a major source of colonization for both bacterial and fungal communities. Consistently, some of the most abundant bacterial taxa and some of those significantly varying with pH along the chronosequence (Polaromonas, Granulicella, Thiobacillus, Acidiferrobacter) are known to be actively involved in rock-weathering processes due to their chemolithotrophic metabolism, thus suggesting that the early phase of the chronosequence could be mainly shaped by the biologically controlled bioavailability of metals and inorganic compounds. Fungal communities were dominated by ascomycetous filamentous fungi and basidiomycetous yeasts. Their role as cold-adapted organic matter decomposers, due to their heterotrophic metabolism, was suggested.

5.1. Introduction

Cold habitats (characterized by an average annual temperature <5◦C) represent ∼80% of the Earth's environments; the most relevant ones are deep seas, cold deserts, ice sheets and Arctic, Antarctic and high mountain glaciers (Anesio et al. 2012; Buzzini et al. 2013). A number of studies documented that these habitats, exhibiting extreme ecological conditions, host viable and metabolically active prokaryotic and eukaryotic microbial communities (Boetius et al. 2015; Buzzini et al. 2012; Siles et al. 2016; Buzzini et al. 2013). Both psychrophilic and psychrotolerant microorganisms living in cold habitats are subjected to a combination of stress factors (Rothschild et al. 2001), therefore they have developed multiple physiological adaptations allowing them to successfully colonize these habitats where they are considered active players in biogeochemical cycles (Margesin et al. 2003; Buzzini et al. 2018). Since the end of the last century, cold habitats worldwide have been under threat due to ongoing climate change. One of the most outstanding consequences of the impact of global warming on environment is glaciers retreat, which opens up newly exposed habitats for terrestrial life, and determines an important transition from glacial to pedogenic ecosystems (Boy et al. 2016). This phenomenon has been increasingly observed over the last few decades, and has determined the exposition of increasing portions of deglaciated barren areas. In this context, the

process of colonization of the areas released by glacier retreat by pioneer microbial and plant communities along a well-defined chronosequence is particularly interesting for understanding the response of early successional stages to climate change (Martiny et al. 2006; Tedersoo et al. 2012; Siles et al. 2016). Since the 2000s, some deglaciated Alpine areas have been used as *in situ* models to investigate the dynamics of pioneer microbial and plant communities in barren substrates released by glaciers retreat (Bernasconi et al. 2011; Zumsteg et al. 2012; Bajerski et al. 2013; Rime et al 2016; Edwards et al. 2014). Despite the above studies, a complete model of the colonization processes and dynamics of microbial and plant communities is far from being achieved, due to site specificity and different length and time resolution of the studies reported in current literature. Particularly, most of the studies published so far considered chronosequences spanning tens of years or even centuries, where the earliest times are represented by one or a few samples (Tian et al. 2017; Bernasconi et al. 2011). In contrast, very few studies investigated the early ecological succession stages that take place in the first years after glaciers retreat (Fernández-Martínez et al. 2017). It is therefore worth studying the early succession pattern in order to assess the ecological processes driving the taxonomic structure of early microbial and plant communities and their temporal changes. To do so, however, sampling early stages at a high temporal resolution is necessary, as early successional stages following glacier retreat are predicted to change rapidly. Forni Glacier (Ortles Cevedale group, Northern Italy) is one of the major storages of ice in the Italian Alps and is currently suffering a quick ablation process due to global warming, like almost all glaciers in the Italian Alps (Smiraglia et al., 2015; Franzetti et al., 2017a). Recent detailed glaciological studies have investigated the mass balance and the origin and distribution of supraglacial debris of Forni Glacier and the surrounding area (Azzoni et al. 2016; Fugazza et al. 2015). Therefore, Forni Glacier presents an ideal location for observing the impact of climate change on Alpine ice masses. Indeed, its location on the Italian (southern) side of the Alps makes it much more prone to environmental, ecological and biological changes than glaciers located on the northern side (Rustad et al. 2001; Rustad 2008; Margesin et al. 2009; Djukic et al. 2010; Frey et al. 2010; Blankinship et al. 2011; Schindlbacher et al. 2011; Smittenberg et al. 2012; Shen et al. 2014; Siles et al. 2016; Rime et al. 2016). Most of the studies investigating the biological communities of the habitats associated with Alpine glacier ecosystems (e.g. barren soil, cryoconites, meltwaters, supraglacial and subglacial debris) documented temporal changes in the ecological communities (i.e. bacteria, yeasts, filamentous fungi, plants and arthropods). Most of them focussed on temporal changes along the ablation season, and found that autotrophic populations typically dominated communities after

snowmelt, while heterotrophic populations increased in abundance later in the season (Buzzini et al. 2005; Gobbi et al. 2006; Turchetti et al. 2008; Franzetti et al. 2017; Franzetti et al. 2017b). Some studies investigated plant colonization of deglaciated areas and showed that communities undergo a gradual increase in structural complexity, biomass, species diversity and ecosystem interaction along the ecological succession (Odum 1969; Milner et al. 2007; Matthews 1993). Moreover, sediment availability and soil characteristics evolve as terrestrial succession progresses, changing from soils with a characteristically high sediment availability and simple structure to soils with complex structure and low sediment availability, stabilised by vegetation growth at a later successional stage (Klaar et al. 2015). Despite these studies, researchers are far from providing a clear and complete picture of the biological diversity colonizing proglacial areas and of the changes in the interactions between different taxa that occur along the ecological succession. Indeed, studies conducted on other Alpine glaciers (Rime et al. 2015; Ohtonen et al. 1999; Zhang et al. 2013; Boy et al. 2016; Tian et al. 2017; Rime et al. 2016) showed that a clear picture of the ecological processes driving the succession of pioneer microbial organisms (both prokaryotic and eukaryotic) and plants colonizing the area around the glacier can be gained only by an integrated analysis considering all taxa simultaneously. The aim of this study is to fill these gaps of knowledge for the area of Forni Glacier, which can be considered a model ecosystem for glaciers on the southern Alpine side, by addressing the following questions. (i) What is the early chronological succession of microbial and plant communities colonizing newly exposed barren areas after glacier retreat? (ii) Which bacterial, fungal and plant taxa are characteristic of areas located at increasing distances from the glacier front (and therefore in areas that have been free of ice for a longer time)? (iii) Which environmental factors may putatively affect microbial and plant taxonomic assemblages of these ecosystems?

5.2. Material and methods

5.2.1. Site description and sampling

Forni Glacier (Fig. 5.1) is one of the largest glaciers in the Italian Alps, with an area of 10.38 km2 based on the 2016 new Alps Glacier Inventory (Paul et al., 2019), an altitudinal range between 2501 and 3673 m a.s.l. and a north–northwesterly aspect. Its extent underwent a strong reduction; in 1869, at the end of the Little Ice Age, its area was 18.99 km2 and its tongue has retired ~2 km in the last century. The glacier shrinkage rate has shown a quick acceleration over the past three decades, typical of valley glaciers in the Alps (Azzoni et al. 2018),that exposed increasing portions of barren areas and, in 2015, caused a fragmentation into two or three separate glacial bodies (Smiraglia et al. 2015; Franzetti et al. 2017a). The proglacial area of the Forni Glacier is not characterized by a

homogeneous slope, but it consists of two steps separated by a steep rockwall. In the lower part of the proglacial area, the glacier has been present from the end of Little Ice Age to the 1980s, while the recent glacier retreat took place in the upper part of the proglacial area (last 20 years), with a gap of ~20 years (1980–2000) when the glacier terminus was characterized by an unstable icefall. This study includes a detailed annual chronosequence of 18 years (from 1998 to 2015, excluding 1999 and 2000). The position of the glacier terminus in each year during the timeframe analysed was reconstructed along three different transects (named west, central and east) based on terminus variation data published after the annual glaciological campaign held by the Italian Glaciological Committee (Baroni, Bondesan, and Mortara 2016). Note that the central transect had to be shifted eastwards to avoid the forefield area highly disturbed by the river originating from the glacier (Fig. 5.1). Soil samples were collected in August 2015 in 31 plots in stable positions along the three transects, excluding areas where fluvial erosion had occurred. Since some sites could not be sampled (e.g. because of exposed bare rocks) the three transects resulted in different lengths and no sites were sampled where the area was released by the glacier in 1999 and 2000. For each plot, we collected three samples from the top 5 cm of the substrate, for both biomolecular and pedological analyses.

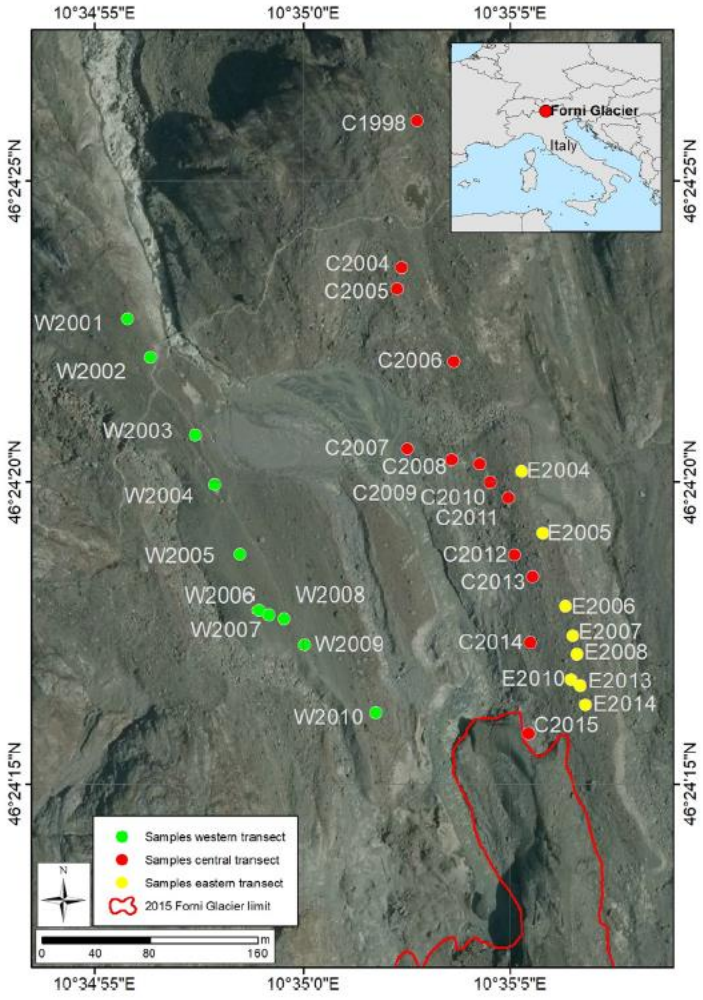

Figure 5.1 Forni Glacier proglacial area with the location of sampling points.

5.2.2. Soil analysis and plant occurrence

Collected samples were subjected to analytical procedures, summarized as follows. Grain size analyses (Gale et al. 2011) were performed after removing organics using hydrogen peroxide (130 vol) treatment; sediments were wet-sieved (diameter from 1000 to 63 μm), then the finer fraction (63 μm) was determined by aerometer on the basis of Stokes's law. Humified organic carbon was identified by means of the Walkley and Black (1934) method, using chromic acid to measure the oxidizable organic carbon (titration). Soil samples were diluted in distilled water and pH was measured with a digital pH-meter. Plants (both herbaceous and arboreous) present in each plot were classified and reported as presence/absence.

5.2.3. DNA extraction and structure of bacterial and fungal communities

Soil samples for molecular analyses were transferred at 4◦C to the laboratory in<12 h and stored at−20◦C until DNA extraction. DNA was extracted from soil samples with a FastDNATM SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA) according to the manufacturer's instructions. The V5–V6 hypervariable regions of bacterial 16S rRNA gene were amplified by PCR as previously reported

(Pittino et al. 2018b). Fungal Internal Trascribed Spacer 1 (ITS1) region was amplified by PCR using the primers ITS1F and ITS2R as suggested in (Mello et al. 2011). The amplicons obtained were sequenced by Illumina MiSeq (Illumina Inc., San Diego, CA, USA) using a 2 × 250 bp paired-end protocol as previously reported (Pittino et al. 2018b). The obtained sequences were demultiplexed according to the indices and the internal barcodes used. The Uparse pipeline was used for subsequent elaborations (Edgar 2013). Forward and reverse reads were merged with perfect overlapping and quality filtered with default parameters. Suspected chimeras and singleton sequences (i.e. sequences appearing only once in the whole data set) were removed. Operational taxonomic units (OTUs) were defined on the whole data set clustering the sequences at 97% similarity and defining a representative sequence for each cluster. OTU representative sequences were classified using the RDP (Ribosomal Database Project) classifier tool (Cole et al. 2009) with the RDP database for bacteria and UNITE database for fungi. The abundance of each OTU was estimated by mapping the sequences of each sample against the OTU representative sequences.

5.2.4. Statistical analyses

OTUs appearing in one sample only were removed from all the analyses because they can inflate the variance explained by redundancy analyses (RDAs) (Borcard et al. 2011). The removed OTUs had abundance <1.8% (bacteria) and <0.2% (fungi) in each sample. Bacterial and fungal OTUs were aggregated into genera if their classification confidence was >0.9.

Alpha-diversity

Chao I index calculated on all sequences for each sample was used to estimate the total number of OTUs assigned to bacterial and fungal taxa. In contrast, the Shannon diversity index (base *e*) and the Gini inequality index (Gini 1912) were calculated after rarefying bacterial and fungal sequences to 2000 and 7500 sequences per sample, i.e. slightly less than the minimum number of sequences per sample. The Gini index is commonly used in ecology as an index of evenness, whose increasing values indicate lower evenness (Wittebolle et al. 2009; Ambrosini et al. 2017). Variation in alpha-diversity in both fungi and bacteria according to year of glacier retreat, debris pH and transect (three-level fixed factor) was estimated by linear regression. These predictors were chosen as we found that they significantly affected beta-diversity (see Results). For the Chao I index, regression was weighted by the inverse of the index variance in each sample. Variation in the number of plant species was investigated by generalized linear models (GLMs) assuming a Poisson distribution and corrected for overdispersion. To check the robustness of the results to small deviation of model assumptions, all the analyses were re-run by assessing significance with a randomization procedure

(999 permutations), which, however, always confirmed the results of previous models. We therefore reported only those results.

Beta-diversity

Beta-diversity was investigated by RDAs run separately on the non-rarefied, Hellinger-transformed bacterial and fungal OUT abundances and on presence/absence of plant species. The Hellinger distance was chosen as it reduces the double-zero problem in ecological community analyses and gives larger relevance to occurrence than to abundance and can be applied to both abundance and presence/absence data (Legendre et al. 2012). RDAs were performed with a forward selection procedure with the double-stopping criterion (Blanchet et al. 2008) to control for Type-I error rate during the variable selection procedure. The predictors entered in the models were year since glacier retreat, pH, amount of organic matter and the proportion of clay, silt and sand in the sediment. The amount of gravel was discarded as it was strongly and negatively correlated with the amount of silt (Pearson-r = −0.88) and its inclusion would have determined problems highly correlation of the independent variables (collinearity) in the analyses. Preliminarily, we checked for variation in the multivariate variance among transects using the betadisper function in the *vegan* package of R. Since we found significant differences among transects in both the analyses of bacterial and fungal communities (see Results), we tested the significance of RDA models by restricting permutations within transects. Variation of the relative abundance of the 20 most abundant bacterial genera according to transect, pH and year was tested by Poisson GLMs corrected for overdispersion. In these models, the total abundance of each genus (i.e. the sum of the abundances of all the OTUs belonging to each genus) was entered as a dependent variable, with transect, pH and year since glacier retreat as predictors. The log of the coverage of each sample was also entered as an offset. With this parameterization, the GLMs model the variation in the relative abundance of each genus according to the predictors (Zuur et al. 2015). The presence of plant species that occurred in at least 3 (10%) of the sampling sites was investigated by binomial GLMs with the same predictors as above. The significance of these models was adjusted with the Benjamini–Hockberg procedure to account for inflation of type-I error rate due to multiple tests (Benjamini et al 1995).

Network analyses

Non-random co-occurrence patterns among bacterial and fungal genera with the checkerboard score (C-score) (Stone et al. 1990) was first tested, under a null model preserving both site and species frequencies (sim9 algorithm in the R package *EcoSim*) (Strona et al. 2014). A checkerboard

unit is a 2 × 2 matrix where both taxa occur once but on different sites. Then network analysis was used to investigate the patterns of covariation between the abundance of bacterial and fungal genera simultaneously. To reduce network complexity, this analysis was restricted to the 20 most abundant bacterial and the 20 most abundant fungal genera. In order to infer a parsimonious direct microbial interaction network from these community composition datasets, the method of sparse inverse covariance matrix estimation within and between multiple compositional datasets (multi-SPIEC-EASI procedure in the *SpiecEasi* package of R) was used. This procedure identifies a network where unconnected taxa (here bacterial or fungal genera) are conditionally independent, which means that, given the abundance of all the other taxa in the network, the abundance of one taxon does not provide any additional information on that of the other. In contrast, the abundances of two connected taxa are not conditionally independent, i.e. there is a relationship between them that cannot be better explained by a relationship with the abundance of any other taxon (Kurtz et al. 2015). This method thus reduces the number of spurious relations among the taxa, and outperforms other commonly used methods for assessing networks like Pearson correlation among abundances and co-occurrence analysis (Kurtz et al. 2015; see also Supporting Information in Musitelli et al. 2018 for a detailed description of a similar approach). These analyses were performed with the Meinshausen–Buhlmann's neighbourhood selection method. Nodes were then classified according to their topological role in the network using the method proposed by Guimera et al. (2005). This method allows the separation of nodes that connect different modules of the network (hub nodes) from those with connection mostly within their module (non-hub nodes). In addition, it classified hub nodes into three and nonhub nodes into four categories according to the number and distribution of their links (see online supplementary material for further details). For instance, a peripheral node has some links to nodes in other modules, while an ultra-peripheral one has all its link within its module. All analyses were performed in R 3.6.2 (R Core Team 2019) with the *vegan* (Oksanen et al. 2016), *EcoSimR* (Strona et al. 2014), *multtest* (Pollard, Dudoit, and van der Laan 2005), *SpiecEasi* (https: //github.com/zdk123/SpiecEasi) and *igraph* packages (Csardi et al. 2006).

5.3. Results

5.3.1. Taxonomic assemblages of bacterial, fungal and plant communities

Overall, 1 987 046 bacterial and 8 280 798 fungal sequences were obtained, which clustered in 3211 bacterial and 1569 fungal OTUs. The number of bacterial OTUs at each sampling site ranged from 196 to 528, and that of fungi from 87 to 296. Further details are reported in Table S5.1 (see online supplementary material). Rarefaction curves reported in Fig. S5.1 (see online supplementary

material) showed that the diversity of some microbial communities was not fully described with the actual sequencing depth. OTU tables are shown in Tables S5.2 and S5.3 (see online supplementary material) for bacteria and fungi, respectively. Fig. S5.2 (see online supplementary material) reports the barplots representing the relative abundance of bacterial and fungal genera grouped by year since deglaciation (A: bacteria; C: fungi) and by transect (B: bacteria; D: fungi). Fig. S5.3 (see online supplementary material) reports the barplots representing the relative abundance of bacterial and fungal classes grouped by year since deglaciation (A: bacteria; C: fungi) and by transect (B: bacteria; D: fungi). The 20 most abundant bacterial genera were *Thiobacillus*, *Mucilaginibacter*, *Saccharibacteria* genera incertae sedis, *Sphingomonas*, *Acidiferrobacter*, *Granulicella*, *Acidipila*, *Methylobacterium*, *Phenylobacterium*, *Geobacter*, *Gaiella*, *Polaromonas*, *Aquabacterium*, *Bradyrhizobium*, *Aeribacillus*, *Enterococcus*, *Gallionella*, *Nakamurella*, *Rhizobacter* and *Ralstonia*. The genus *Thiobacillus* appeared with a relative abundance >1% only in the areas released by the glacier in 2004 or in more recent years. *Mucilaginibacter* was present with a relative abundance >1% in 6 years (2002, 2003, 2005, 2006, 2008 and 2010), *Saccharibacteria* genera incertae sedis in 2004, 2006 and 2007, *Sphingomonas* in 2003 and 2010, *Acidiferrobacter* in 2006, 2008 and 2010, *Granulicella* in six years (2002, 2003, 2006, 2007, 2008 and 2010), *Acidiphila* in five years (2004, 2006, 2007, 2008 and 2010), *Methylobacterium* in 2008 and 2015, *Phenylobacterium* in four years (2001, 2004, 2005 and 2010), *Geobacter* in 2005, 2007 and 2013, *Gaiella* in eight years (2004–08 and 2012– 14), *Polaromonas* in 9 years (2004, 2006–08, 2010, 2011, 2013–15), *Aquabacterium* in 2008, 2009 and 2013, *Bradyrhizobium* in four years (2004, 2005, 2007 and 2013), *Aeribacillus* in 2008 and 2009, *Enterococcus* in 2009 and 2010, *Gallionella* in six years (2006–08, 2010, 2013 and 2014), *Nakamurella* in four years (1998, 2001, 2002 and 2006), *Rhizobacter* in 2004 only and *Ralstonia* in 2008 and 2009. *Saccharibacteria* genera incertae sedis, *Geobacter, Gaiella, Polaromonas, Aquabacterium* and *Nakamurella*were absent in transect W, *Granulicella, Acidipila, Sphingomonas* and *Aeribacillus* were absent in transect E, *Acidiferrobacter* and *Phenylobacterium* were present in transectWonly, *Methylobacterium* was present in transect C only, *Rhizobacter* was present in transect E only, while all the other genera were present in all the three transects. Chloroplasts were also abundant, accounting for 1.6% of the sequences; however, they were discarded from subsequent analyses. The 20 most abundant fungal genera (including filamentous and yeast forms) were *Mortierella*, *Penicillium*, *Cryptococcus* g1, *Heterobasidion*, *Suillus*, *Cladophialophora*, *Acarospora*, *Thelephora*, *Tetracladium*, *Microdochium*, *Paecilomyces*, *Rhizoscyphus*, *Psilocybe*, *Calycina*, *Venturia*, *Alternaria, Lecanicillim, Preussia, Leptosphearia* and

Kurtzmanomyces. The genus *Mortierella* appeared with relative abundance >1% in all the sampled soil, with high percentage (>10%) in 18 samples of the total 30 studied. All the other genera appeared with a relative abundance >1% only in some of the total samples. The genus *Thelephora* showed relative abundance >1% in 5 years (2003, 2006, 2007, 2009 and 2010), *Rhizoscyphus* in years from 2001 to 2006 and in 2007, 2010 and 2012, *Venturia* in 7 years (1998, 2001–03, 2005, 2006, 2009), *Cladophialophora* in 1998, 2001, 2005, 2007, 2009, 2010 and 2015, *Lecanicillium* in years from 2006 to 2009 and in 2012, 2013 and 2015, *Calycina* in 2007 and 2009, *Tetracladium* in 3 years (2004, 2006, 2010), *Microdochium* in 2001 and 2005, *Suillis* in 2013 and 2015. Some genera exhibited relative abundance >1% only in soil sampled in one year: *Preussia* in 2005, *Leptosphaeria* in 2004, *Penicillium* in 2003, *Cryptococcus* g1 in 2001, *Alternaria* in 2015, *Kurtzmanomyces* in 2009 and *Psilocybe* in 2003. The other genera showed relative abundance <1% in all the samples. Considering the transects, *Paecilomyces*, *Acarospora*, *Heterobasidion* were present with relative abundance <1% in all the samples; *Preussia*, *Leptosphaeria*, *Alternaria* showed relative abundance >1% only in transect C, *Microdochium*, *Penicillium*, *Kurtzmanomyces* and *Psilocybe* only in transect W; while *Mortierella*, *Thelephora*, *Rhizoscyphus*, *Cladophialophora* and *Lecanicillium* in all the transects. Twelve plant species (*Agrostis rupestris, Agrostis schraderana, Cerastium pedunculatum, Cerastium uniflorum, Deschampsia caespitosa, Gnaphalium supinum, Leucanthemopsis alpina, Luzula alpino-pilosa, Poa alpina, Sagina saginoides, Salix appendiculata, Salix helvetica*) occurred in at least three different sampling sites. *Agrostis rupestris, A. schraderana, C. pedunculatum, L. alpino-pilosa*, and *P. alpine* were observed in all the transects and overall in >11 samples. On the other hand, *Avenella flexuosa, Betula pendula, Cardamine resedifolia, Cerastium uniflorum, Deschampsia caespitosa, Doronicum clusii, Euphrasia minima, Geum reptans, Gnaphalium supinum, Juncus trifidus, Larix decidua, Oxyria digyna, Phleum alpinum, Sagina saginoides, Salix appendiculata, Salix helvetica, Salix herbacea, Saxifraga bryoides, Tussilago farfara* and *Veronica alpine* were sporadically observed, occurring in only 1–4 different sampling sites.

5.3.2. Analysis of alpha-diversity

Significant increases of both alpha-diversity and evenness of bacterial communities with greater time since deglaciation were observed (Chao I index: coef. = −82.97 ± 17.85 SE, $F_{1,25}$ = 21.615, P < 0.001; Fig. 5.2a; Shannon index: coef.=−0.122 ± 0.028 SE, $F_{1,25}$ = 18.542, P < 0.001; Gini index: coef. = 0.004 ± 0.001 SE, $F_{1,25}$ = 16.038, P < 0.001, note that increasing values of Gini index indicate decreasing evenness). In contrast, the alpha-diversity index did not change according to pH ($F_{1,25}$ ≤ 1.928, P = 0.177) or transect ($F_{2,25}$ ≤ 2.131 P = 0.140). On one hand, the Chao I index of fungal

diversity differed among transects ($F_{2,25}$ = 5.313, P = 0.012, Fig. 5.2b): it was lower in the western transect than in the central and eastern ones ($t_{25} \leq -2.598$, $P \leq 0.037$). On the other hand, this index did not vary according to year ($F_{1,25}$ = 1.694, P = 0.205) or pH ($F_{1,25}$ = 2.931, P = 0.099). Shannon and Gini indices of fungal diversity did not vary according to any predictor ($F_{1,25} \leq 1.520$, $P \geq 0.229$). The number of plant species followed the same pattern observed for bacterial diversity: it increased with greater time since deglaciation (coef. = −0.09± 0.03 SE, $F_{1,25}$ = 12.408, P = 0.002, Fig. 5.2c), while it did not vary according to pH ($F_{1,25}$ = 0.671, P = 0.421) or among transects ($F_{2,25}$ = 0.144, P = 0.866).

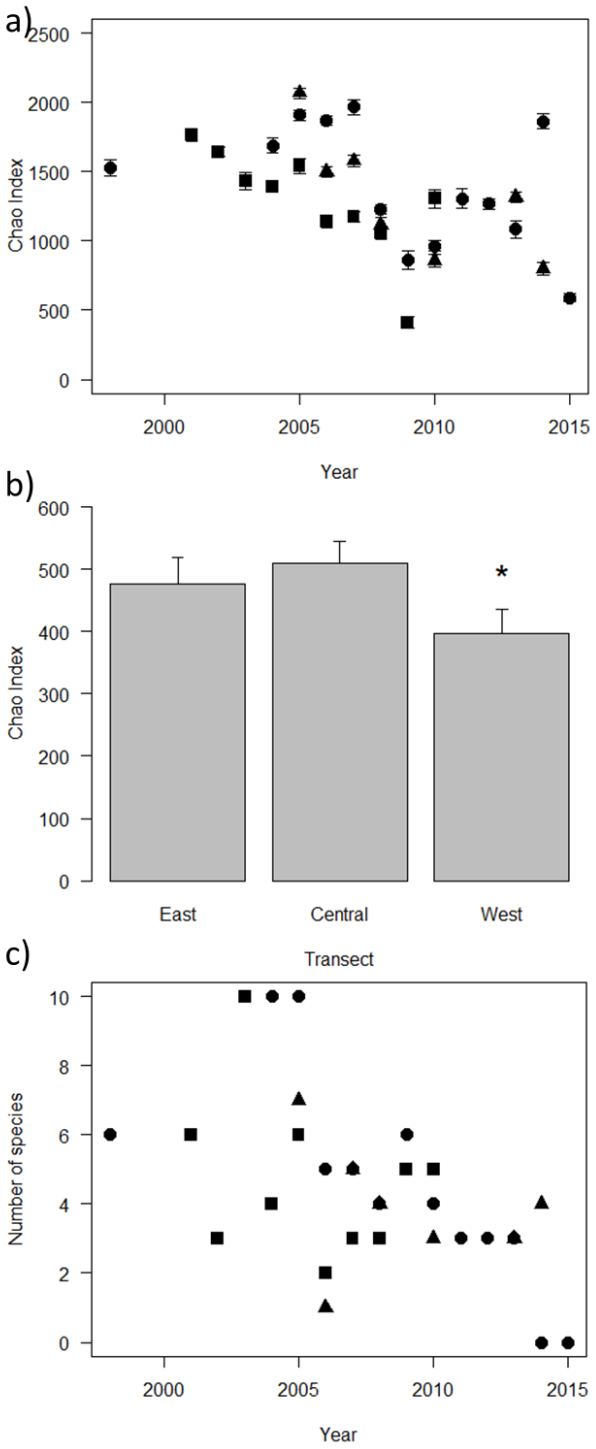

Figure 5.2 Variation of bacterial and fungal diversity (Chao I index) and of the number of plant species. (a) bacteria; (b) fungi; (c) plants. Triangles, east transect; circles, central transect; squares, west transect.

5.3.3. Analysis of beta-diversity

RDAs on Hellinger-transformed abundance of bacterial and fungal OTUs are reported in Fig. 5.3. Multivariate variance of both bacterial and fungal communities significantly differed among transects (betadisper analysis: $F_{2,27} \geq 4.506$, $P \leq 0.020$). For bacteria, the east transect showed lower dispersion than the west and the central ones (Padj ≤ 0.032 in Tukey *post hoc* tests; Fig. 5.3a), while for fungi the west transect showed lower dispersion than the central one (Padj = 0.004 in Tukey *post*

hoc tests; Fig. 5.3b). RDA of Hellinger-transformed abundance of bacterial OTUs showed that the structure of bacterial communities changed significantly with both year since glacier retreat and pH (Tab. 5.1, Fig. 5.3a). In contrast, the structure of fungal communities appeared to be affected only by the year since deglaciation, despite the forward selection procedure also retaining pH as a predictor (Tab. 5.1, Fig. 5.3b). The structure of plant communities did not vary according to any variable (details not shown). The relative abundance of OTUs belonging to the bacterial genera *Thiobacillus*, *Gaiella*, *Polaromonas*, *Aquabacterium* and *Rhizobacter* significantly increased with pH, while those of *Saccharibacteria* genera incertae sedis, *Acidiferrobacter*, *Acidipila* and *Phenylobacterium* decreased ($|t_{27}| \geq 3.260$, $P_{FDR} \leq 0.005$). In particular, the abundance of OTUs belonging to *Thiobacillus* increased in sites free from ice for a longer time, while that of OTUs belonging to *Gallionella* was higher in more recently released sites ($|t_{27}| \geq 3.738$, $P_{FDR} \leq 0.001$). The relative abundance of OTUs belonging to fungal genera did not show significant variation with year since deglaciation ($|t_{28}| \leq 3.116$, $P_{FDR} \geq 0.285$).

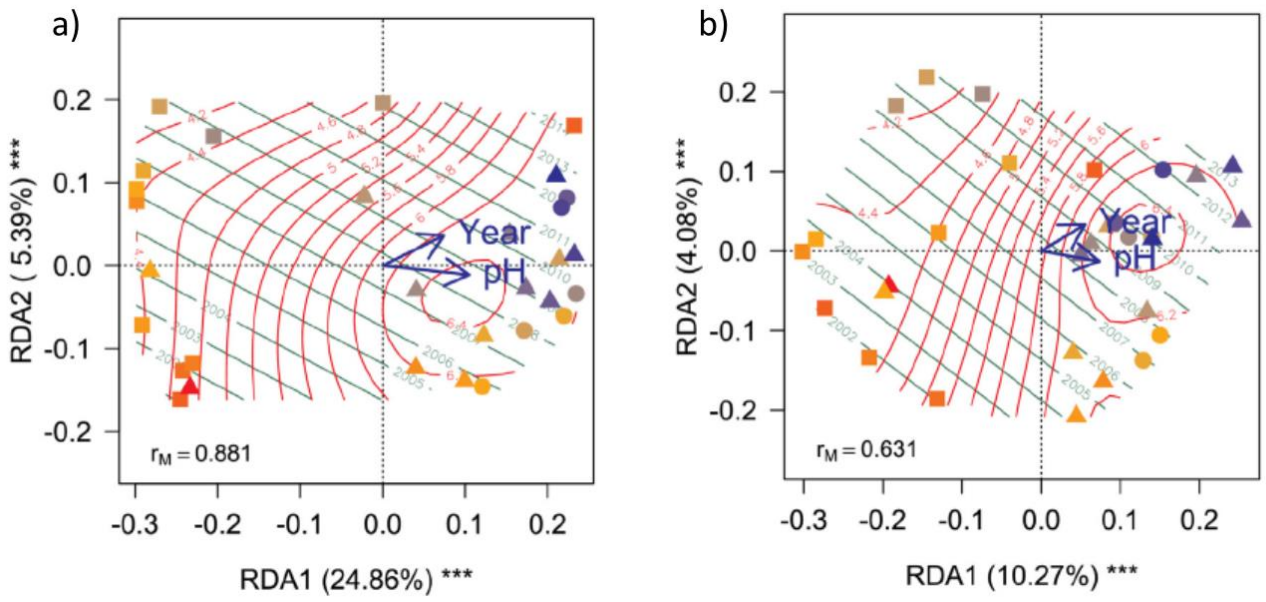

*Figure 5.3 RDAs of Hellinger-transformed abundance of bacterial and fungal OTUs. (a) RDA of bacterial OTUs; (b) RDA of fungal OTUs. Symbols denote sampling sites. Colours from blue to red indicate the pattern of the time since deglaciation: blue recently deglaciated sites, red deglaciated formerly; dots = east transect, triangles = central transect, squares = west transect. Arrows, constraining variables. Contours represent the gradient of significant predictors (red contours, pH; green contours, year). Variance explained by the first two axes are reported as well as axis significance (***P < 0.001). rM is the Mantel correlation coefficient among Hellinger distances among samples and Euclidean distances among points representing each sample in the plot. Value close to one indicate that the plot accurately represents distances among samples.*

Table 5.1 Final RDA on Hellinger-transformed abundance of bacterial and fungal OTUs.

Variable	df	Variance	F	P
Bacterial OTUs				
pH	1	0.097	7.579	0.046

Year	1	0.039	3.049	0.001
Residuals	27	0.344		

$F_{2,27}$ = 7.347, P = 0.001, Adjusted-R^2 = 0.304

Fungal OTUs

pH	1	0.063	3.357	0.116
Year	1	0.041	2.169	0.005
Residuals	26	0.509		

$F_{2,27}$ = 3.431, P = 0.001, Adjusted-R^2 = 0.144

5.3.4. Network analysis

The relationships between the taxa forming bacterial, fungal and plant communities were investigated using network analysis. To reduce network complexity, the analysis was focussed on the 20 most abundant genera for both bacteria and fungi. Nonrandom co-occurrence patterns were found (C-score = 2.571, $P < 0.001$), which were further inspected using network inference. Multi-SPIEC-EASI (Kurtz et al. 2015) identified a parsimonious network including 20 edges and 40 nodes representing either bacterial or fungal genera (Fig. 5.4). The average network distance between all pairs of nodes (average path length) was 2.848 edges with a diameter (longest distance between nodes) of 5 edges. The modularity index was 0.661 (values >0.4 indicate modularity, Newman 2006), suggesting the existence of clusters of tightly connected taxa. Importantly, the network exhibited a positive and strong assortativity coefficient (+0.875) due to a prevalence of bacteria vs bacteria and fungi vs fungi connections (homophily) (Fig. 5.4). This value was >1000 assortativity coefficients obtained by randomization. The classification of nodes according to their topological role (Guimera and Amaral 2005) indicated that no node in the network could be considered a hub. Among the non-hub nodes, none were connectors or kinless nodes, five were peripheral and 15 were ultra-peripheral. The peripheral nodes were the bacterial genera *Granulicella*, *Thiobacillus*, *Acidipila*, *Polaromonas* and *Phenylobacterium*. On the other hand, fungal genera (including both yeast and filamentous forms) reported in the network analysis were classified as either ultra-peripheral or isolated nodes (Fig. 5.4).

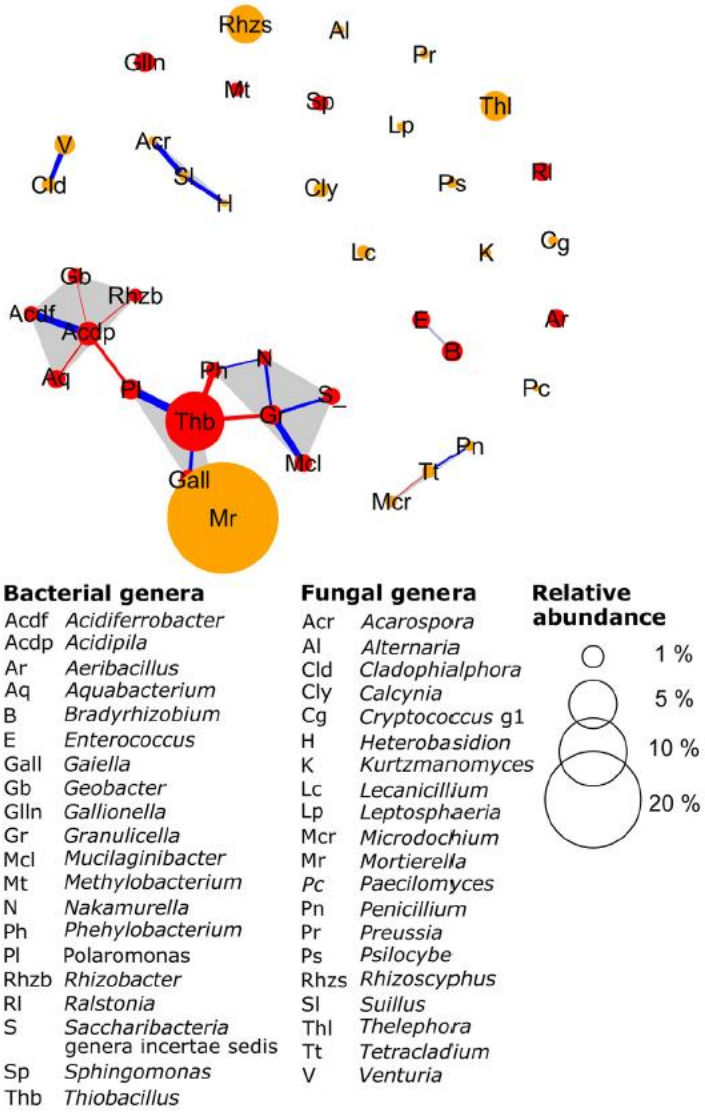

Figure 5.4. Network representation of conditionally non-independent abundant bacterial and fungal genera. Red circles, bacterial genera; orange circles, fungal genera. Circle size is proportional to the normalized genus coverage. Blue lines, positive correlation among nodes; red lines, negative correlations. Line width is proportional to the strength of the correlation among nodes. Grey polygons, modules including at least three nodes.

5.4. Discussion

The primary ecological succession occurring in the forefield of a retreating Alpine glacier has been studied by a few authors (Brown et al. 2014; Jiang et al. 2018). The present study investigated a yearly chronosequence spanning a period of 18 years (from 1998 to 2015), which allowed a detailed description of the earliest phases of colonization and succession of newly released proglacial areas by bacterial, fungal and plant populations. Analysis of the most abundant bacterial genera suggested that the studied area was probably firstly colonized by microorganisms arriving from multiple environmental sources. Interestingly, the high relative abundance of the genus *Gallionella* in sites recently released by the glacier and closer to the terminus might indicate a freshwater origin. Indeed, the genera *Aquabacterium* and *Gallionella* are generally found in fresh waters (Chen et al.

2012; Hallbeck et al 2015; Pham et al. 2015). This, however, did not occur for *Aquabacterium,* whose abundance was associated with pH. The common presence of the genera *Mucilaginibacter, Acidipila* and *Gaiella* in soil (Jiang et al. 2012; Jiang et al. 2016; Naumova et al. 2015; Liu et al. 2017), and that of *Bradyrhizobium*, *Rhizobacter* and *Nakamurella* in the rhizosphere (Miller et al. 1996; Wei et al. 2015; Meena et al. 2018; Liu et al. 2019), and the general abundance of broad varieties of archaea, bacteria and fungi known as inhabitants of the rhizosphere (Odelade et al. 2019) suggests that plants could be a major source of colonizing bacteria. This hypothesis was also supported by the presence of a large abundance of chloroplast sequences in studied samples (details not shown). Moreover, the presence of the mainly faecal genus *Enterococcus* suggests a contribution from animal associated microorganisms (Aarestrup et al. 2000). Regardless of the potential source, however, the ecology of the most abundant bacterial populations suggests that the communities appeared to be mostly shaped by local environmental conditions, typical of the glacier forefields. Indeed, some of the most abundant genera are common inhabitants of cold habitats, such as *Polaromonas, Granulicella, Sphingomonas* and *Methylobacterium* (Miteva 2008). Some of them, particularly *Sphingomonas* and *Methylobacterium,* are also known to be adapted to harsh environmental conditions, such as high UV radiation or desiccation (Hugenholtz et al. 1995; Fredrickson et al. 2008; Marizcurrena et al. 2017; Madueño et al. 2018). The high abundance of the thermophilic genus *Aeribacillus* could be justified by supposing its transportation in the troposphere by aerial circulation and its subsequent release in the cool areas, as previously reported for other thermophiles (Rahman et al 2004). Interestingly, some bacterial genera among the most abundant in our samples (such as *Thiobacillus, Gallionella, Acidiferrobacter)* are known to be actively involved in rock weathering processes, mainly because of their acid-producing metabolism. For example, in aerobic environments rich in sulfide-containing minerals, the genus *Thiobacillus* can oxidize sulfide or elemental sulfur to sulfate, which, in the presence of water, is converted into sulfuric acid (Pronk et al. 1990), while *Gallionella* and *Acidiferrobacter* are common iron-oxidizers (Hedrich et al. 2011). This may lead to a local acidification of the substrate that can support the selective growth of other acidophilic bacteria, such as *Granulicella* and *Acidipila* (Huber et al. 2017) that are abundant members of the studied bacterial communities. Acid production contributes both to rock weathering and metal solubilization (Paces 1986; Mitani et al. 1991), thus increasing metal concentrations in debris. This may lead to environmental conditions that, in turn, can select for metal-tolerant bacteria, such as *Mucilaginibacter, Phenylobacterium* and members of phylum *Saccharibacteria,* which are commonly retrieved in soils containing high concentrations of metals

(Cáliz et al. 2013; Ni et al. 2016; Tang et al. 2016). These observations are consistent with the fact that the abundance of the genera *Acidiferrobacter, Acidipila* and *Phenylobacterium* increased at low pH values. Moreover, other bacterial genera, such as the iron-reducer *Geobacter,* which was associated with *Acidiferrobacter* according to the network analysis herein reported, can also take advantage of high concentrations of Fe^{3+} because this metal is used as an electron acceptor in their anaerobic respiration (Champine et al. 2000). The genus *Polaromonas* has been found in supraglacial debris in the Italian Alps (Franzetti et al. 2013) and was found among the weathering-associated bacteria observed in a Swiss glacier forefield (Frey et al. 2010). Overall, the present results seem to indicate that the bioavailability of metals retrieved from the former glacier bedrocks could favour the growth of both chemolithotrophic and chemoorganotrophic bacterial populations. Rock-weathering bacteria influence pH (and are influenced by it) and promote the release of metals that could also be made available for other microorganisms. This is consistent with the RDA analysis, which indicates that the bacterial community is significantly affected by pH, and with the role of peripheral nodes assigned to the genera *Granulicella*, *Thiobacillus*, *Acidipila*, *Polaromonas* and *Phenylobacterium* by the network analysis, where these genera exhibited a high number of connections (Fig. 5.4). Overall, fungal communities changed according to year since deglaciation, but none of the most abundant fungal genera changed significantly according to this variable. This suggests that community structure variation identified by RDA may be related to variation in the presence and abundance of rare taxa, despite this analysis usually giving more relevance to abundant taxa (Legendre et al. 2012). Analysis of the most abundant fungal taxa documented the presence of both filamentous and yeast forms, such as *Mortierella, Cladophialophora, Tetracladium, Penicillium* and *Cryptococcus*, which could act as cold-adapted organic matter decomposers due to their heterotrophic status, but no fungal node in the network could be considered a hub and all fungal genera were classified as either ultra-peripheral or isolated nodes. Among them, the dominance of ascomycetous filamentous fungi and basidiomycetous yeasts was found, consistent with current knowledge on fungal dominance in worldwide cold ecosystems (Onofri et al. 2007; Brunner et al. 2011; Buzzini et al. 2012; Buzzini et al. 2017; Sannino et al. 2017; Durán et al. 2019). The remarkable proportion of fungal OTUs that cannot be classified (unclassified fungi) is common to other fungal metabarcoding studies using ITS1 (Garrido-Benavent et al. 2020). The lack of sequence data in the most common databases for some fungi already described, and the presence in the examined samples of DNA belonging to non-cultivable taxa, not yet isolated and described, could justify the abundance of sequences not attributable to any known taxa. Special attention

should be paid to the yeast genus *Cryptococcus.* Recent reviews have described the unequivocal prevalence of species belonging to this phenotypic basidiomycetous genus in worldwide cold habitats (Buzzini et al. 2012; Connell et al. 2014; Zalar and Gunde-Cimerman 2014). However, the recent taxonomic revision of the Tremellomycetes taxon (Liu et al. 2015) positioned species previously included in this genus in many new basidiomycetous taxa. Therefore, such modification suggests reconsidering the ecological significance of this genus in cold ecosystems worldwide, because a closer look at the OTUs classified in this taxon, indeed revealed that some OTUs formerly classified in the genus *Cryptococcus*, well known for its psychrophilic and psychrotolerant aptitude, are now classified inside the genera *Goffeauzyma*, *Solicoccozyma* and *Vishniacozyma* (Buzzini et al. 2017; Sannino et al. 2017). Most of the filamentous fungal genera found in this study, namely *Paecilomyces* and *Alternaria*, have been commonly found in Antarctic air samples, and species of the genus *Penicillium* were isolated in many cold environments and were described as producers of anti-freeze proteins, an efficient strategy to colonize cold ecosystems (Cuthbertson et al. 2017; Kawahara 2017)b. Likewise, the cosmopolitan genus *Mortierella* includes a few species (e.g. *Mortierella alpina* and *Mortierella antarctica*) that are currently considered part of Antarctic fungal communities (Onofri et al. 2007). Interestingly, some of the filamentous fungal genera found in this study are well-known either for their positive association (e.g. root-symbiosis) with plants, such as *Rhizoscyphus*, or for their ability to act as phytopathogens, entomophatogens or decomposers of plant materials like *Microdochium*, *Lecanicillium*, and *Mortierella*, respectively (Badali et al. 2008; González-Domínguez et al. 2017; Fehrer et al. 2019). Pioneer plants may therefore promote colonization of barren debris released after glacier retreat by both bacterial and fungal communities. On the other, both bacteria and fungi can promote plant growth by syntrophic and symbiotic associations, namely root-associated bacteria diazotrophs (e.g. *Rhizobium* spp. And *Frankia* spp.) (Nash et al. 2018) and fungal ectomycorrhizae (Landeweert et al. 2001). Brown and Jumpponen (2014) also reported that bacterial and plant communities followed the same colonization pattern leading to an increasing species richness during the primary succession that occurred >90 years after the retreat of the Lyman Glacier (Washington State, USA). This suggested two alternative hypotheses on how either bacteria or plants (or even both) can promote the development of the other: (i) plant development could increase niche heterogeneity that, in turn, could promote bacterial diversity; and (ii) bacteria could promote plant establishment by accumulating organic carbon and accelerating pedogenesis. Despite that the present study does not directly assess relationships among assembling plant and microbial communities, alpha-diversity of

both plant and bacterial communities increased along the chronosequence. These results are, therefore, consistent with both hypotheses, which should not be considered strictly as alternatives, because both processes may occur concomitantly in natural ecosystems. Alpha-diversity and evenness of bacterial and plant communities increased significantly at increasing distance from the glacier, in agreement with the results of Kazemi et al. (2016), but in contrast with those of Y. Jiang et al. (2018), who found that bacterial diversity did not significantly increase along a two century chronosequence in the forefield of Hailuogou Glacier (China), while plant diversity was highly responsive to time (2- fold increase in two centuries). In the present study, fungal richness did not exhibit a clear relationship with the year of deglaciation. Previous studies on fungal communities colonizing deglaciated barren areas over longer chronosequences reported contrasting results: Brown and jumpponen (Brown et al. 2014; Brown et al. 2015) and Bradley et al. (2014) found no relationship with time, whereas other studies reported that fungal diversity increased over the successional time (Jumpponen et al. 1999; Blaalid et al. 2012; Cutler et al.2014) or even exhibited fluctuations around a central sampling point (Tian et al. 2017). The inconsistencies found among different literature data, and between them and the results found in the present study, suggest that the ecosystem of barren debris released after glacier retreat is extremely complex and can largely differ among glaciers worldwide. This could be due to different geographical, geological, physical, chemical and biological features, which might somehow drive the taxonomic assemblage of microbial prokaryotic and eukaryotic communities of deglaciated forefields (including their ecological successions), although the available information is currently too little and scattered to identify any general pattern. Microbial richness may be controlled by both bottom-up processes like nutrient and carbon quantity and quality and top-down grazing processes. A holistic model including all these complex interactions putatively predicting the temporal patterns of microbial prokaryotic and eukaryotic communities and plants in barren soils is currently far from being developed. Among the possible causes that prevent the development of this holistic model, the most relevant are probably the different lengths and the different time resolution used in current literature for studying the investigated chronosequences, which hamper a proper comparison of the results in different deglaciated areas. Previous studies have made several attempts to determine the relative contributions of deterministic and stochastic processes to the assembly of microbial communities in primary and secondary successions of deglaciated areas. In many studies, fungal community structure was mainly shaped by deterministic processes (Tian et al. 2017), whereas Brown et al. (2014) reported a dichotomous condition, where bacterial community was structured by

deterministic processes, while the fungal community was structured by more stochastic processes in primary succession. By considering the whole 18 year chronosequence studied in the Forni Glacier transects, bacterial and fungal (both filamentous and yeast forms) communities showed an ecological succession, as the taxonomic structures of both communities were influenced by the year of deglaciation. On the other hand, plant community structure apparently did not change, despite their diversity increasing with time since glacier retreat. However, the vegetation analysis carried out in this study was limited to the description of the presence/ absence of plants, and more detailed investigations are recommended for evaluating the evolution of plant community structure. Moreover, pH proved to be a major factor driving the taxonomic assemblage of bacterial community, but not that of fungi. As previously mentioned, this might be related to the pH-controlled bioavailability of metals coming from the former glacier bedrocks, which, in turn, may affect bacterial community structure. The oligotrophic conditions of bare rocks might have promoted the growth of chemolitotrophic and rock-weathering bacterial populations. Therefore, deterministic processes might be of great importance even in the early succession stages, when environmental filtering and species sorting effect might select the species arriving on a site. A similar process was supposed by Andrea Franzetti et al. (2013, 2017a) for bacterial communities colonizing cryoconites and supraglacial debris and by some studies addressing fungal communities at the initial stage of succession (Rime et al. 2016; Tian et al. 2017). On the other hand, this process is apparently in contrast with the findings of Brown et al. (2014), who reported that bacterial primary succession was determined by stochastic colonization with increasing determinism as a result of ecosystem development and/or pedogenesis. In conclusion, the high temporal resolution of the data provided a detailed (yearly) picture of early succession processes in glacier forefields and demonstrated that in the early times after glacier retreat the biological communities showed a chronosequence with increasing richness of bacterial, fungal and plant taxa and significant changes of the taxonomic structure of bacterial and fungal communities over time. Our results allow to be put forward the hypothesis that the environmental filtering driving the bacterial community in the early phase of the chronosequence is possibly due to the bioavailability of metals. This bottom-up control could be coupled with a biological control by chemolithotrophic and rock-weathering bacteria, which could promote the bioavailability ofmicronutrients and electron acceptors for other microbial populations and act as key species of the ecosystems.

5.5. Data accessibility

Sequence data were submitted to European Nucleotide Archive (EBI-ENA), study accession number PRJEB38009 (http://www.ebi. ac.uk/ena/data/view/PRJEB38009).

5.6. Acknowledgements

The authors thank Miss Teresa Emma Cutrona for English revision.

Conflict of Interest. None declared.

5.7. Supplementary material

Table S1 is available at

https://academic.oup.com/femsec/article/96/10/fiaa165/5894918#supplementary-data.

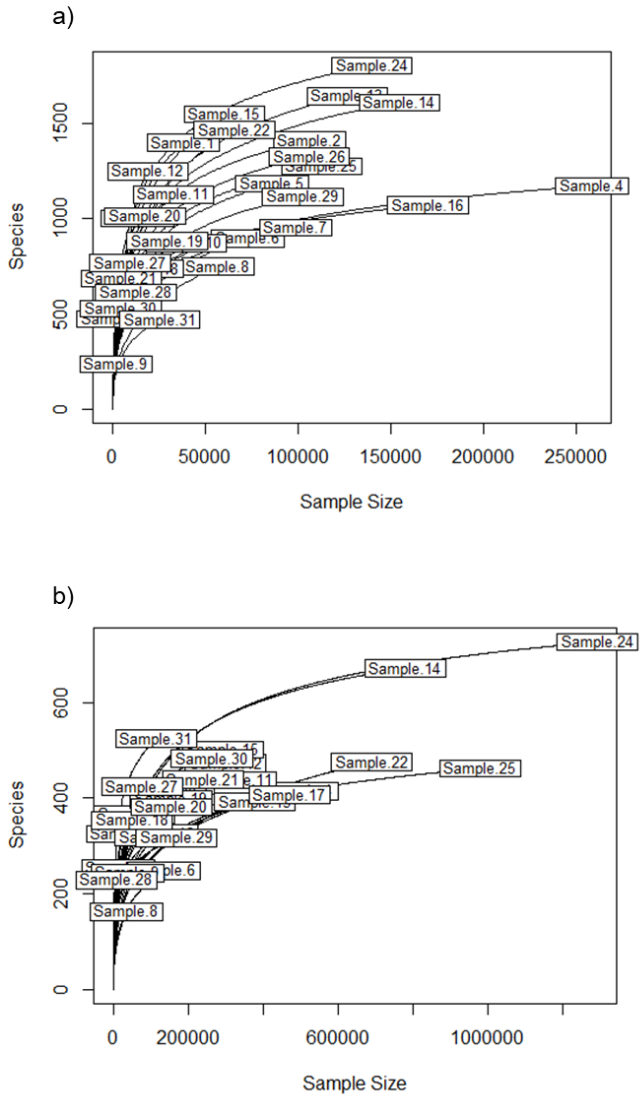

Figure S8.1 - Rarefaction curves for bacterial (a) and fungal (b) samples.

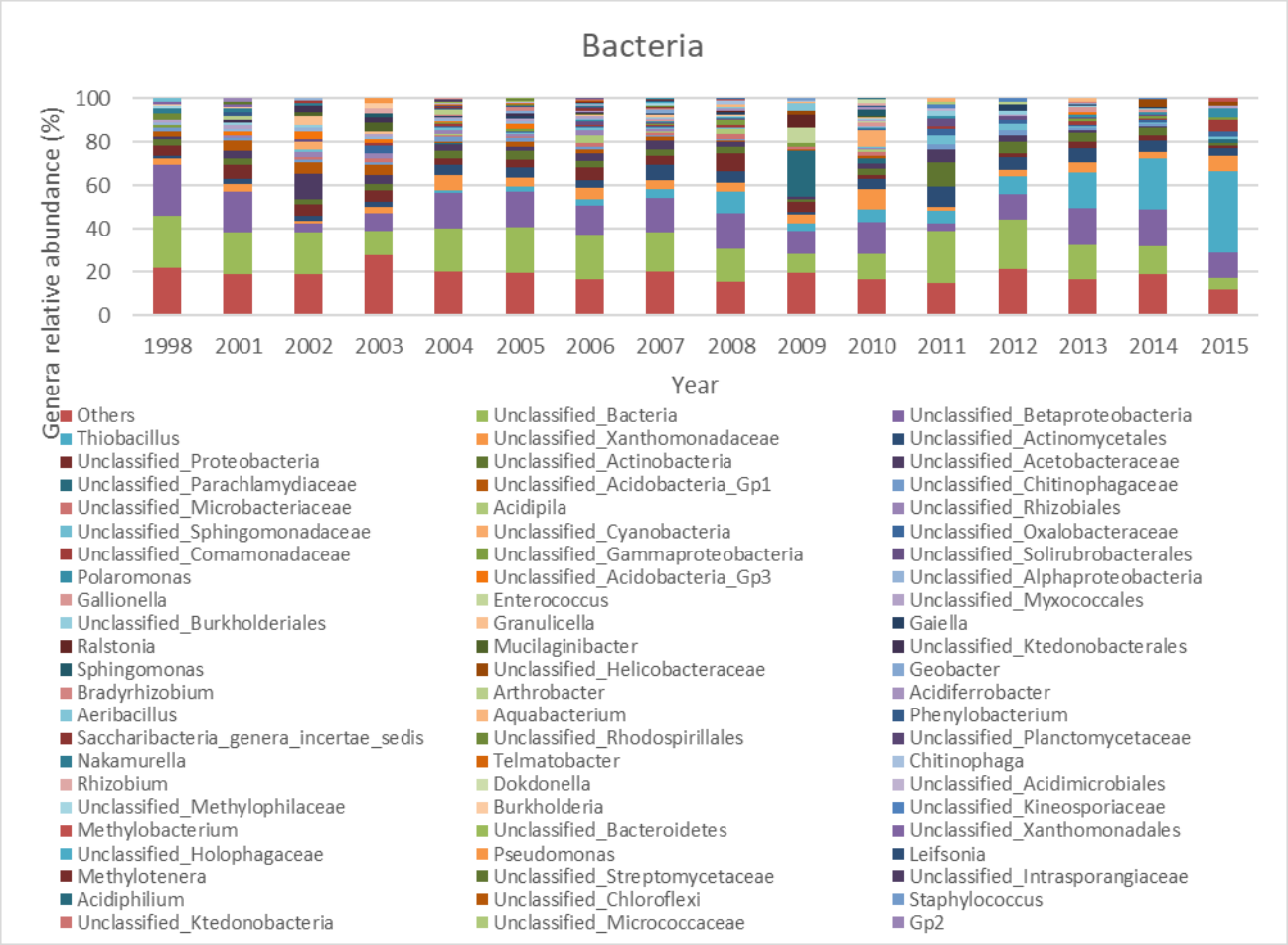

Figure S8.2a Barplot representing the relative abundance of bacterial genera grouped by year. Genera less abundant than 1 % in each sample are grouped in other.

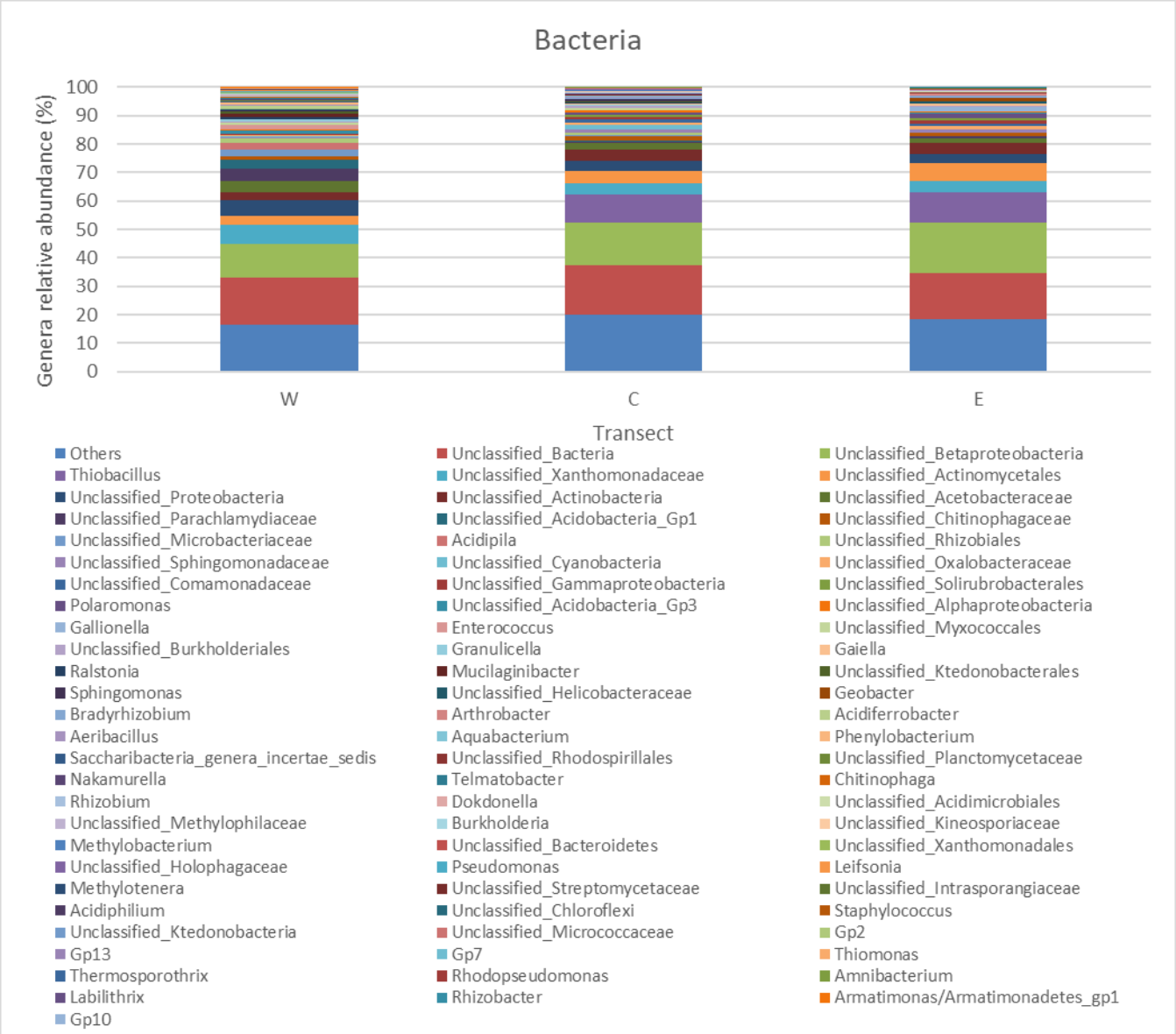

Figure S8.2b Barplot representing the relative abundance of bacterial genera grouped by transect. Genera less abundant than 1 % in each sample are grouped in other.

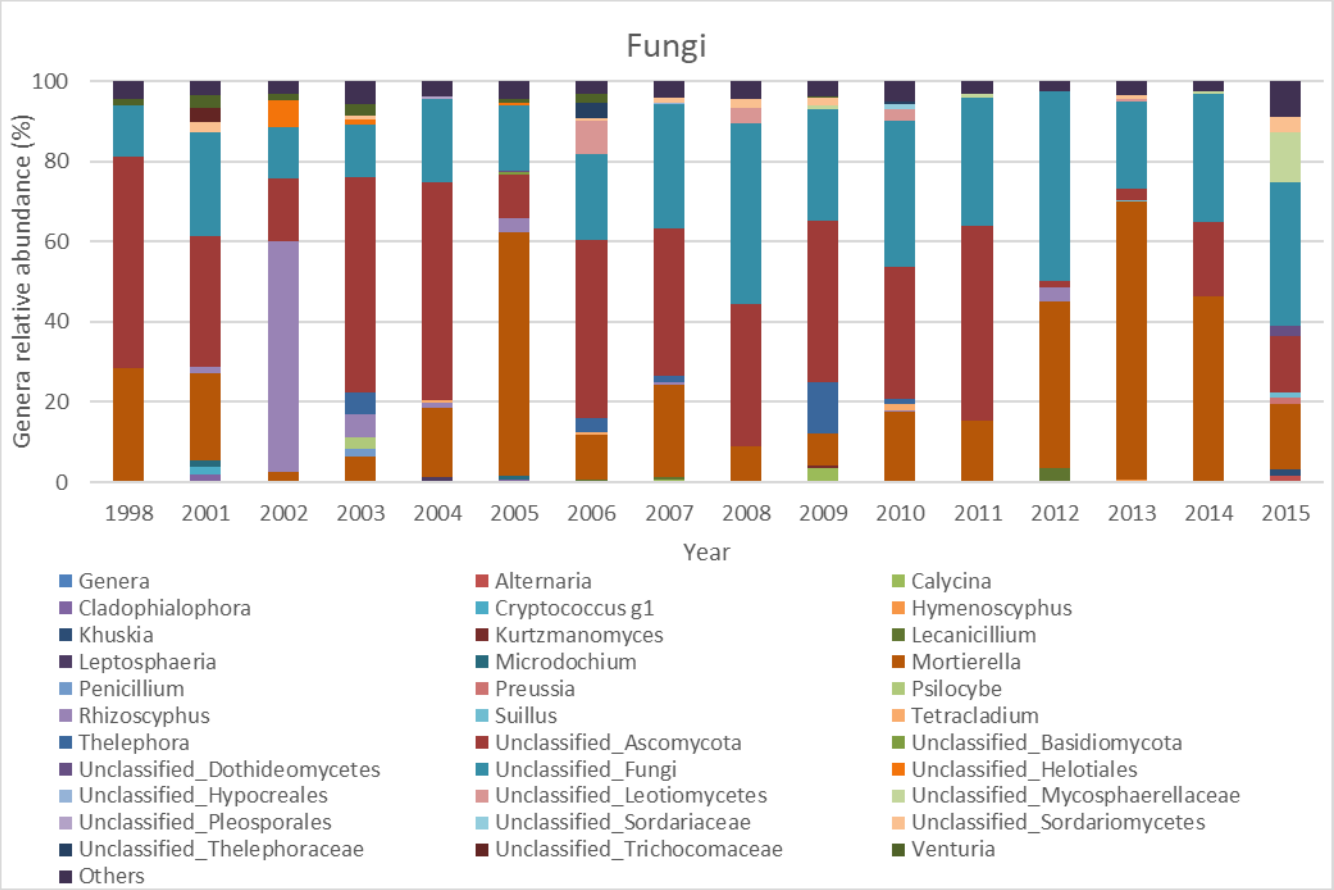

Figure S8.2c Barplot representing the relative abundance of fungal genera grouped by year. Genera less abundant than 1 % in each sample are grouped in other.

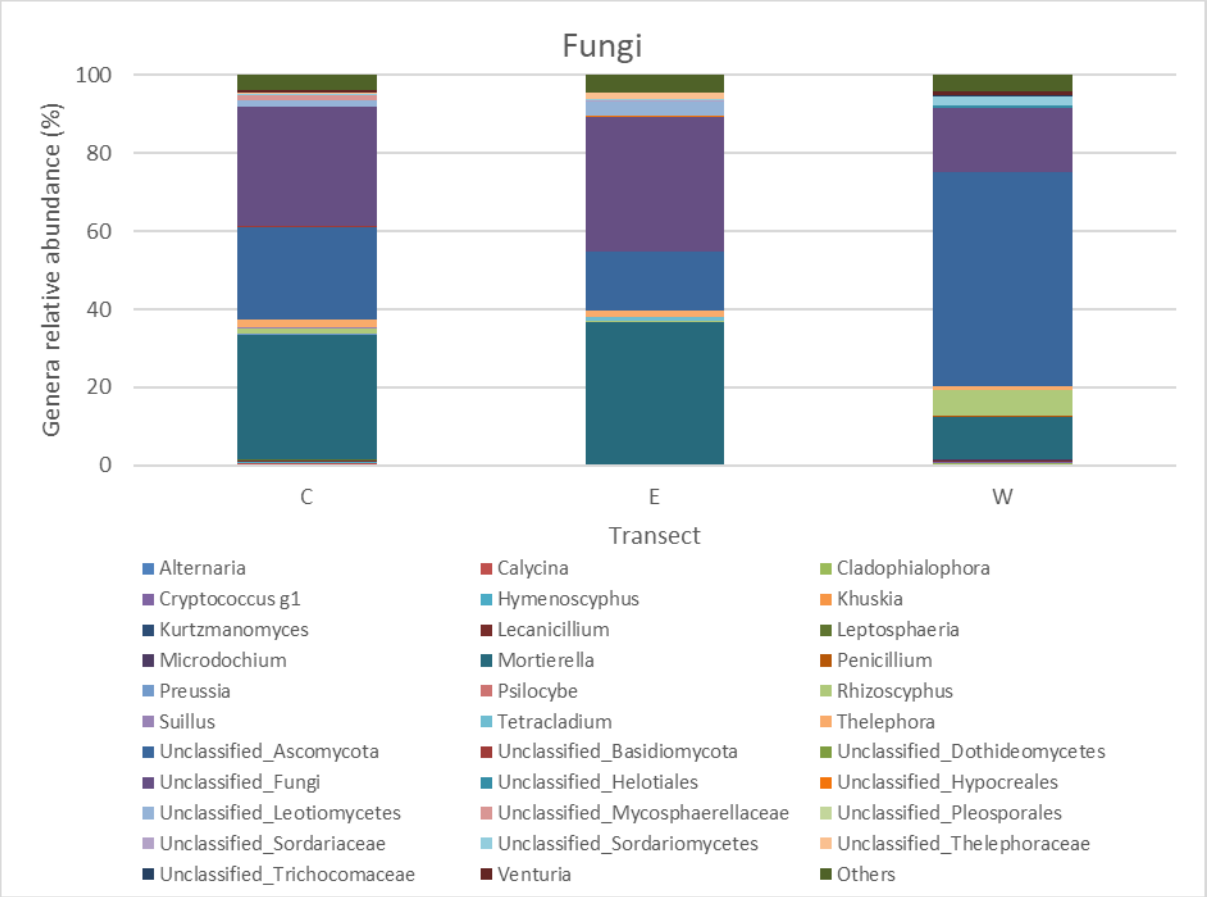

Figure S8.2d Barplot representing the relative abundance of fungal genera grouped by transect. Genera less abundant than 1 % in each sample are grouped in other.

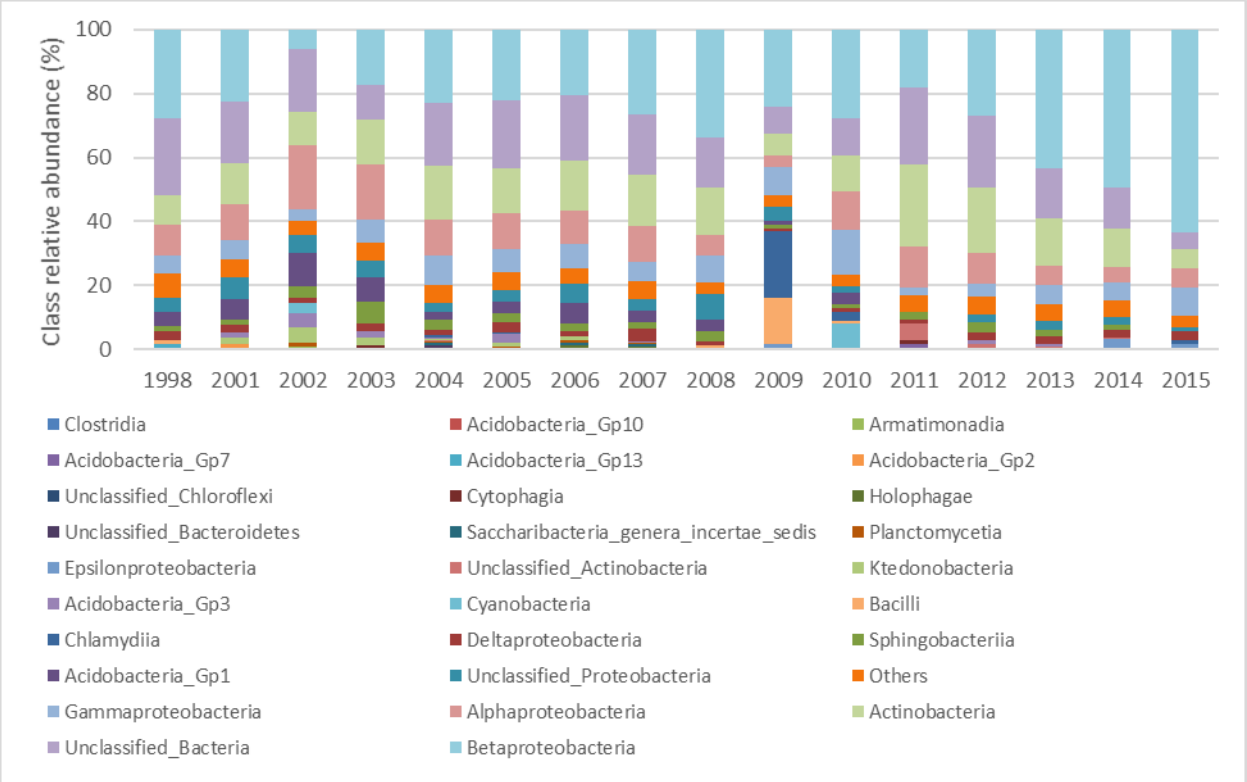

Figure S8.3a Barplot representing the relative abundance of bacterial classes grouped by year. Classes less abundant than 1 % in each sample are grouped in other.

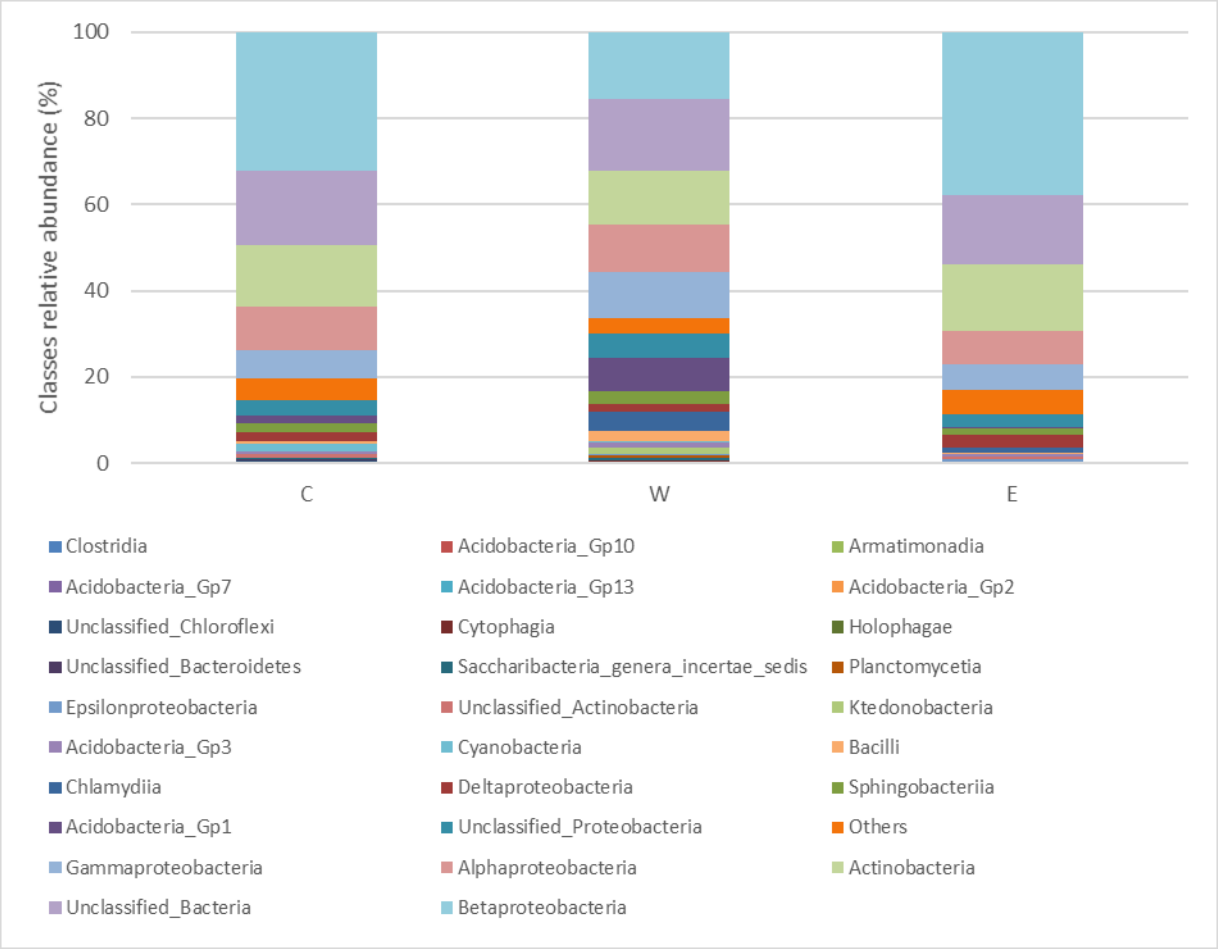

Figure S8.3b Barplot representing the relative abundance of bacterial classes grouped by transect. Classes less abundant than 1 % in each sample are grouped in other.

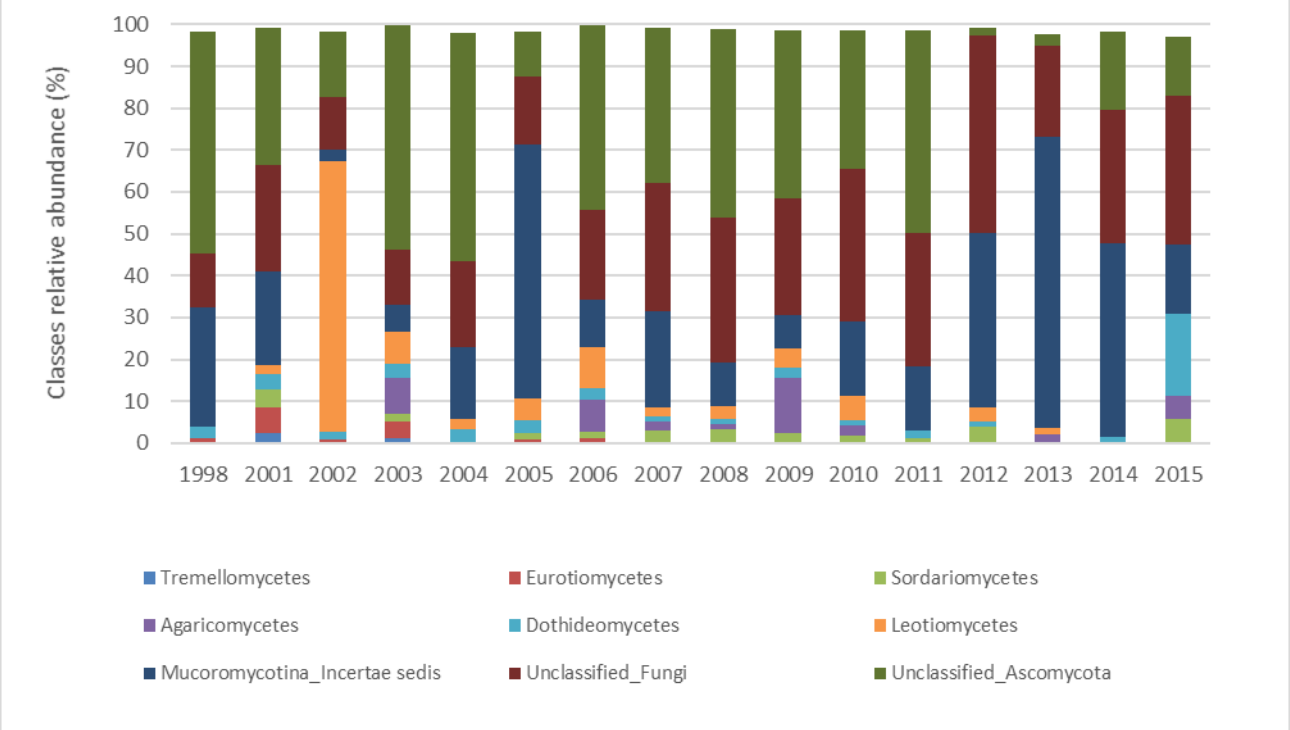

Figure S8.3c Barplot representing the relative abundance of the fungal classes grouped by year. Classes less abundant than 1 % are grouped in other.

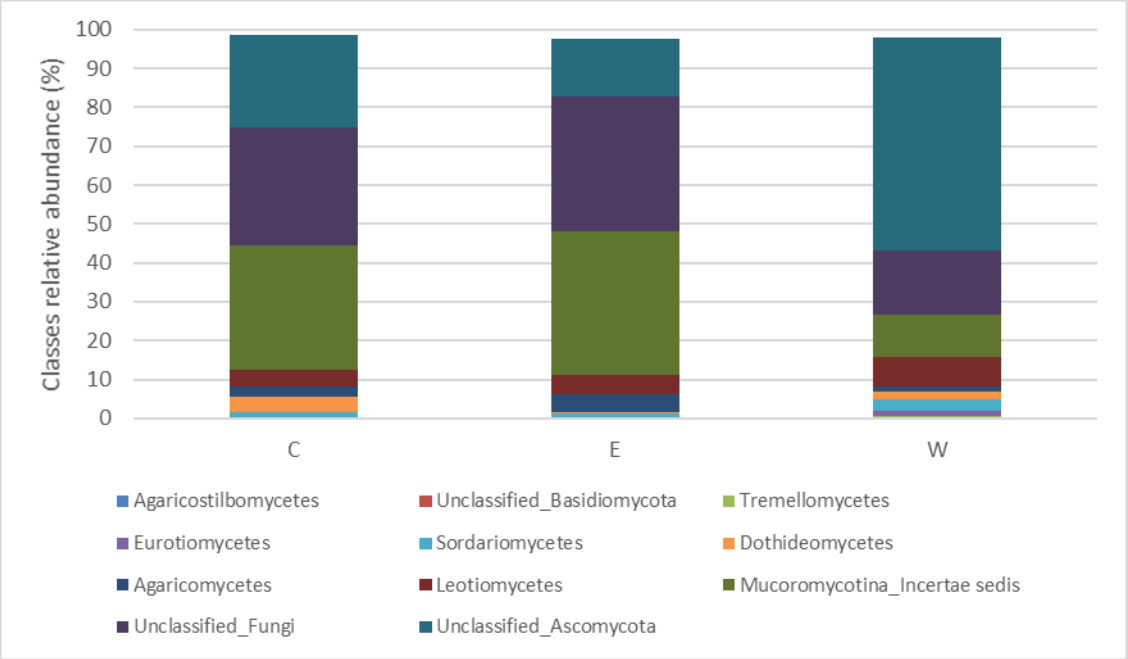

Figure S8.3d Barplot representing the relative abundance of the fungal classes grouped by transect. Classes less abundant than 1 % are grouped in other.

Network node classification

We classified network nodes according to their topological role in the network using the method proposed by (Guimera and Amaral 2005). For each node, we calculated the normalized within-module degree (z) and the participation coefficient (p) and plot values in a (p, z) space. Hubness of a node is defined by its within module degree (z): nodes with $z > 2.5$ are hub, otherwise they are non-hub. Both kinds of nodes are then classified according to the participation coefficient (p). For non-hubs, four classes are defined:

- $0 < p < 0.05$ -> ultra-peripheral node (all its links are within its module)
- $0.05 < p < 0.62$ -> peripheral node (most of its links within its module)
- $0.62 < p < 0.80$ -> connector node (many links to other modules)
- $0.80 < p < 1.00$ -> kinless node (links homogeneously distributed among all modules)

For hubs, three categories are defined

- $0 < p < 0.30$ -> provincial hub (most of its links within its module)
- $0.30 < p < 0.75$ -> connector hub (many links to most of the other modules)
- $0.75 < p < 1$ kinless hub (links homogeneously distributed among all other modules)

Reference

Guimera R and Amaral LAN. Functional cartography of complex metabolic networks. *Nature* 2005;**433**:895–900.

6. SUPRAGLACIAL SPARSE DEBRIS OF NEARBY GLACIERS HOST MORE DIFFERENT BACTERIAL COMMUNITIES THAN CRYOCONITE HOLES

ABSTRACT Supraglacial ecosystems concentrate their microbial communities mostly in cryoconite holes, small pits full of melting water with a sediment on the bottom. The geographical differences of their bacterial communities (among glaciers) are quite ascertained, especially at large scale. Furthermore, so far no data are available to confirm the hypothesis that bacterial communities inhabiting cryoconite holes are different from those that can be found in the sparse debris (the dry debris that is not immersed in the melting water), especially considering that the sparse debris can form cryoconite holes and vice versa. In this study we characterized bacterial communities of the sparse debris of three different glaciers belonging to a quite restricted area (maximum distance < 10 km) of the Ortles Cevedale Group (Italian Alps) and confirm that bacterial communities differ among different glaciers, but not according to their geographic distance. Indeed, lithology seems to have an effect on their composition. Furthermore, we found that bacterial communities of the sparse debris are significantly different from those inhabiting cryoconite holes.

6.1. Introduction

Glaciers are ecosystems teeming with microbial life, whose study has gained interest in the last decade, mostly thanks to the advances in sequencing techniques that have allowed investigating the structures of microbial communities at an unprecedented level of detail. Glacial ecological studies have dealt on a wide range of questions, from the physiological adaptations of organisms to the extreme conditions of ice, to applicative studies on the ability of these communities to degrade pollutants under cold conditions.

Among the different environments present on glaciers, the supraglacial one is by far the most studied, and, within this environment, the majority of the studies focussed on cryoconite holes, small ponds full of melting water and with a fine-grained sediment on the bottom (the cryoconite) that form on glacier surface. Cryoconite origin is mostly atmospheric and it originates mainly from local sources, even if there is a minor fraction of both biotic and abiotic particles that comes from long-range atmospheric transport (Edwards et al. 2011; Franzetti et al. 2017a; Cameron et al. 2016; Sommers et al. 2018). Cryoconite holes are considered hot-spots of biodiversity in glacier environments because they host metabolically active microbial communities (Cook et al. 2016b) typically dominated by Cyanobacteria (Takeuchiet al. 2010), while other typically abundant phyla are: Betaproteobacteria, Actinobacteria, Chloroflexi, Acidobacteria, Bacteroidetes, and Proteobacteria (Boetius et al. 2015; Liu et al. 2017; Christneret al. 2003; Franzetti et al. 2016; Sommers et al. 2018).

On glaciers, the major selective pressures that shape microbial communities are low temperature, intense solar radiation, wind exposition, electrical conductivity, and pH (Pittino et al. 2018b; Mueller et al. 2004; Edwards et al. 2011). For example, on the Baltoro Glacier Sphingobacteriales and

Sphingomonadales were observed to increase significantly with pH (Ambrosini et al. 2017). Cryoconite holes are oligotrophic microhabitats (Takeuchi, Kohshima, and Seko 2010) and nutrient availability is a limiting factor for bacteria. Total organic carbon (TOC), ammonium, and phosphorous seem particularly relevant in shaping bacterial communities of these environments (Mindl et al. 2007; Grzesiak et al. 2015; Zarsky et al. 2013; Mueller and Pollard 2004; Edwards et al. 2011).

Glaciers, and, more generally, high elevation cold environments are considered ideal models for microbial biogeographical studies (Cook, Edwards, Takeuchi, et al. 2016) because they replicate similar general ecological conditions in different geographical areas of the world (Ambrosini et al. 2017; Franzetti et al. 2013). Cryoconite holes, in particular, can be found on glaciers all over the world in both polar and alpine environments. A study on the biogeography of bacterial communities of cryoconite holes conducted at global scale has revealed that the structure of cryoconite hole communities shows a typical decay-by-distance pattern of similarity (Darcy et al. 2018). At smaller spatial scale, cryoconite hole bacterial communities seem to differ from one glacier to a nearby one, and even within a glacier (Ambrosini et al. 2017). The first study that compared cryoconite holes bacterial communities from different glaciers in a restricted geographical area (c.ca 10 km), was written by Edwards et al. (2011), who sampled three different glaciers of Svalbard during two different years. These three glaciers were quite close, but the ecological distance among their bacterial communities did not increase with geographic distance among them. Furthermore, Sommers et al. (2018) investigated differences in bacterial communities of cryoconite holes among glaciers in Antarctica confirming that different glaciers host different bacterial communities. Ambrosini et al. (2017) found that different sampling areas on the Baltoro Glacier (Pakistani Karakoram) host different bacterial communities (see Pittino et al. (2018b) for a further interpretation of the same data). It therefore appears that each glacier hosts peculiar cryoconite hole bacterial communities, and that variation from one glacier to a nearby one is mostly due to local environmental conditions, while the decay-by-distance pattern appears only at larger spatial scales.

On temperate mountain glaciers, cryoconite holes are rather ephemeral structures that continuously form and are dismantled by the strong ablation during the melting season (Pittino et al. 2018b). Interestingly, the within-season temporal dynamic of the bacterial communities seems to proceed on the whole glacier surface independently of the timing of hole formation. Indeed, Pittino et al., (2018b) showed that bacterial communities of cryoconite holes seem to follow an ecological succession during the melting season, with communities richer in Cyanobacteria and

primary producer at the beginning of the melting season, and communities richer in heterotrophic taxa, like Bukholderiales and Sphingomonadales late in the season. However, cryoconite communities typical of late successional stages were observed late in the season also in newly formed holes. This suggests that when a hole is dismantled by the ablation and the cryoconite is dispersed on the glacier surface, the bacterial community can survive, and the succession process proceeds once the cryoconite has formed a new hole (Pittino et al. 2018b).

Therefore, sparse (dry) sediment on glacier surface, i.e. the cryoconite, potentially mixed with coarser sediment, that is broadly dispersed and has not formed a hole, may host bacterial communities similar to those of cryoconite holes. However, this type of sediment has been poorly investigated to date. Indeed, only a minority of papers investigated bacterial communities of supraglacial debris, and most of them focussed on the debris of debris covered glaciers, which is a thick sediment layer whose superficial part is not in direct contact with glacier ice (Darcy et al. 2017; Franzetti et al. 2013). Even less studies focussed on supraglacial sparse debris in direct contact with ice. Two studies showed that microbial communities of dirt cones (type of depositional glacial feature) are more similar to those of moraines than to those of cryoconite holes both in the Arctic (Stibal et al. 2006) and on the Alps (Franzetti et al. 2017a). However, dirt cone sediment differs from that of cryoconite holes, because it is generally thicker and typically originates in crevasses or moulins (Swithinbank, 1950). In contrast, the fine-grained sparse debris that occurs on glacier surface may host bacterial communities more similar to those of cryoconite holes, because it mixes with the cryoconite dispersed on the glacier surface when a hole is dismantled by ablation.

So far, a comprehensive study that has investigated and compared the bacterial community composition of supraglacial sparse debris of different glaciers of the same study area has not been conducted yet. To fill this gap, we investigated bacterial communities of the supraglacial environments of three glaciers located in a rather small geographical area (they are < 6 km to one another) of Italian Alps. We formulated, in particular, two main hypotheses. First, if bacterial communities of supraglacial sparse debris derive, at least partly, from cryoconite holes dismantled by ablation, and cryoconite hole communities differ among glaciers, we expect also bacterial communities of supraglacial sparse debris to differ from one glacier to another. Second, if cryoconite hole bacterial communities are released on glacier surface because of ablation, we also expect communities of supraglacial sparse debris to be more similar to those of cryoconite holes of the same glacier than to those of supraglacial sparse debris of nearby glaciers.

6.2. Materials and methods

6.2.1. Sampling sites and samples collection

Both cryoconite and supraglacial sediment were sampled on three glaciers of the Stelvio National Park: West Zebrù (WZB), Gran Zebrù and Cedec (CED) (Fig. 6.1). West Zebrù is the northernmost glacier, covers 0.99 km2 and ranges from 2816 m a.s.l. to 3268 m a.s.l. This glacier lays on a sedimentary bedrock (dolomite). Gran Zebrù is located about 3 km south from WZB, has a surface area of 0.79 km2 and ranges from 2957 m to 3380 m a.s.l. This ice body has a particular geologic setting: its accumulation basin lies on the Zebrù tectonic line that divides sedimentary bedrocks to the North from metamorphic bedrocks to the South. Consequently, its supraglacial debris is composed of both limestone and micaschist. Moreover, this glacier is divided into two tongues by a rock ridge. In the present study, we considered samples collected on the two tongues of this glacier separately, and we called them, respectively West Gran Zebrù (WGZ) and East Gran Zebrù (EGZ). Cedec Glacier lies on a metamorphic bedrock about 3 km south-east from Gran Zebrù, covers an area of 2.07 km2 with an altitudinal range from 2687 to 3761 m a.s.l.(Azzoni et al. 2018).

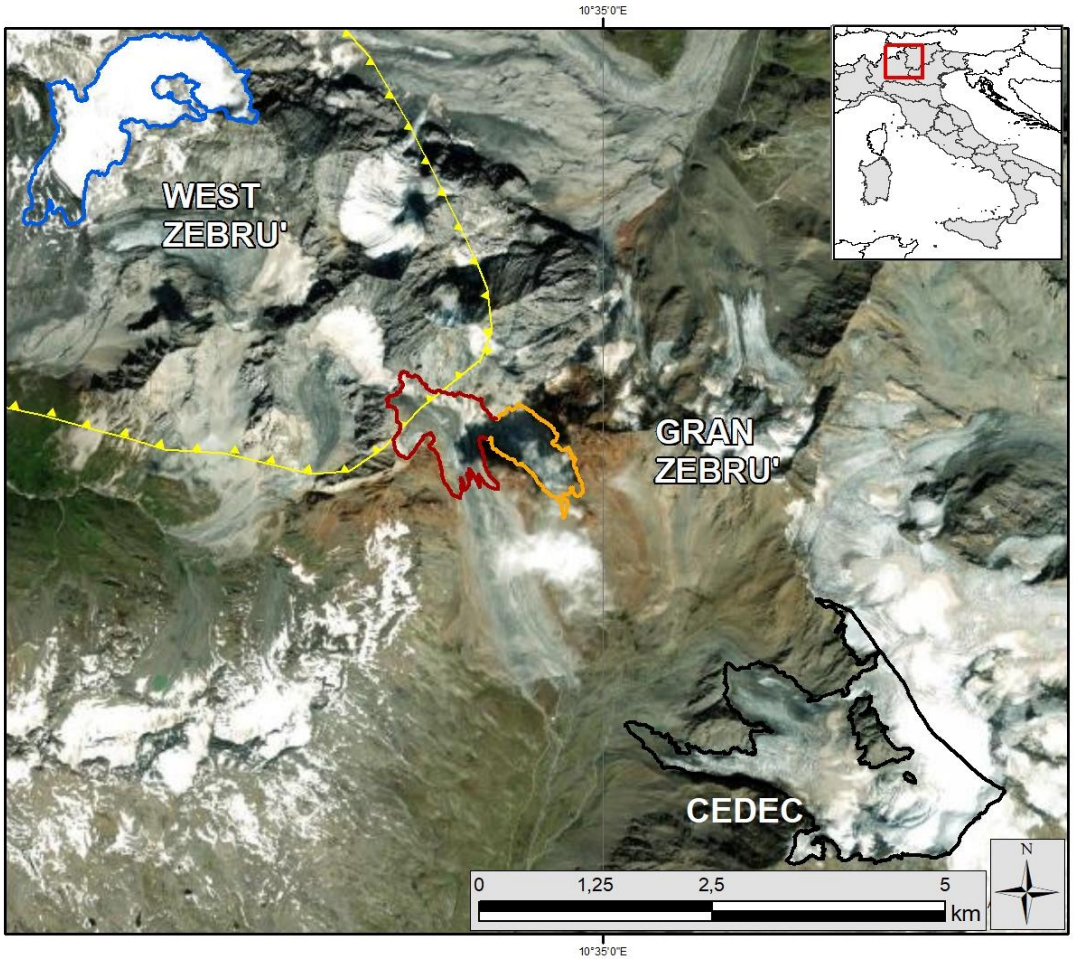

Figure 6.1 Location map of the studied glaciers with 2015 glacier limits (Stelvio Park, Ortles-Cevedale Group, Lombardy Sector, Italian Alps). The yellow line indicates the Zebru` Tectonic Line that divides sedimentary bedrocks in the North from metamorphic bedrocks in the South.

Supraglacial sparse debris samples were aseptically collected in sterile plastic bags every ~ 20 m along transects crossing the glacier tongues at approximately the same elevation (~ 3100). Cryoconite samples were aseptically collected and stored in 50 mL FalconTM tubes in the ablation areas of each glacier. Cryoconite holes were rare on WGZ and EGZ, and sample size from these glaciers was insufficient for any statistical analyses (Tab. 6.1). Therefore only CED and WZB were included in the analyses of cryoconite holes. All the samples were kept at 4 °C during transport to the laboratory (~ 8 hours), where they were stored at -20 °C.

Table 6.1 Sampling date and number of samples collected on each glacier.

Glacier	Date	Supraglacial sediment samples	Cryoconite samples
West Zebrù (WZB)	30/08/2017	9	11
East Gran Zebrù (EGZ)	19/08/2017	12	2
Cedec (CED)	31/08/2017	12	11
West Gran Zebrù (WGZ)	19/08/2017	12	4

6.2.2. Chemical analyses

For each sample, we estimated the pH value and the total organic carbon (TOC) content. Soil samples were diluted in distilled water and pH was measured with a digital pH-meter (HACH LANGE HQ40D, Loveland, CO, USA). TOC was estimated by loss on ignition (Heiri et al. 2001) with an uncertainty margin of 0.1 %: samples were air-dried and organic matter was oxidized at 500–550 °C to carbon dioxide and ash; the samples were weighed before and after heating to calculate the mass loss.

6.2.3. Molecular analyses and sequences elaboration

DNA was extracted from 0.7 g of sediment of each sample using the FastDNA Spin for Soil kit (MP Biomedicals, Solon, OH) according to the manufacturer's instructions. DNA samples and library preparation were performed as reported in Pittino et al. (2018) amplifying the V5-V6 hypervariable region of the 16S rRNA gene and sequencing was performed at "Consorzio per il centro di Biomedicina Molecolare (CBM)" (Trieste, Italy). The reads were demultiplexed according to the indexes. Sequences were grouped in Amplicon Sequence Variants (ASVs) using DADA2 (Callahan et al. 2016) and classified with RDP classifier (Wang et al. 2007). Cyanobacteria were not classified at order level because the RDP classifier does not report the order level for this phylum (Garrity et al. 2007; Wilmotte et al. 2015). When working at phylum level we therefore kept the Cyanobacteria/Chloroplast definition given by rdp. Since we removed those Cyanobacteria/Chloroplast that were classified as Chloroplast, the difference between phylum and

order is attributed to the presence of Unclassified Cyanobacteria/Chloroplast, that are those that rdp is not able to distinguish and are therefore not classified at more specific taxonomic levels. Therefore, considering the phylum Cyanobacteria/Chloroplast we may be including likely algal ASVs, as algae are an important component in cryoconite (Stibal et al. 2006).

6.2.4. Statistical analyses

Analyses were performed with R 3.5.1 (R Core Team, 2014) with the VEGAN, BIODIVERSITYR, MULTTEST, MULTCOMP packages. Singletons (ASVs present only once in the dataset) were removed and Hellinger distance was used to compute the distance among the samples, which depends on the differences in ASVs proportion among them, decreases the importance of ASVs over their occurrence and avoids the double-zero problem (De Cáceres et al. 2010; Legendre et al. 2001). Alpha-diversity was investigated calculating the Shannon diversity index, which accounts for both the richness and the evenness of the species (Shannon 1948), and Gini inequality index which is an index of inhomogeneity largely used in economics (Gini 1912). Redundancy analysis (RDA) and variation partitioning (VP) were used to investigate the variation of community structures. Predictors were: glacier (four-level factor), the type of sediment (either cryoconite or sparse debris, two level factor) and TOC. This latter variable was mean-centred within glacier (i.e. from TOC values at each sample we removed the mean TOC values of all samples of that glacier). We did not include pH because it was strongly collinear with TOC (Pearson-r = 0.8) and it is influenced by the lithology, which has been included in the analysis as characteristic of each glacier. It is likely that the reason of the correlation is that a higher amount of organic matter caused a more acidic pH, thus pH is a consequence of not only the lithology, but also the organic matter content. Different sets of predictors and their interactions were entered in different models (details in results). Significance was assessed with 99,999 permutations. Helmert contrasts were used to assess the amount of variance explained by each variable and by their interaction (Borcard, Gillet, and Legendre 2008). Post-hoc tests were also performed to assess pairwise differences between glaciers while correcting P-values for multiple statistical tests according to the false discovery rate (FDR) procedure (Benjamini and Yekutieli 2001). Taxa (either phyla or orders) with > 30,000 reads in all samples (excluding cryoconite from EGZ and WGZ) were considered abundant taxa. Variation in the abundance of Cyanobacteria and of all the most abundant orders according to the variables that significantly affected the structure of bacterial communities identified by the RDAs was investigated by generalized linear models (GLMs) assuming a Poisson distribution corrected for overdispersion (Zuur et al. 2009). Also in these cases, P-values were corrected using the FDR procedure. Analysis of

variance (ANOVA) and linear models (LMs) assuming a Gaussian error distribution, eventually followed by Tukey post-hoc tests, were used to investigate changes in alpha diversity indices and chemical features of the sediments according to the same predictors. Model assumptions were checked using standard methods.

6.3. Results

6.3.1. Chemical features of the sediment

Results from chemical analyses showed that both pH and TOC varied among glaciers and between type of sediment. In each glacier, pH and TOC were strongly collinear ($r > 0.8$) except on CED ($r = 0.3$). pH values of supraglacial debris were more acidic on CED and more alkaline on WGZ while WZB and EGZ had intermediate pH values, the cryoconite was more acidic on CED than on WZB (Fig. 6.2a). TOC content in supraglacial debris was higher in WZB samples than in the other glaciers, which did not differ to one another. Cryoconite TOC was also higher on WZB than on CED (Fig. 6.2b).

Cryoconite samples were consistently more acidic than supraglacial sediment samples on both CED ($t_{16.15} = -8.78$, $p < 0.001$) and WZB ($t_{10.96} = -6.283$, $p < 0.001$) (Fig. 6.2a), and TOC content was always higher in cryoconite samples than in supraglacial debris (CED: $t_{11.05} = 4.635$, $p < 0.001$; WZB: $t_{11.69} = 3.464$, $p = 0.005$; Fig. 6.2b).

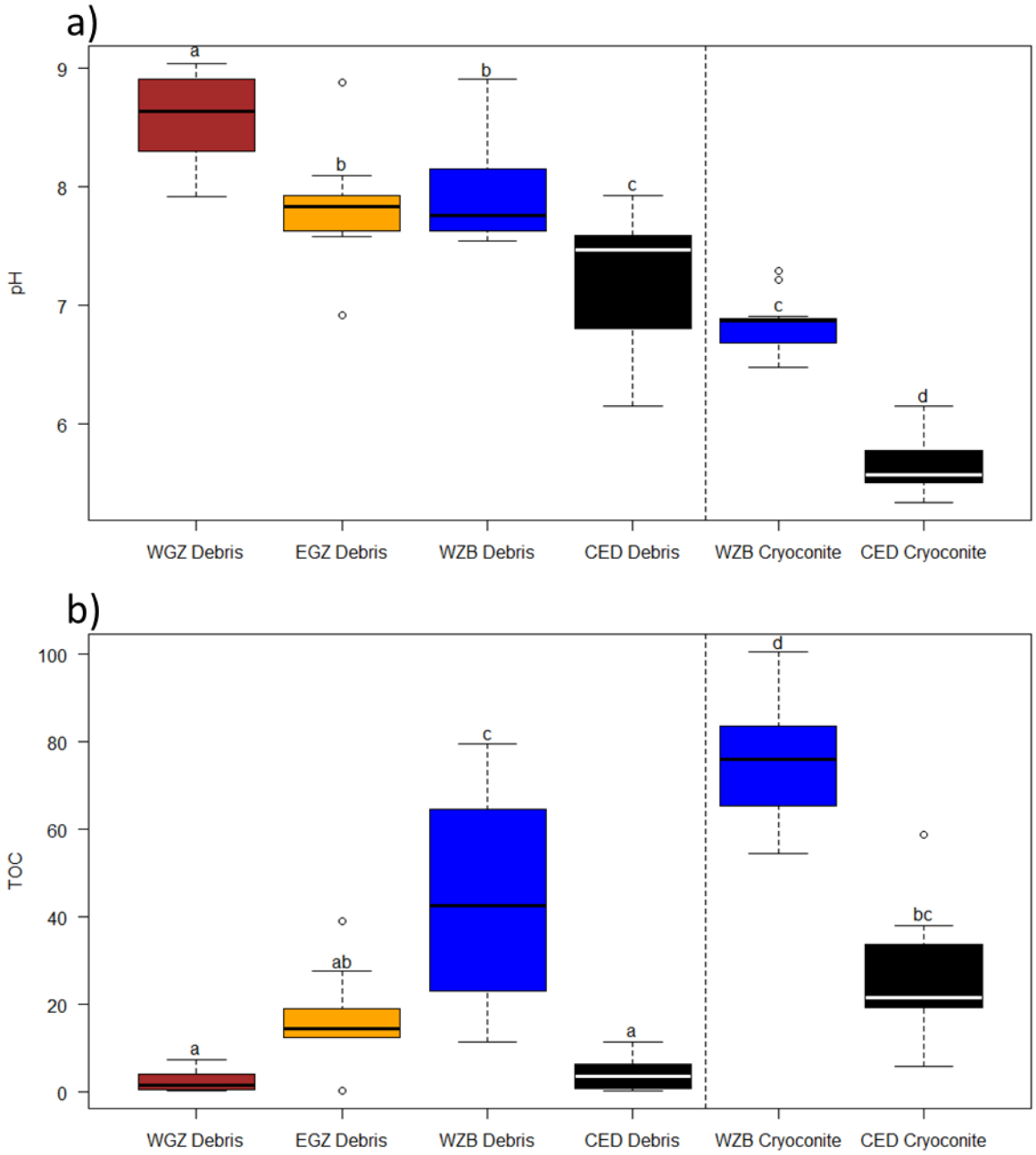

Figure 6.2 Boxplots representing pH (a) and TOC (b) of supraglacial sediment on WGZ (brown), EGZ (orange), WZB (blue) and CED (black), and of cryoconite of WZB (blue) and CED (black). The thick lines represent the median, boxes upper and lower limits the 25th and the 75th percentiles respectively, whiskers the data that go beyond the 5th percentile (lower whisker) and the 75th percentile (upper whisker), dots represent the outliers and different letters indicate significant differences (P < 0.05) between the mean values of different groups at post hoc-tests.

6.3.2. Bacterial communities

The number of sequences we obtained varied from 7,458 to 113,151 per sample.

The orders with more than 30,000 of total sequences were considered as the most abundant and were: Burkholderiales, Actinomycetales, Sphingobacteriales, Enterobacteriales, Pseudomonadales, Sphingomonadales, Cytophagales. Cyanobacteria class sequences were also > 30,000 (Fig. 6.3). They

were analysed together with the other orders since the RDP taxonomy does not provide the classification at order level for them (see Pittino et al. (2018) for a similar approach).

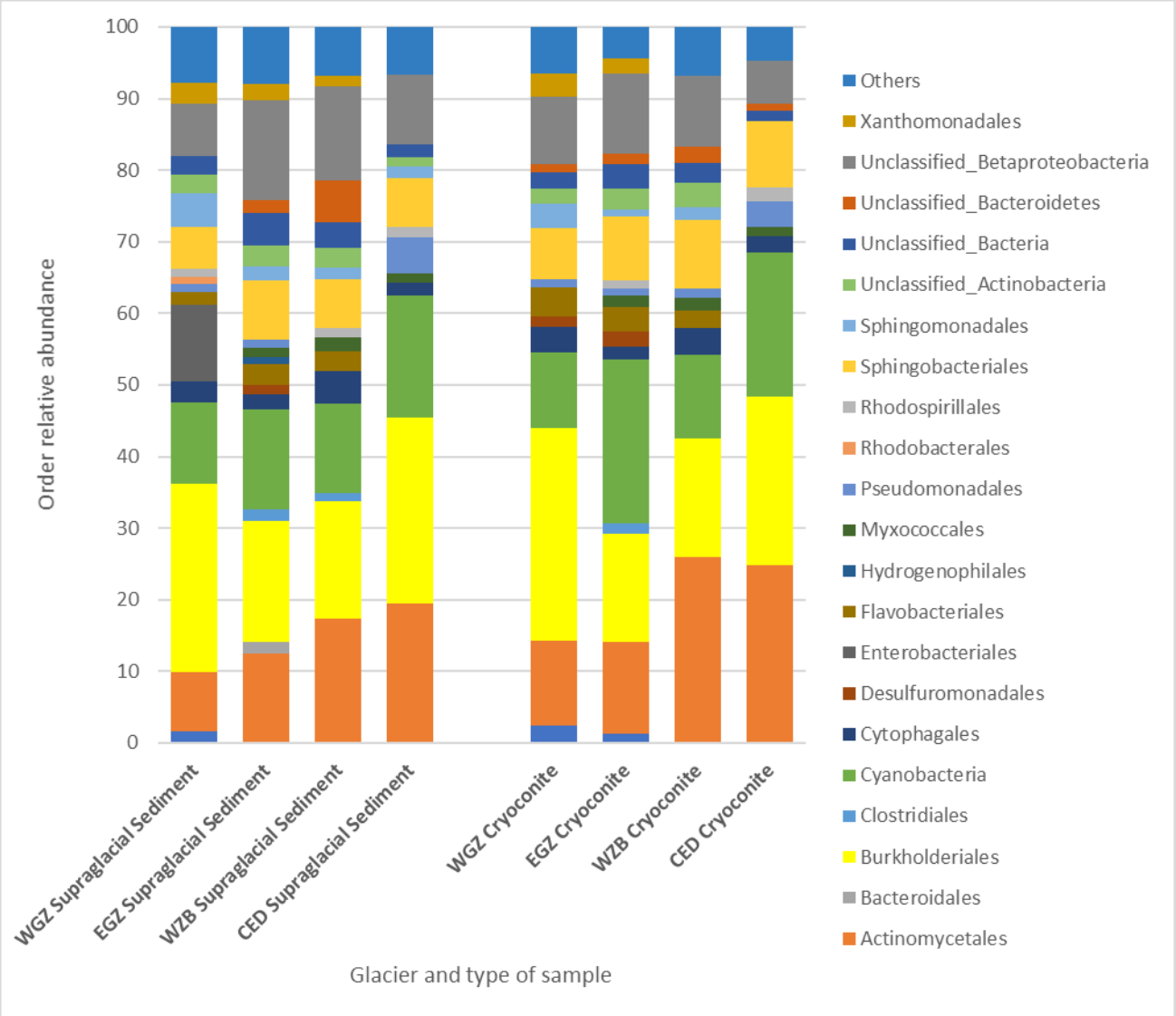

Figure 6.3 Barplot showing the relative abundance of the orders in each glacier in cryoconite and in supraglacial sediment. Orders less abundant than 1 % were grouped in others. Cyanobacteria *are reported among the orders since rdp does not provide the classification at order level for them. Cryoconite samples from GZ were included here but not in statistical analyses because of the low number of samples.*

Abundant phyla (i.e. > 30,000 reads) were: Proteobacteria, Actinobacteria, Cyanobacteria and Bacteroidetes (Fig. 6.4).

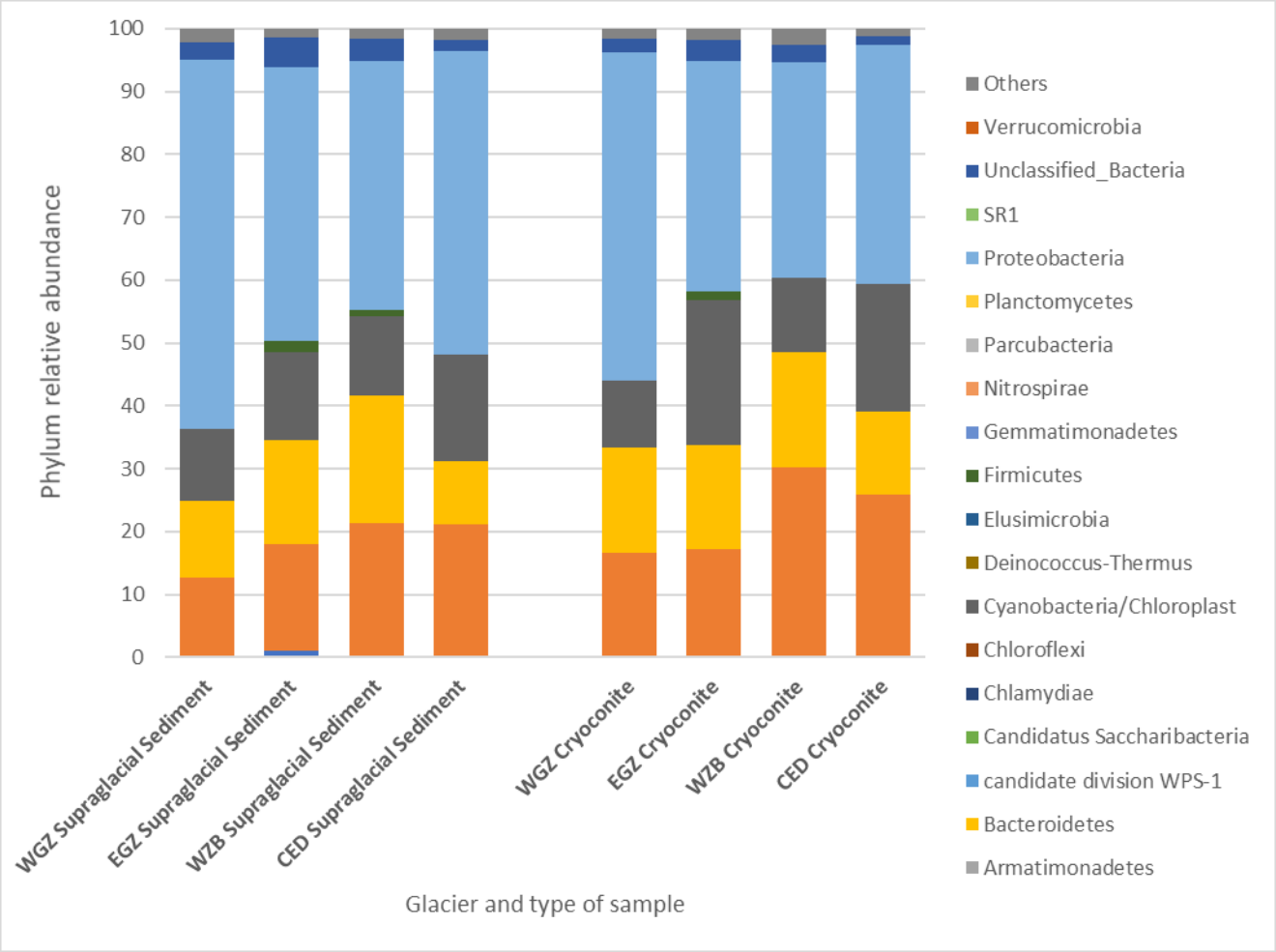

Figure 6.4 Barplot showing the relative abundance of the phyla in all the glaciers in cryoconite and supraglacial sediment. Phyla less abundant than 1 % were grouped in others. Cryoconite samples from GZ were included here but not in statistical analyses because of the low number of samples.

6.3.3. Bacterial communities of sparse supraglacial debris

RDA on supraglacial sediment samples and post-hoc tests showed that bacterial communities differed significantly among glaciers (Tab. 6.2), with significant differences between each pair of glaciers ($F_{2,40} \geq 4.57$, $P_{FDR} \leq 0.025$). In addition, TOC significantly affected bacterial community structures (Tab. 6.2, Fig. 6.5a). VP showed that glacier explained 49 % of variance, while TOC accounted for 1.7 % only (Fig. 6.5b).

Table 6.2 RDA of Hellinger-transformed bacterial ASVs abundances of supraglacial sediment samples according to the glacier and TOC.

Variable	df	Variance	F	P
Glacier	3	0.207	14.974	< 0.001
TOC	1	0.011	2.394	0.018
Residuals	40	0.184		
$F_{4,40}$ = 11.829, P < 0.001, Adjusted R^2 = 0.496				

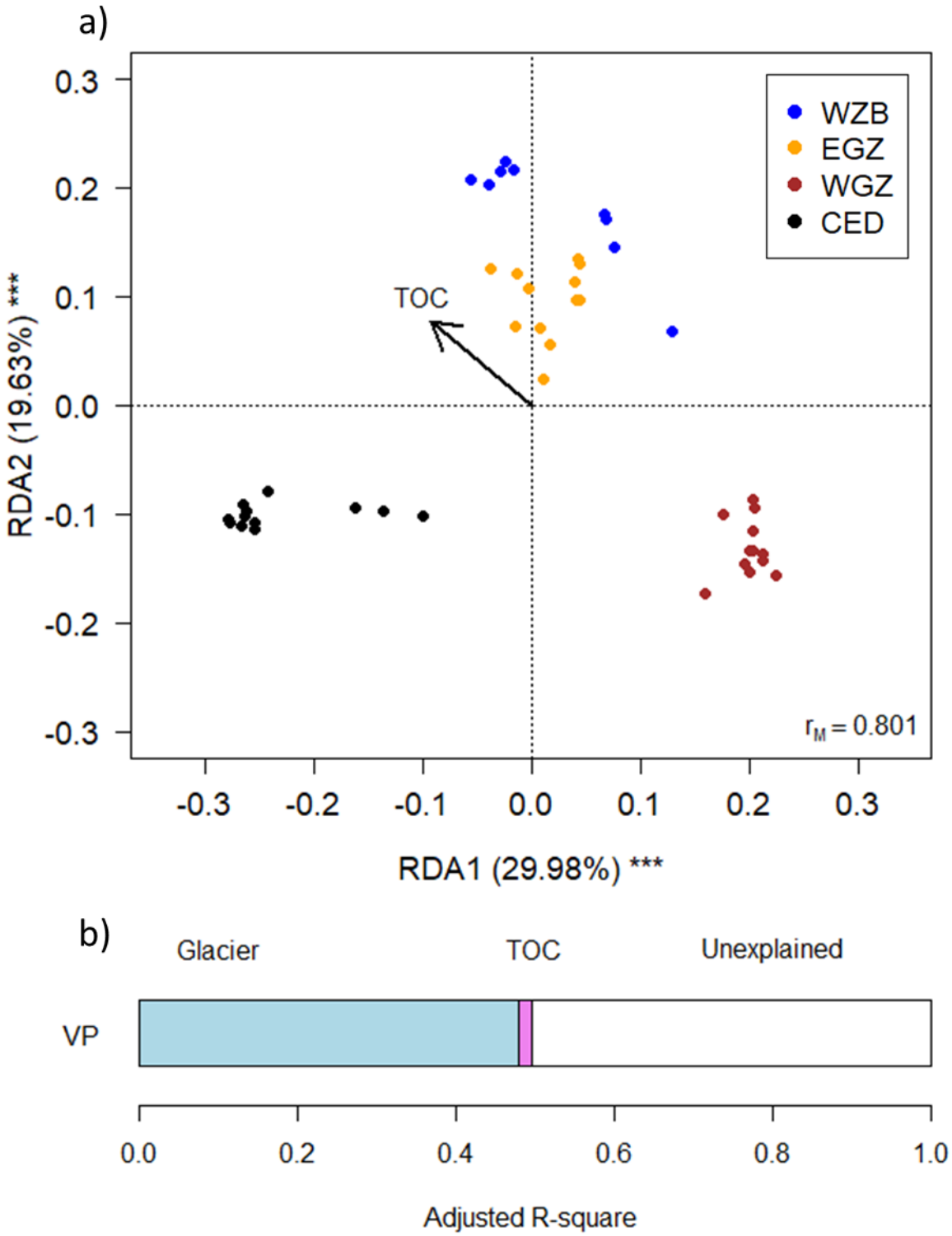

*Figure 6.5 (a) Biplot from RDA on Hellinger-transformed bacterial ASVs abundances in the supraglacial sediment of glacier and TOC. The percentage of variance explained by each axis and their significance (***: P < 0.001) is reported. (b) Results from the Variation Partitioning (VP) showing the amount of variance explained by each predictor of the RDA. The joint contribution of glacier and TOC was null.*

Analyses of most abundant taxa showed that their relative abundance significantly changed among glaciers ($F_{3,40} \geq 7.419$, $P_{FDR} \leq 0.002$). Burkholderiales were more abundant on CED and WGZ ($F_{3,40}$ =

10.301, $P_{FDR} < 0.001$; Fig. S6.1a), Actinomycetales on CED and WZB and less abundant on WGZ ($F_{3,40}$ = 17.646, $P_{FDR} < 0.001$;Fig. S6.1b), Sphingomonadales ($F_{3,40}$ = 18.750, $P_{FDR} < 0.001$;Fig. 6.1c) and Pseudomonadales were more abundant on WGZ ($F_{3,40}$ = 27.211, $P_{FDR} < 0.001$;Fig. S6.1d) and Cytophagales on WZB ($F_{3,40}$ = 7.419, P_{FDR} = 0.002;Fig. S6.1e). Abundance of Sphingobacteriales and Enterobacteriales did not change among glaciers ($F_{3,40} \leq 1.23$, P_{FDR} = 1). TOC did not affect the abundance of any of the most abundant taxa ($F_{1,40} \leq 8.65$, $P_{FDR} \geq 0.11$).

GLMs performed on the most abundant phyla showed that Proteobacteria were more abundant on WGZ and CED than on the other glaciers, ($F_{3,40}$ = 6.32, P_{FDR} = 0.004; Fig. S6.2a), Actinobacteria were more abundant on CED and WZB ($F_{3,40}$ = 6.708, P_{FDR} = 0.004; Fig. S6.2b), and Bacteroidetes on WZB and EGZ ($F_{3,40}$ = 6.857, P_{FDR} = 0.004; Fig. S6.2c). Abundance of Cyanobacteria/Chloroplast did not change significantly among glaciers ($F_{3,40}$ = 1.083, P_{FDR} = 0.765). Cyanobacteria/Chloroplast ($F_{1,40}$ = 8.651, P_{FDR} = 0.023) increased with TOC (Fig. S6.3a), while Proteobacteria ($F_{1,40}$ = 9.706, P_{FDR} = 0.023) decreased (Fig. S6.3b).

6.3.4. Comparison of bacterial communities of cryoconite holes and sparse supraglacial debris

The RDA performed on the Hellinger-transformed ASV abundances of cryoconite and supraglacial sediment samples from CED and WZB showed that bacterial communities varied according to type of sediment, glacier and their interaction (Tab. 6.3, Fig. 6.6). Partial adjusted R^2 showed that the type of sample explains 45.9% of the variance, the glacier 3.4%, and their combined effect 2.8%.

Table 6.3 RDA of Hellinger-transformed bacterial ASV abundances of both cryoconite and supraglacial sediment samples of Cedec and Zebrù Est according to the glacier, type of sample and their interaction.

Variable	Df	Variance	F	P	Partial Adjusted R^2
Glacier	1	0.014	3.684	< 0.001	0.034
Type	1	0.137	36.992	< 0.001	0.460
Glacier × Type	1	0.012	3.172	< 0.001	0.028
Residuals	39	0.144			
$F_{3,39}$ = 14.42, P < 0.001, Adjusted R^2 = 0.489					

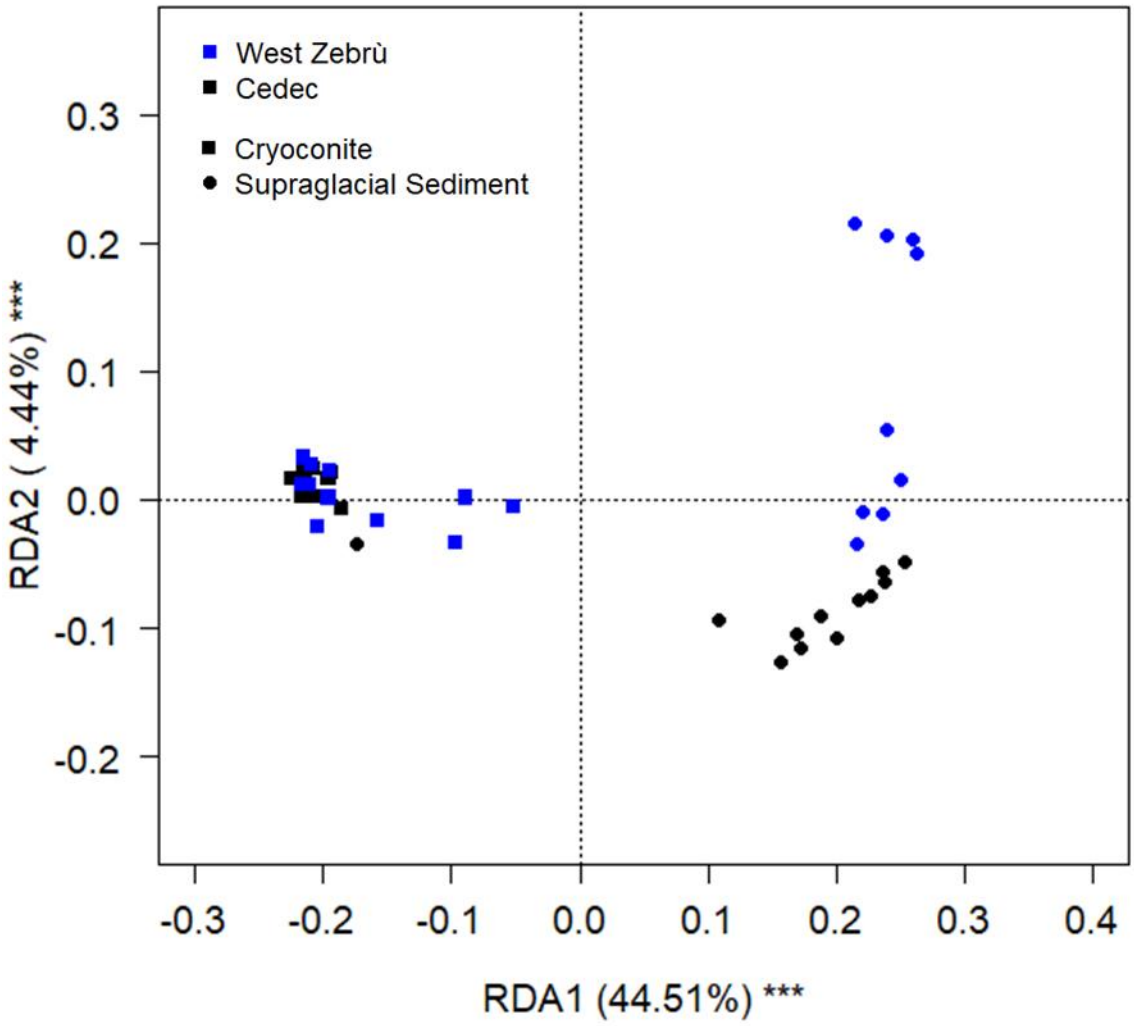

*Figure 6.6 Biplot from RDA on Hellinger-transformed bacterial ASV abundances on glacier and type of sample. The percentage of variance explained by each axis and their significance (***:P < 0.001) is reported.*

Even if in the RDA biplot (Fig. 6.6) cryoconite samples of the two glaciers look similar, post-hoc tests revealed that they were significantly different, indeed bacterial communities resulted different both between glaciers and between type of sediment ($F_{1,39} > 2.708$, $P_{FDR} < 0.038$).

GLMs performed on the most abundant orders and phyla according to type of sediment, glacier and their interaction showed that only relative abundance of Cyanobacteria varied according to the type of sediment ($F_{1,39} = 12.199$, $P_{FDR} = 0.022$) and that they were more abundant in cryoconite samples than in supraglacial sediment samples (Fig. S6.4).

6.3.5. Alpha diversity

GLMs performed on alpha diversity indexes according to the glacier and TOC, showed that the Shannon index ($F_{3,40} = 5.020$, $P = 0.005$) and Gini index ($F_{3,40} = 7.228$, $P < 0.001$) changed among

glaciers but not with TOC ($F \leq 0.950$, $P \geq 0.336$). In particular, Shannon index was higher on EGZ and WGZ and lower on CED and WZB (Fig. 6.7a) and Gini index was higher on CED and WZB and lower on EGZ (Fig. 6.7b).

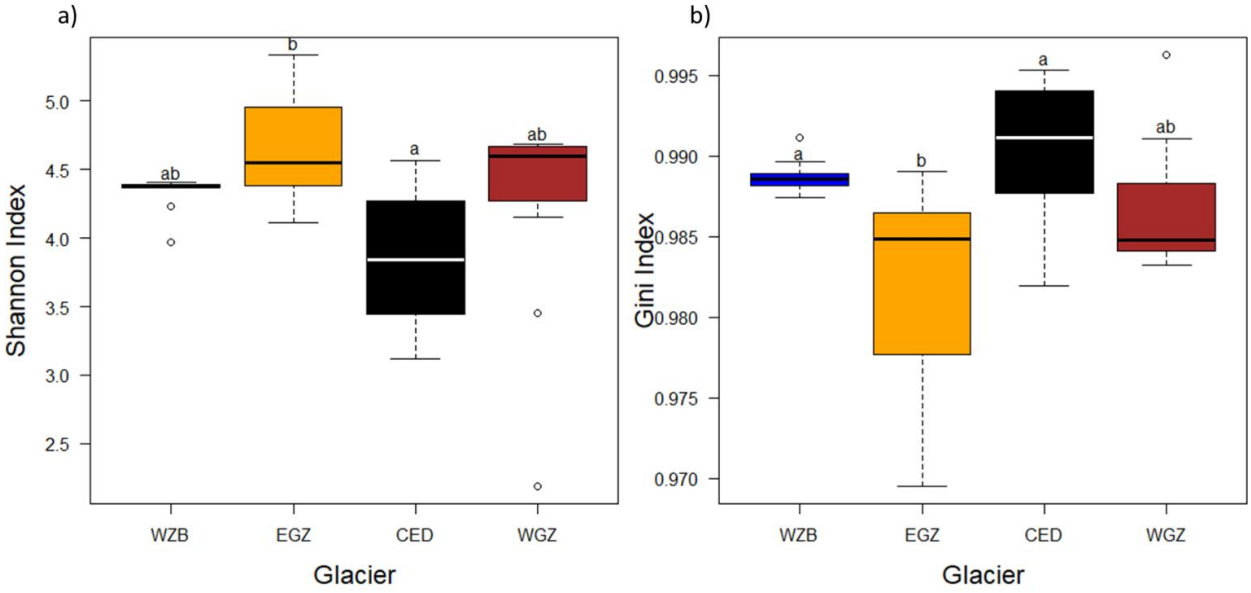

Figure 6.7 Shannon (a) and Gini index (b) at each glacier. The thick lines represent the median, boxes upper and lower limits the 25th and the 75th percentiles respectively, whiskers the data that go beyond the 5th percentile (lower whisker) and the 75th percentile (upper whisker), dots represent the outliers and different letters indicate differences between the mean values of different groups.

GLMs on the alpha diversity indexes according to type of sediment, glacier and their interaction showed that Shannon index changed significantly according to glacier only ($F_{1,39} = 47.17$, $P < 0.001$) with higher values on the WZB (Fig. 6.8a). Gini index changed significantly among glaciers ($F_{1,39} = 21.822$, $P < 0.001$), with higher values on CED (Fig. 6.8b). In addition, it varied also with the interaction between glacier and type of sample ($F_{1,39} = 8.071$, $P = 0.007$), with higher values in both cryoconite and sparse supraglacial debris of CED and the lowest value in cryoconite of WZB (Data not shown).

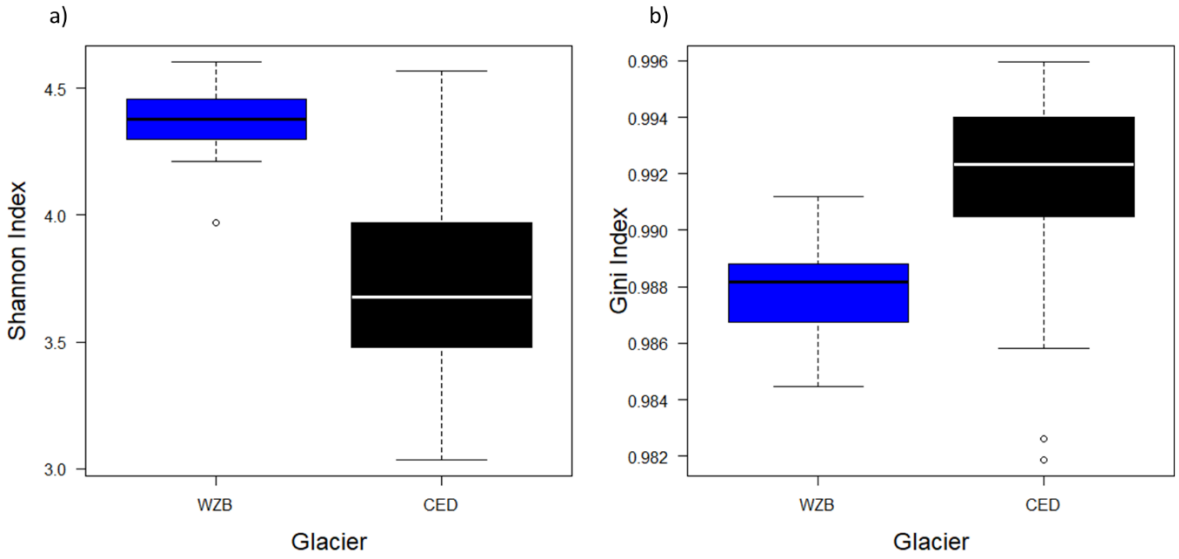

Figure 6.8 Boxplots representing differences of Shannon index (a) and Gini index (b) on WZB and CED. The thick lines represent the median, boxes upper and lower limits the 25th and the 75th percentiles respectively, whiskers the data that go beyond the 5th percentile (lower whisker) and the 75th percentile (upper whisker), dots represent the outliers and different letters indicate differences between the mean values of different groups.

6.4. Discussion

The glaciers we studied are all within a quite limited geographical area, being at a maximum of 6 km to one another. They are therefore a useful model to compare bacterial diversity between glaciers and, within glacier, between cryoconite holes and sparse supraglacial debris. The Ortles-Cevedale area is geologically heterogeneous, with a close contact between sedimentary and metamorphic rocks (Montrasio et al. 2008). In particular, the Zebru` Tectonic Line separates Pre-Permian mica schist and paragneiss, in the Southern area, from the Rhaetian dolomite and limestone, outcropping in the Northern part of the region (Montrasio et al. 2008). The local bedrock, and consequently the debris originating from it, exhibits different colors, which has different effects on the albedo and on heat absorption (Hall et al. 2005); metamorphic rocks are mostly dark grey, brown, or reddish brown, whereas sedimentary rocks feature a light gray to whitish color.

Despite we inspected almost all the ablation area of the Gran Zebrù glacier (i.e. both EGZ and WGZ), we could not find a sufficient number of cryoconite holes to allow any meaningful comparison of their bacterial communities with those of the other glaciers. We therefore only reported a description of the bacterial communities composition of the cryoconite holes of these glaciers. In contrast, we compared the features and the bacterial communities of the supraglacial debris of all glaciers, and we compared bacterial communities of supraglacial debris and cryoconite holes on CED and WZB only.

CED supraglacial debris showed the lowest pH values, followed by EGZ and WZB, while WGZ had the highest values (Fig. 6.2a). These differences can be related to differences in the lithology of the rocks surrounding each glacier. CED is surrounded mainly by mica schist with chlorite and sericite. EGZ lays under the steep south face of Gran Zebrù mountain, which is composed of dolomite as the rocks surrounding WZB. Dolomite clasts therefore reach EGZ in large abundance (our personal observations), which may explain why pH values of EGZ supraglacial debris was similar to that of WZB. WGZ receives less dolomite clasts then EGZ, because of the rock ridge that separates them, and is surrounded by metamorphic rocks enriched in iron that gives them a red-brown colour. TOC content of supraglacial debris was higher on WZB than on all other glaciers, and showed also larger variance. Maybe carbonate debris can favour higher productivity, which may also explain the slightly higher TOC values of EGZ, whose debris is enriched in dolomite, than WGZ and CED. However, abundance of Cyanobacteria, which are the main primary producers in glacier environments and are expected to lower the pH, did not differ significantly among glaciers.

Generally, bacterial communities of both cryoconite from cryoconite holes and supraglacial sparse debris were dominated by Cyanobacteria, Burkholderiales, and Actinomycetales. Other abundant orders were Sphingobacteriales, Pseudomonadales, Clostridiales, Rhodospirillales, Cytophagales. These orders are typical of glacial environments and among the most abundant in cryoconite (Pittino et al. 2018b; Sommers et al. 2019; Edwards et al. 2016; Franzetti et al. 2017b). Indeed, also in the few cryoconite samples of EGZ and WGZ we found these orders (Fig. 6.3).

Despite the taxonomic similarity, the structure of bacterial communities of supraglacial sparse debris differed among glaciers, and this held true also for EGZ and WGZ, which are two tongues of the same glacier. Interestingly, Figs. 6.3 and 6.5a show that bacterial communities of EGZ are more similar to those of WZB than to those of the WGZ. Sparse debris of WZB and EGZ have similar pH values maybe because of the inputs of dolomite debris. WGZ and CED communities were also separated from those of the other glaciers and to one another. This latter difference can be explained by the difference in pH values among glaciers. Indeed, the first RDA axis seems to represent a gradient of the pH values of supraglacial debris of our glaciers (Fig. 6.5a), which, unfortunately, we could not include directly in the analysis because of its strong collinearity with TOC. In contrast, the second axis seems to separate glaciers with and without carbonatic inputs. WGZ, EGZ and WZB communities in the RDA are also along the TOC gradient of the glaciers, while CED ones seem to deviate. Indeed, CED supraglacial debris showed the lowest values of alpha diversity, the highest evenness (Fig. 6.7), and the highest relative abundances of Actinomycetales,

Burkholderiales and Pseudomonadales. In contrast, WZB showed both low alpha diversity values and high relative abundance of Actinomycetales and Cytophagales. These orders are chemorganotrophic bacteria and their presence is consistent with the high TOC values present on this glacier that decrease also the presence of metals because of its sorption capacities, decreasing metals bioavailability for lithotrophic bacteria (Reith et al. 2012; Risueno et al. 2020). This is consistent also with the fact that for example Acidimicrobiales, that are chemolithotrophic, were more abundant on the WGZ that had very low TOC values, so mobile metals were more bioavailable (Risueno et al. 2020). WGZ showed also higher alpha diversity than the other glaciers, with abundant Sphingobacteriales and Burkholderiales that may have been favoured by the alkaline pH.

Despite in our RDA model TOC was significantly related to bacterial community variation of supraglacial debris, VP analysis showed that it explained 1.7% of variance only (Tab. 6.2). TOC is known to affect bacterial communities in soils of different environments (Banerjee et al. 2016; Zeng et al. 2016; Song et al. 2011). For instance, one study reported an increase in Actinobacteria relative abundance and a decrease of Betaproteobacteria and Gammaproteobacteria with TOC in mountain soils (Shen et al. 2013). However, the actual effects of TOC on bacterial communities of the supraglacial sparse debris are unknown. In our study, it did not affect significantly neither the abundance of Cyanobacteria nor that of the most abundant orders. At phylum level instead we found a significant effect on Cyanobacteria/Chloroplast and Proteobacteria. In contrast, differences among glaciers accounted for 49% of observed variability in bacterial communities, which strongly suggests that, even within a small geographical area, bacterial communities are well separated from one glacier to another.

The fact that Cyanobacteria/Chloroplast abundance is positively correlated with TOC might be due to their autotrophic activity that is important for cryoconite grains production (Takeuchi et al. 2010). Indeed, organic carbon in supraglacial environments can have three main sources: aeolian transport, the wash-away of supraglacial debris and biological carbon transformation mostly in cryoconite holes played by Cyanobacteria (Stibal et al. 2010). Furthermore, it has been demonstrated that only a small fraction of organic carbon is consumed by the heterotrophic community, explaining the increase and accumulation of TOC in parallel with the high relate abundance of Cyanobacteria/Chloroplast (Anesio et al. 2010).

Proteobacteria are a phylum characteristic of soils with a wide variety of metabolisms involved in the global carbon, nitrogen and sulfur cycles (Spain et al. 2009). This phylum has been reported as one of the most abundant in many studies of soils in extreme environments (e. g. supraglacial

environments, snow, arid and semiarid soils and also highly polluted soils) (Connon et al. 2007; Bastida et al. 2016; Nogales et al. 1999; Azzoni et al. 2018; Edwards et al. 2013a; Pittino et al. 2018b) and also it was demonstrated to be one of the first to colonize new environments (Esposito et al. 2013). Their decrease with the increase in TOC is an unexpected result similar to the one obtained by Shen et al. (2013). It is unlikely that this phylum is not a good colonizer of soils (or supraglacial sediment in our case), as in other studies the opposite was demonstrated (Bastida et al. 2015; Goldfarb et al. 2011). However, this phylum is composed by a very heterogeneous group of bacteria, it is therefore difficult to find an ecological explanation of their correlation with TOC (Spain et al. 2009).

These results therefore show that the structure of bacterial communities of supraglacial sediment changes from one glacier to another. This is consistent with the results reported in a study by Sommers et al. (2018b), which showed that the source of the sediment plays an important role in shaping supraglacial bacterial communities. This may occur because the sediment source determines its mineralogical and chemical composition, which, in turn, may affect the structure of the bacterial communities that develop in cryoconite holes and supraglacial sediment.

Our results also showed that, at local scale, there are differences among bacterial communities of the supraglacial environment that do not correlate with geographic distances (i. e. EGZ and WGZ that are two tongues of same glacier are not the most similar). This is consistent with previous studies reporting that other factors, likely edaphic variables, are mostly responsible for bacterial communities' composition in soils (Fierer et al. 2006).

Bacterial community composition of mountain soils at phylum level is comparable to the one of supraglacial sediments (Labbé et al. 2007; Nemergut et al. 2005) albeit these two habitats area clearly different (Edwards et al. 2013b). In our case, pH did not affect bacterial communities of supraglacial sediment, while its effect in soil bacterial communities has been largely reported (Shen et al. 2013). For example the phylum Acidobacteria, characteristic of acidic soils that can be affected by elevation (Bryant et al. 2008; Shen et al. 2019), in our study was found only on EGZ with a relative abundance close to 1%, but not on CED that resulted to be the most acidic. Only few studies investigated the effect of pH on supraglacial sediment (cryoconite) (Edwards et al. 2011; Ambrosini et al. 2017) but not much is known about how does it affect bacterial communities. What we can conclude so far is that supraglacial sediment bacterial communities do not show any evident responses to differences in pH in our samples.

The second aim of this study was to compare cryoconite collected in cryoconite holes and sparse debris. Due to the low number of cryoconite holes on EGZ and WGZ, this comparison could be done on CED and WZB only. Cryoconite collected in cryoconite holes showed lower pH and higher TOC values than the sparse debris of the same glacier. In addition, both CED cryconite and sparse debris were more acidic than WZB ones, consistently with the different lithology of the surrounding rocks. Interestingly, RDA showed that cryoconite communities from the two glaciers are closer to one another, while sparse debris communities look more different (Fig. 6.6), even if this was not supported by the results of post-hoc tests, the effect of the type of sediment (either cryoconite from cryoconite holes or sparse debris) was predominant (it explained 45.9% of variance) with respect to that of glacier (3.4%). This suggests that cryoconite holes provide a peculiar microhabitat that differs from the sparse supraglacial debris. Generally, cryoconite holes are considered microhabitats where many microorganisms can find protection from harsh wind, extremely cold temperatures and high UV radiation that characterize the glacier environment (Takeuchi et al. 2000). These conditions allow the establishment and development of bacterial communities typical of these microhabitats, which increase the organic carbon content of the sediment and decrease pH. This may explain the similarity between bacterial communities of cryoconite holes of these nearby glaciers, which however largely differ for the lithology of the surrounding mountains. Furthermore, the fact that bacterial communities of cryoconite holes of different glaciers are more similar than bacterial communities of the sparse debris, brings support to the hypothesis that these communities (the cryoconite holes ones) inhabit a microhabitat that select similar taxa and brings the communities to a climax situation. This hypothesis was already supposed in Pittino et al. (2018), where two different trends along two different ablation seasons on the same glaciers were investigated, and from the RDA biplot appears that in the late ablation season communities are more similar than in the early stages.

The higher alpha diversity and the higher evenness of cryoconite than sparse debris observed on WZB is consistent with this hypothesis. However, on CED, the opposite pattern was found. A possible explanation is that on this glacier cryoconite biodiversity is lower because of the low pH of cryoconite samples (< 6). Alpha diversity was also generally higher on WZB than on CED. However, on CED alpha diversity was higher in the supraglacial debris, while on WZB it was higher in cryoconite (Fig. 6.8). Only Cyanobacteria abundance changed between the two type of samples, and they were more abundant in cryoconite samples (Fig. S6.4), consistently with the general assumption that this

phylum is typical of cryoconite holes and a keystone taxon on these environments, as it is one of the first colonizers and the responsible of cryoconite grains formation (Takeuchi et al., 2010).

To the best of our knowledge, no studies so far compared bacterial communities between cryoconite holes and supraglacial sparse debris. Stibal et al. (2006) compared bacterial communities of cryoconite holes with those of supraglacial kames that are characterized by a thicker sediment. They found that Cyanobacteria were in the same range in both supraglacial kames and cryoconite holes, and acidic pH had a positive effect on bacteria growth. However, they concluded that cryoconite was a more suitable environment for bacteria. Another study by Stibal et al. (2012) described the bacterial communities of supraglacial environments, but without distinguishing between cryoconite holes and sparse supraglacial debris, as they investigated differences among what they called “ecological zones”: bare ice, marginal ice and slush zones. Their results showed that the ecological zone is one of the main drivers of variation in bacterial communities. The authors also hypothesized that bacterial communities may be mostly influenced by physical factors rather than chemical ones (Stibal et al. 2012b).

Our results are only partly consistent with those of the above mentioned studies because we did not find a generally higher biodiversity in cryoconite holes than in sparse debris, but opposite patterns in the two glaciers. However, the debris considered in the 2006 study by Stibal and colleagues was rather different than the sparse supraglacial debris we investigated in this study while the 2012 study did not separate cryoconite holes from other types of debris. In addition, we found similar communities in the cryoconite holes of the two glaciers, and both communities differed from those of the supraglacial debris of the respective glacier, which also differed to one another. This in only partly consistent with our hypothesis that cryoconite released from cryoconite holes dismantled by ablation contributes to the bacterial communities of sparse debris, because a stronger similarity among cryoconite and sparse debris bacterial communities was expected in that case. Supraglacial sparse debris seems therefore to host more diverse bacterial communities, largely different from glacier to glacier, also within the same small geographic area. These communities may derive partly from long-range transport, but also from the surrounding environments, and therefore be affected by the lithology of the glacier bedrock and of the surroundings, which also affects sediment glacier lithology. In contrast, cryoconite hole environment seems to favour typical environmental communities that benefit from the presence of liquid water and more protected conditions. For instance, these communities are enriched in Cyanobacteria, which produce organic matter and are able to increase TOC content and maybe, decrease pH of cryoconite.

6.5. Conclusions

Bacterial communities of the supraglacial sparse debris that covers almost all glacier surface seem strongly variable and may be linked to the characteristic of the surrounding environment, including lithology. Cryoconite and supraglacial sparse debris host different bacterial communities. In particular, cryoconite samples, even from different glaciers, host bacterial communities that are more similar to one another than those of sparse debris, likely because of the peculiar feature of cryoconite hole microhabitat. Our hypothesis that bacterial communities of cryoconite holes affects those of the sparse supraglacial debris because they are released on the glacier surface when the holes are dismantled from the ablation found therefore little support. In contrast, we can confirm that different glaciers host different bacterial communities in their supraglacial debris. We must stress that we investigated and quantified biodiversity by sequencing the rDNA genes, so we could not separate active from inactive bacteria in the communities we studied. However, our results suggest that supraglacial sparse debris can host an even larger biodiversity than cryoconite holes. Glacier bacterial biodiversity can therefore be much larger than that of cryoconite holes, which are considered hotspots of biodiversity on glaciers. In a period of global warming, when glaciers are quickly disappearing, we may therefore be losing a larger amount of biodiversity than previously considered.

6.6. Supplementary material

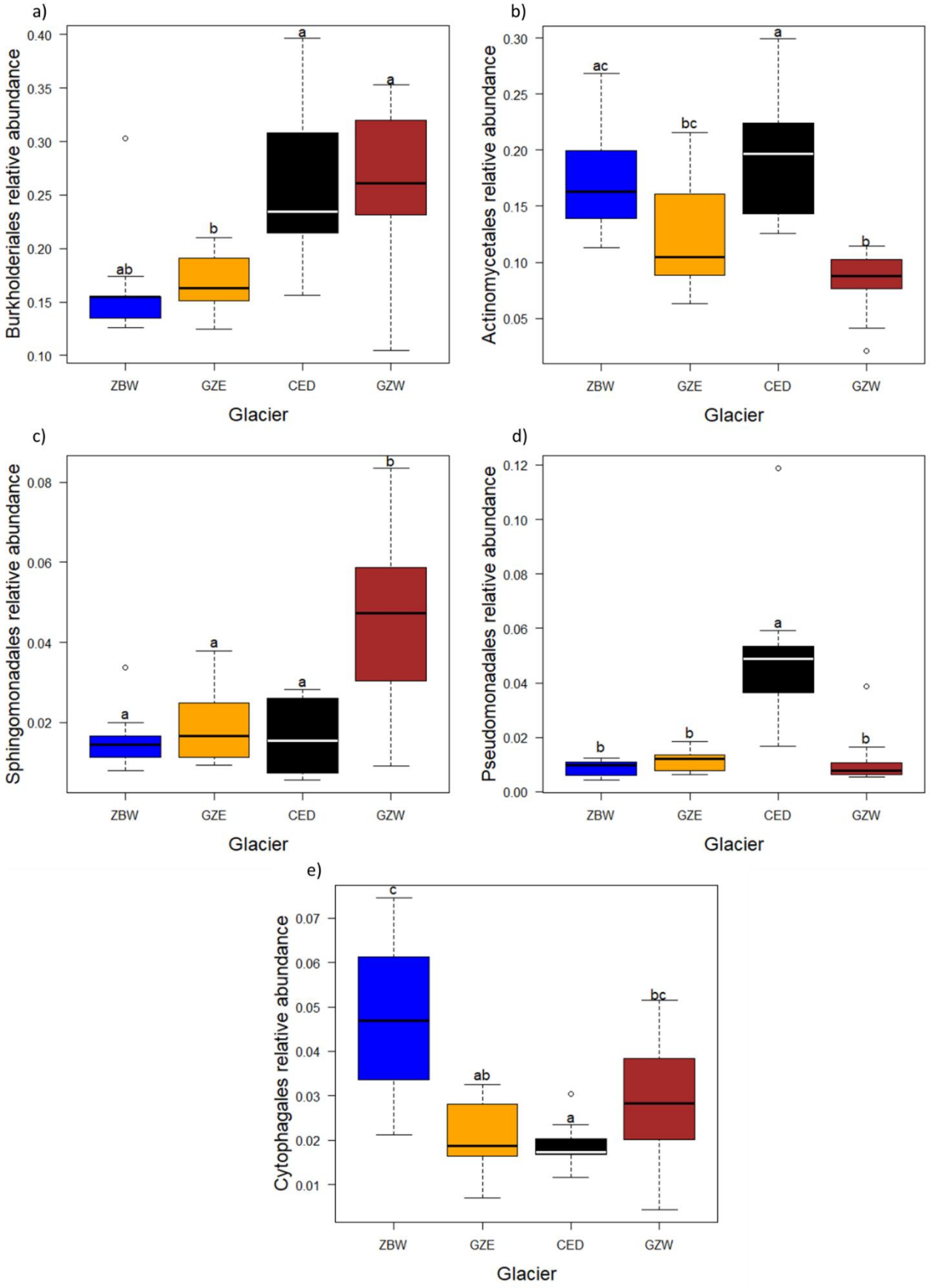

Figure S6.1 Boxplots of the relative abundances of Burkholderiales (a), Actinomycetales (b), Sphingomonadales (c), Pseudomonadales (d) and Cytophagales (e) on the four glaciers. The thick lines represent the median, boxes upper and lower limits the 25th and the 75th percentiles respectively, whiskers the data that go beyond the 5th percentile (lower whisker) and the 75th percentile (upper whisker), dots represent the outliers and different letters indicate differences between the mean values of different groups.

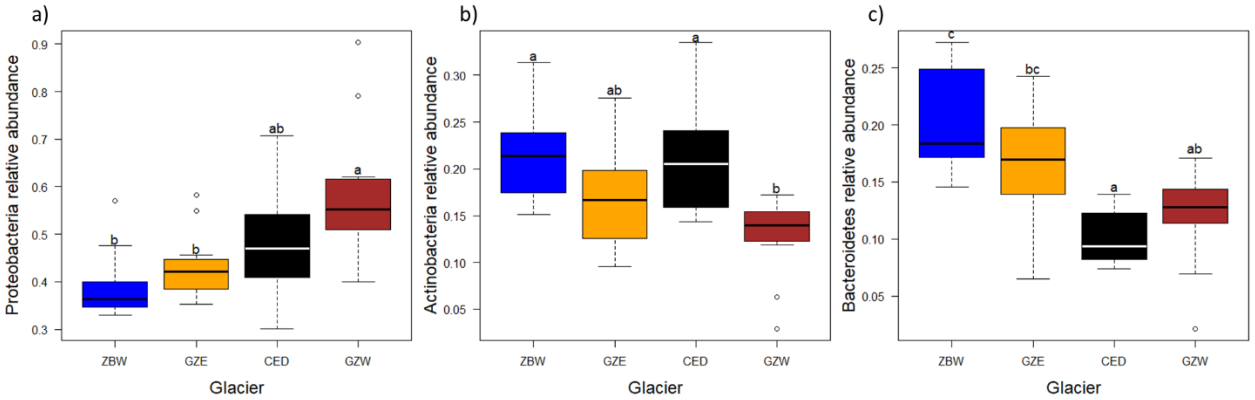

Figure S6.2 Boxplots showing differences in relative abundances of Proteobacteria (a), Actinobacteria (b) and Bacteroidetes (c) on the four different glaciers. The thick lines represent the median, boxes upper and lower limits the 25th and the 75th percentiles respectively, whiskers the data that go beyond the 5th percentile (lower whisker) and the 75th percentile (upper whisker), dots represent the outliers and different letters indicate differences between the mean values of different groups.

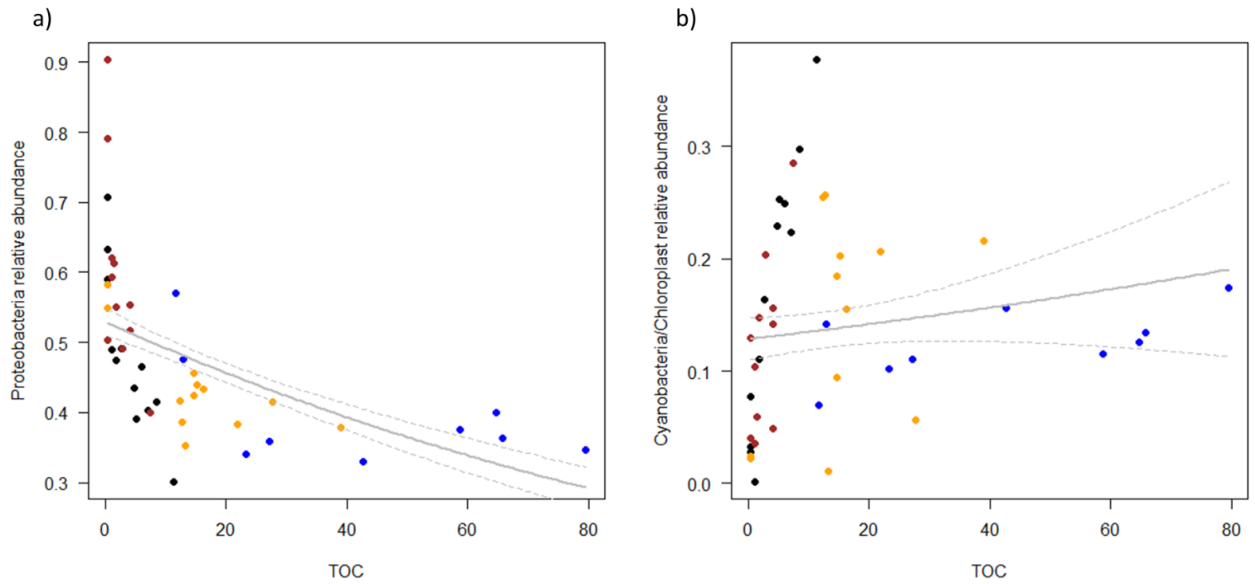

Figure S6.3 Biplot showing how Cyanobacteria (a) and Proteobacteria (b) relative abundances change with TOC. Different colours represent samples from the different glaciers: CED (black), ZBW (blue), GZW (brown) and GZE (orange).

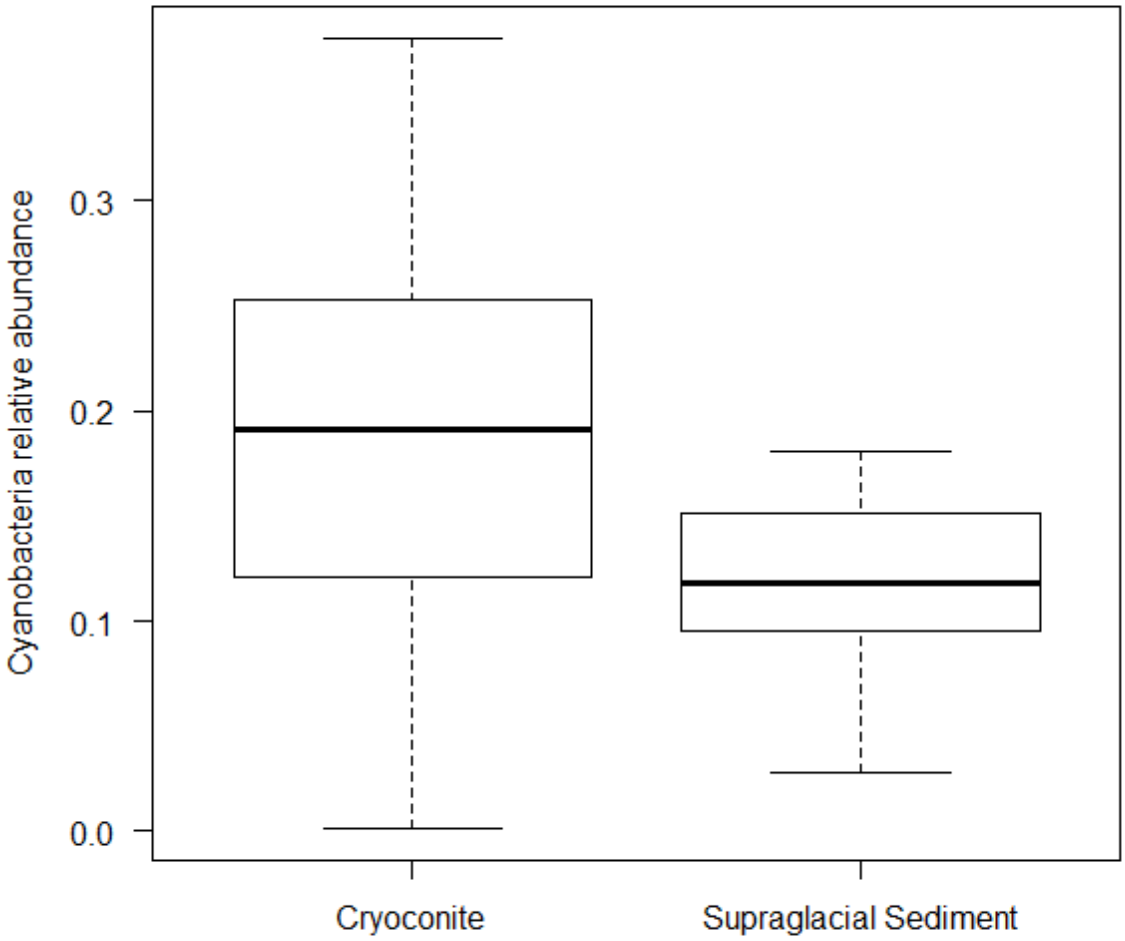

Figure S6.4 Boxplot representing the different relative abundance of Cyanobacteria in cryoconite and supraglacial sediment. The thick lines represent the median, boxes upper and lower limits the 25th and the 75th percentiles respectively, whiskers the data that go beyond the 5th percentile (lower whisker) and the 75th percentile (upper whisker), dots represent the outliers.

7. GEOGRAPHICAL VARIABILITY OF BACTERIAL COMMUNITIES OF CRYOCONITE HOLES OF ANDEAN GLACIERS IN CHILE AND ARGENTINA

ABSTRACT Cryoconite holes, ponds full of melting water with a sediment on the bottom, are hotspot of biodiversity of glacier surface. They host a metabolically active bacterial community that is involved in different dynamics concerning glacier ecosystems. Indeed, they are responsible of organic matter production and with other microorganisms establish a real microecosystem. Cryoconite holes have been described in different areas of the world (*e.g.*, Arctic, Antarctic, Alps, Himalaya), and with this study we will provide the first description of bacterial communities of cryoconite holes of the Andes in South America. We collected samples on three high elevation glaciers of the Andes (Iver, Iver East and Morado glaciers) and two Patagonian glaciers located at sea level (Exploradores glacier and Perito Moreno). Results show that the most abundant orders are Burkholderiales, Cytophagales, Sphingobacteriales, Actinomycetales, Pseudomonadales, Rhodospiarillales, Rhizobiales, Sphingomonadales and Bacteroidales, which have been reported on glaciers of other areas of the world, Bacterial communities change from one glacier to another and both water pH and O_2 concentration affect bacterial communities composition.

7.1. Introduction

Glaciers and ice sheets have been recognized as a biome in their own right as they host viable populations of organisms (Anesio et al. 2012). Glacier ecosystems are mostly dominated by microorganisms that have been found in all parts of the glacier environment, including the englacial and the subglacial zones (Anesio et al. 2012). The supraglacial zone is the most biodiverse among glacier ecosystems (Hodson et al. 2008) and, for practical reasons, is also the most studied. Here, small depressions of the glacier surface filled by meltwater, the cryoconite holes, form as a consequence of the atmospheric deposition of a fine-grained sediment, cryoconite, that locally decreases the albedo and melts the underlying ice. The main source of this sediment is the surrounding environment, but a small part of it is deposited after long-range atmospheric transport (Franzetti et al. 2017a). In the extreme glacier environment, cryoconite holes are protected habitats where general conditions are more favourable for life than in the other parts of a glacier, as they provide liquid water and act as a protective barrier against the intense UV radiation characteristic of the glacier environment (McIntyre 1984). At low temperature, an ice lid can form on the water surface in a cryoconite hole, which further protects this microhabitat. In fact, underneath this cover, water remains liquid because solar radiation can penetrate the ice lid and be absorbed by the dark cryoconite; only in case of extremely cold temperatures the water inside the hole completely freezes (Fountain et al. 2004a).

Cryoconite holes are present on glaciers in almost all areas of the world; so far they have been described in the Arctic, Antarctica, on the Alps, in Tien Shan and in Himalaya (Takeuchi, et al. 2001;Mueller et al. 2004; Telling et al. 2010; Uetake et al. 2016; Franzetti et al. 2017a). However,

they do not show the same features in all the different geographic areas where they occur. Polar cryoconite holes, in particular, are more stable than those that form on temperate mountain glaciers. On Himalayan glaciers they can sometimes survive more than one year, but this does not always occur (Takeuchi et al. 2000; Takeuchi et al. 2010). Indeed, in most temperate mountain glaciers they are mostly ephemeral structures that can be destroyed and form again because of the strong ablation that can quickly dismantle them and wash away the sediment and the intense solar radiation that can form new holes in a few days (Fountain et al. 2004a; Cook et al. 2016b; Pittino et al. 2018b).

Communities inhabiting these microhabitats also differ between polar and temperate mountain cryoconite holes. In polar environments bacterial communities in cryoconite holes are quite stable (Musilova et al. 2015), while on the Alps, the only temperate mountain range where ecological successions studies have been conducted on cryoconite holes, they change along the ablation season (Franzetti et al. 2017b; Pittino et al. 2018b). In particular, filamentous Cyanobacteria and other phototrophs are the first colonizers of cryoconite holes after their formation at the beginning of the ablation season, while at its end bacterial communities are mostly heterotrophic (Franzetti et al. 2017b). Such a difference between stable polar and seasonally varying temperate cryoconite hole communities may be due to the more stable temperatures along the ablation season in the polar regions. Indeed Pittino et al. (2018) reported an effect of temperature variation along the ablation season on bacterial communities composition on an Alpine glacier.

However, investigations of the temporal variability of cryoconite hole bacterial communities of the same glacier along one or even more ablation seasons were seldom conducted, mostly because of the difficulties and the costs of visiting repeatedly glaciers, which usually occur in remote areas. Most often, the description of the bacterial communities of cryoconite holes is conducted with snapshot studies (Edwards et al. 2013; Stibal et al. 2006; Hodson et al. 2010), which allowed a general description of their biotic communities, even if they cannot include the whole biodiversity of these environments (Franzetti et al. 2017b; Pittino et al. 2018b). These studies showed that filamentous Cyanobacteria are the main responsible of cryoconite grain formation (Takeuchi et al. 2001; Stauch-White et al. 2017; Uetake et al. 2016) and the other most abundant bacterial phyla found in these microhabitats are Proteobacteria (Alfa and Beta), Actinobacteria, Chloroflexi, Acidobacteria and Bacteroidetes (Boetius et al. 2015; Liu et al. 2017; Christner et al. 2003; Franzetti et al. 2016). At order level instead we can mention: Sphingobacteriales, Pseudomonadales, Rhodospirillales, Burkholderiales and Clostridiales (Franzetti et al. 2017b; Kaczmarek et al. 2015). So

far, snapshot studies have been used to describe bacterial communities inhabiting cryoconite holes on glaciers in the Alps (Edwards et al. 2013; Franzetti et al. 2017a), Greenland (Musilova et al. 2015; Uetake et al. 2016), the Arctic (Singh et al. 2014; Stibalet al. 2006), Antarctica (Sommers et al. 2018; Christner et al. 2003), Himalaya (Sanyal et al. 2018), Karakoram (Ambrosini et al. 2017) and China (Takeuchi et al. 2001).

To the best of our knowledge, no published information is currently available about cryoconite bacterial communities from South American glaciers. Only one study by Tacheuki et al. (2001) described cryoconite characteristics of the Tyndall Glacier, but from a physico-chemical point of view, not reporting bacterial communities composition. A study reported some data about bacterial communities of glaciers surface describing the gut microbiome of the glacier stonefly (*Andiperla willinki*) that is likely to feed on supraglacial bacteria (Murakami et al. 2018), but no more studies report bacteria inhabiting this environment in South America.

The aim of this work is to provide a first description and an evaluation of the geographical variability of bacterial communities of cryoconite holes of South America, based on samples collected on four Chilean and one Argentinian glaciers. Cryoconite samples were collected from five different glacier of the South American Continent (Fig.7.1). Three glaciers are located on the Chilean Andes close to Santiago de Chile (Morado, Iver and East Iver glaciers). Two other glaciers (Exploradores Glacier in Chilean Patagonia and Perito Moreno in Argentinian Patagonia) are extremely different compared to the first three mainly due to the different climate setting of the area where are located (Pfeffer et al. 2014).

Despite this study was based on snapshot sampling of five glaciers, we are confident that our results can help filling this gap of knowledge on the glacier biodiversity of South America.

7.2. Materials and methods

7.2.1. Study areas and sample collection

Morado Glacier is a valley glacier located about 60 kilometres South East from Santiago, Chile. It covers 1.1 km^2 and its altitudinal range is between 4604 m a.s.l. and 3535 m a.s.l. It is a small glacier, highly crevassed and partly debris-covered in the ablation area, terminating in a small lake.

Iver Glacier can be classified as a mountain ice body and is located about 30 kilometres North West from Santiago, Chile. It has a surface area similar to Morado, covering 1.64 km^2 and its altitudinal range is between 5358 m a.s.l. and 4296 m a.s.l. It is a steep glacier, partly covered in the ablation area. During the Little Ice Age, this glacier was probably connected to East Iver Glacier that is a small glacier covering 0.3 km^2 located few hundred meters from the Iver Glacier.

Exploradores Glacier is a valley glacier located in the Chilean Patagonia. It covers 85 km^2, it is 19 km long and its altitudinal range is between 3735 m a.s.l. and 158 m a.s.l. The frontal area of the ablation zone is debris-covered.

Perito Moreno Glacier is a valley glacier located in the Argentinian Patagonia. It covers 263 km^2, it is more than 32 km long and its altitudinal range is between 2800 m a.s.l. and 190 m a.s.l. where the glacier snout ends in a lake, losing most of its mass through calving processes.

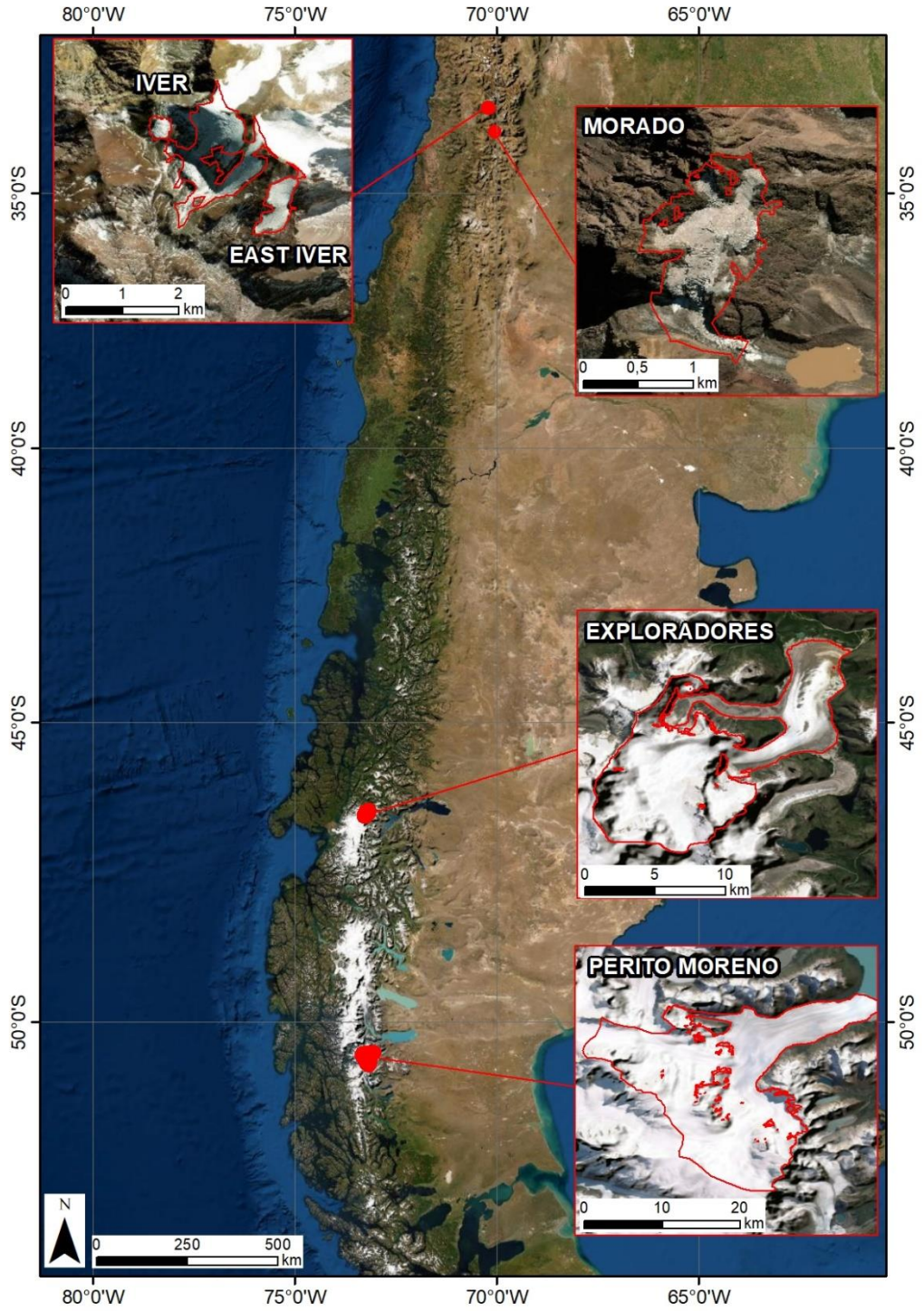

Figure 7.1 Location Map of Andean glaciers located in Chile and Argentina analysed in this report. Glacier limit and morphological parameters are obtained from Randolph Glacier Inventory 6.0 (RGI Consortium 2017).

Cryoconite samples were aseptically collected in falcon tubes on five glaciers in 2017 and 2018 (Table 7.1). We collected samples from 15 cryoconite holes per glacier and, at each hole, we also recorded dissolved O_2 concentration and pH with a portable oximeter/pH meter (HACH LANGE HQ40D, Loveland, CO, USA).

Table 7.2. Date of sampling of each glacier.

Glacier	Date
Morado	18/02/2018
Iver	21/02/2018
East Iver	22/02/2018
Exploradores	01/03/2018
Perito Moreno	29-30/03/2017

7.2.2. DNA extraction and sequencing

DNA was extracted from 0.7 g of cryoconite with the FastDNA® Spin for Soil kit (MP Biomedicals, Solon, OH, USA) according to the manufacturer's instructions. DNA sequencing was performed on the V5-V6 hypervariable region of the 16S rRNA gene as previously described in Pittino et al. (2018b). Sequences were then demultiplexed according to the indexes and clustered in Amplicon Sequences Variants (ASVs) with DADA2 (Callahan et al. 2016). ASVs were then taxonomically classified using rdp classifier (Wang et al. 2007) keeping the full classification only of the taxa attributed with a confidence of 0.8 or higher. Cyanobacteria were classified at phylum level only as rdp classifier does not provide the full classification for them (Garrity et al. 2007; Wilmotte et al. 2015).

7.2.3. Statistical analyses

Analyses were performed with R 3.5.1 (R Core Team, 2014) with the VEGAN, BIODIVERSITYR, MULTTEST, and MULTCOMP packages. Singletons (ASVs present once in one sample only) were removed because they can inflate the variance explained by multivariate tests (Borcard et al. 2011) Alpha-diversity was measured using the Shannon diversity index, which accounts for both the richness and the evenness of the species (Shannon 1948), and the Gini inequality index, which is an index of inhomogeneity largely used in economics (Gini 1912). Gini index ranges from 0 to 1 and low values indicate an homogeneous distribution of the species in the community, while high values indicate an heterogeneous distribution (Gini 1912).

Beta diversity analyses were based on the Hellinger distance, which depends on the differences in the ASV proportion among samples, decreases the importance of ASV abundance over occurrence and avoids the double-zero problem when comparing ASV composition among samples (De Cáceres et al. 2010; Legendre al. 2001). We performed a redundancy analysis (RDA) and a variation partitioning (VP) to quantify the variation of community structures among glaciers (five-level factor), and according to dissolved oxygen concentration and pH of the water above the sediment. The last two variables were mean-centred within glacier before the analyses (i.e. we subtracted from the values recorded at each glacier their mean value). Hereafter, centred variables will be called ΔpH

and $\Delta[O_2]$, respectively. Post-hoc tests were also performed to assess pairwise differences between glaciers while correcting P-values for multiple testing according to the false discovery rate (FDR) procedure (Benjamini et al. 2001). Variation in the abundance of the 9 most abundant orders according to the variables that significantly affected the structure of bacterial communities identified by the RDAs was investigated by generalized linear models (GLMs) assuming a Poisson distribution and correcting for overdispersion. Also in these cases, P-values were corrected using the same FDR procedure as above. Generalized least-square (GLS) models accounting for heterogeneity of variance among glaciers were also used to investigate changes in oxygen concentration, pH and alpha diversity indices according to the same predictors. No deviation from normality assumption was detected during routine model checks (details not shown).

7.3. Results

Both pH ($F_{4,70}$ = 11.87, P = < 0.001) and oxygen concentration ($F_{4,70}$ = 281.9, P < 0.01) in cryoconite holes differed significantly among glaciers. Post-hoc tests highlighted that glaciers could be divided into two groups with respect of pH values, with Exploradores and Morado showing higher values than East Iver and Perito Moreno ($|t_{70}| \geq 0.147$, $P \leq 0.035$; Fig .7.2a), while all glaciers differed to one another in their oxygen concentration ($|t_{70}| \geq 3.480$, $P \leq 0.011$; Fig. 7.2b).

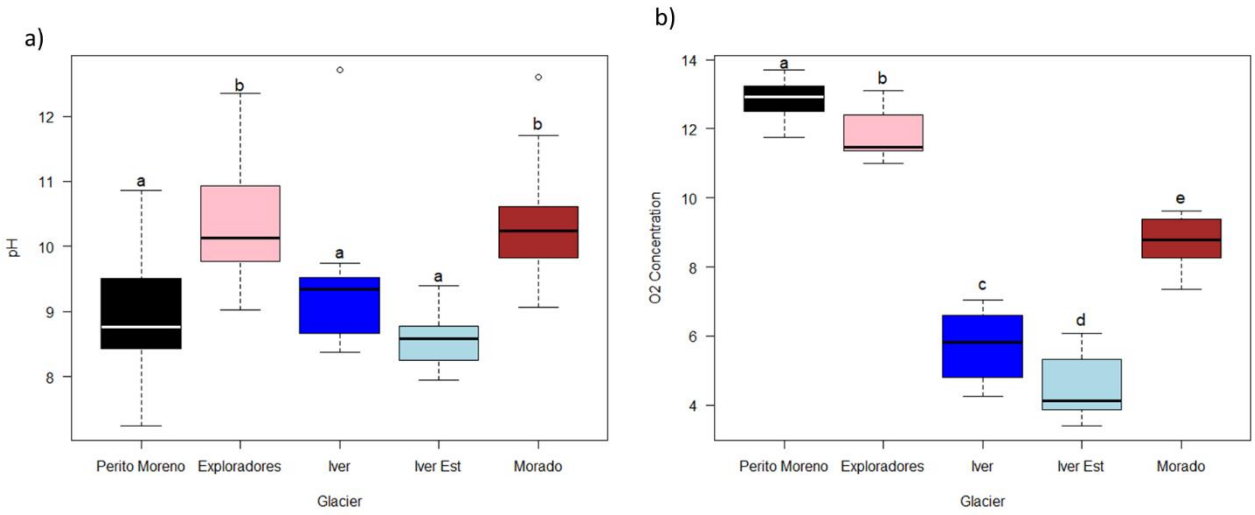

Figure 7.2 Boxplots of pH (a) and oxygen concentration (b) of cryoconite holes in the investigated South American glaciers. The thick lines represent the median, boxes upper and lower limits the 25th and the 75th percentiles respectively, whiskers the data that go beyond the 5th and the 75th percentile, dots represent the outliers and different letters indicate significant differences between glaciers at post-hoc tests.

We obtained 7,600 - 93,425 sequences per sample. Orders with more than 24,000 sequences were considered as the most abundant. They were: *Burkholderiales, Cytophagales, Sphingobacteriales, Actinomycetales, Pseudomonadales, Rhodospiarillales, Rhizobiales, Sphingomonadales* and *Bacteroidales.*

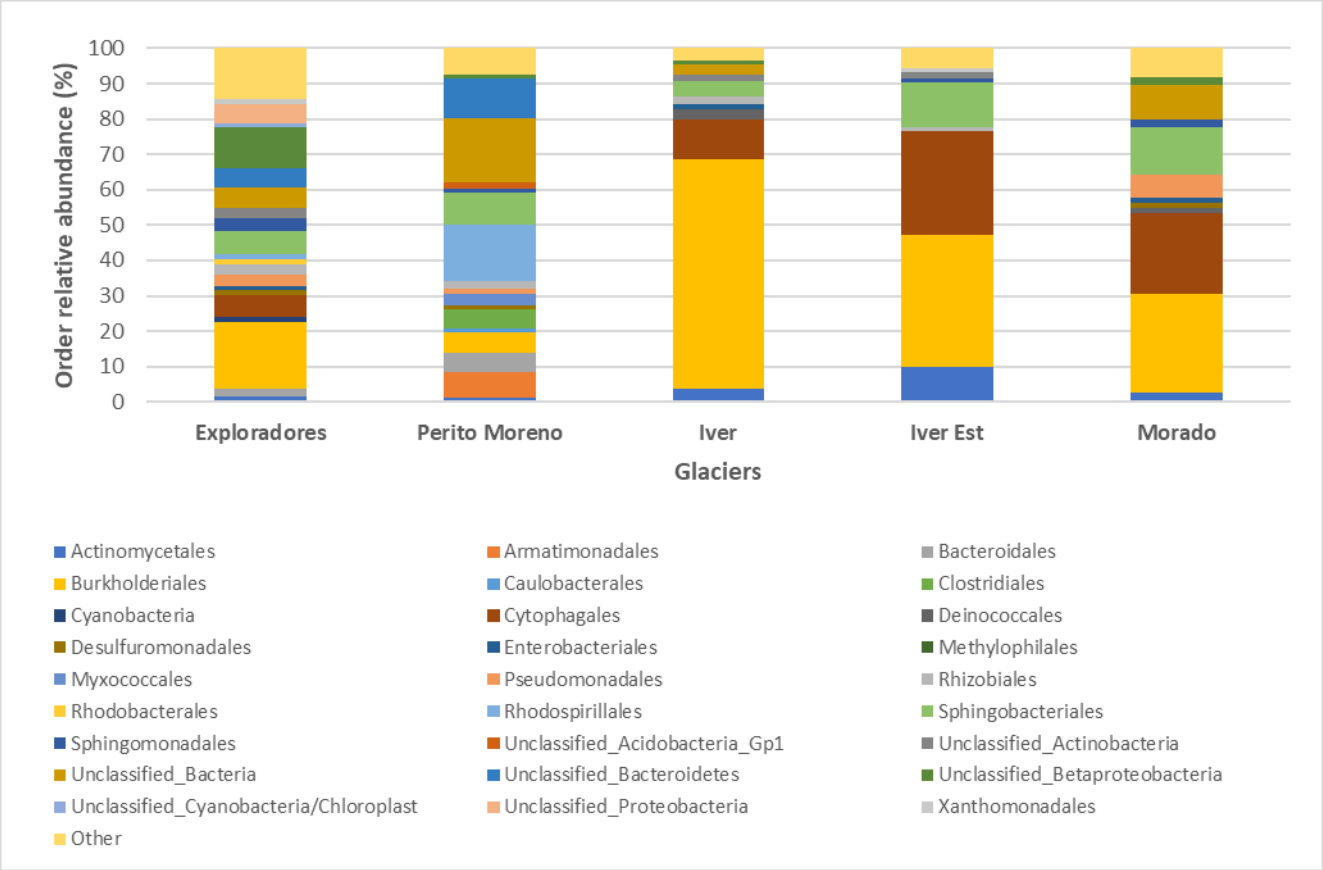

Figure 7.3 Barplot showing the relative abundance of bacterial orders in the cryoconite holes on each glacier. Orders representing less than 1 % of sequences in each sample were grouped in "others". Cyanobacteria are reported as phylum because the rdp classifier does not provide their classification at order level.

The RDA performed on all the samples, showed that bacterial communities differed significantly among glaciers and varied according to ΔpH and $\Delta[O_2]$ (Table 7.2; Fig. 7.4). The biplot also showed that cryoconite hole bacterial communities of the three small glaciers in central Chilean Andes (Iver, East Iver and Morado) seem to vary mostly according to oxygen concentration, while those of the Exploradores glacier seem to be affected by pH.

Table 7.2 RDA of Hellinger-transformed bacterial ASVs abundances of all the samples according to the glacier, pH and oxygen concentration centred according to their mean value per glacier.

Variable	Df	Variance	F	P
Glacier	4	0.445	38.15	0.001
ΔpH	1	0.006	2.110	0.010
$\Delta[O_2]$	1	0.006	2.204	0.001
Residuals	62	0.181		
$F_{6,62}$ = 26.105, P = 0.001, R^2 Adjusted = 0.689				

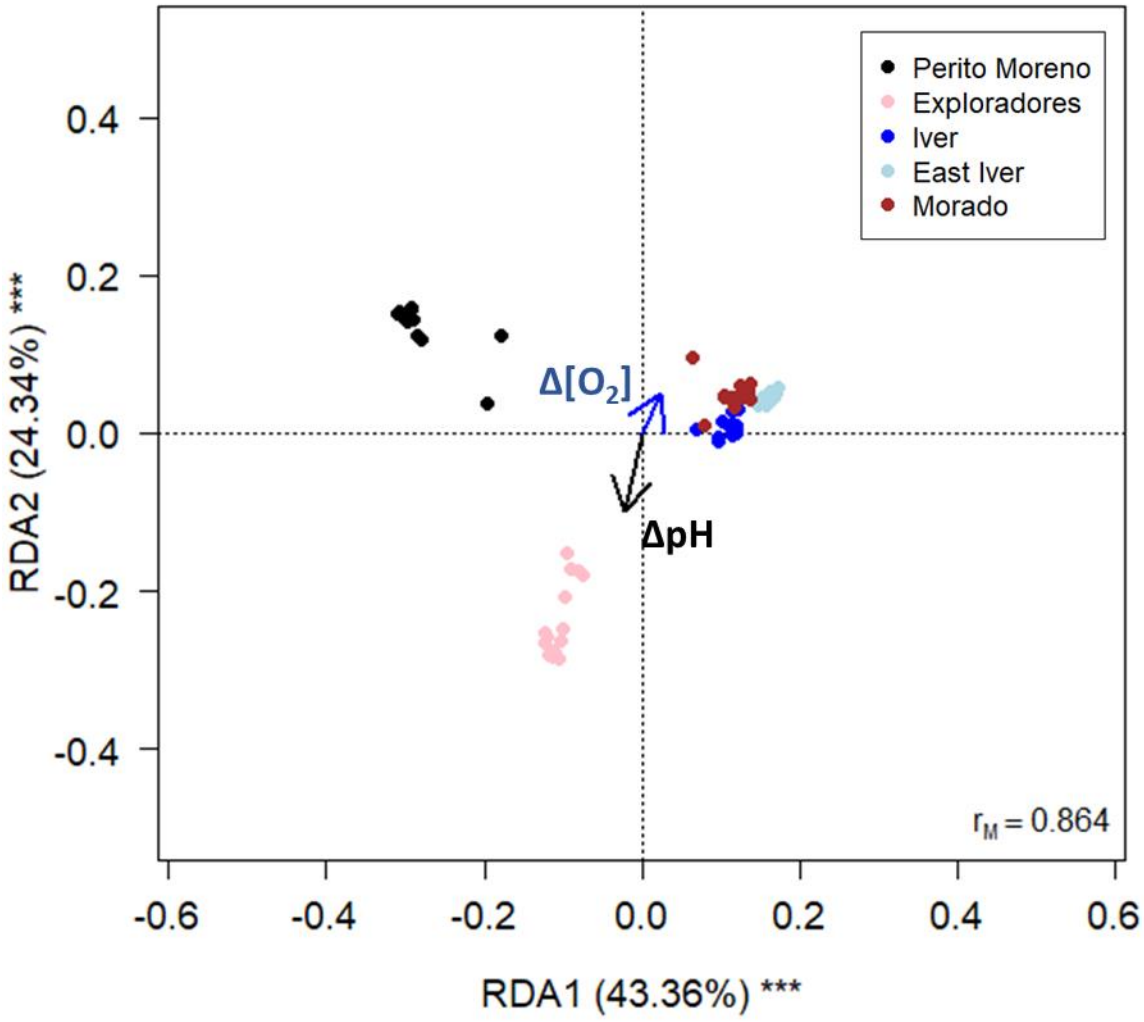

*Figure 7.4 Correlation biplot from RDA on Hellinger-transformed bacterial ASVs abundances of all the samples according to glacier (five level factor), pH and oxygen concentration centred to their mean value per glacier. The blue arrow indicates increasing values of oxygen concentration in each glacier, while the black one increasing pH values in each glacier. The percentage of variance explained by each axis and their significance (***:P < 0.001) is reported. r_M is the Mantel correlation coefficient between the Hellinger distances between samples and the Euclidean distances between the corresponding symbols in the graph. Values close to one indicate that the graph correctly represents the distance between samples.*

Post-hoc tests also revealed that the structure of the bacterial communities of all the five glaciers were significantly different from one another ($|t_{62}| \geq 19.64$, $P_{FDR} \leq 0.003$).

GLSs showed that both the alpha diversity indexes varied significantly among glaciers (Shannon index: $F_{4,73} = 42.147$, $P < 0.001$; Gini index: $F_{4,73} = 33.607$, $P < 0.001$). Post-hoc tests showed that Shannon index was higher in Exploradores samples and lower in Iver and East Iver ones, while Gini index was higher in Iver and in East Iver samples and lower in Exploradores ones (Figure 7.5).

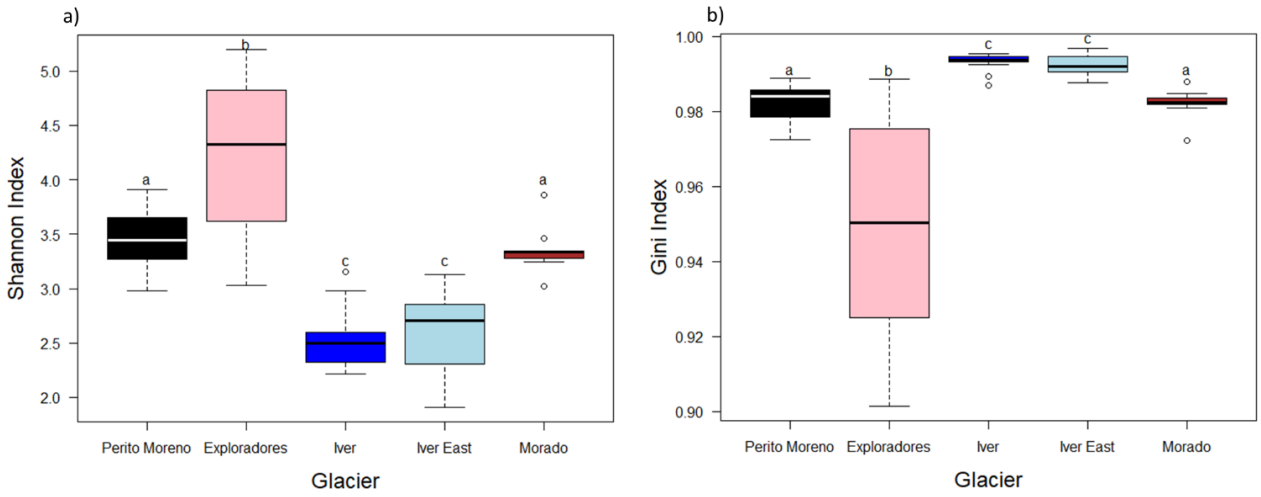

Figure 7.5 Boxplots of Shannon (a) and Gini (b) diversity indices of cryoconite hole bacterial communities. The thick lines represent the median, boxes upper and lower limits the 25th and the 75th percentiles respectively, whiskers the data that go beyond the 5th and the 75th percentile (upper whisker), dots represent the outliers and different letters indicate significant differences at post-hoc tests.

GLMs performed on the most abundant orders showed that the abundances of Burkholderiales, Cytophagales, Sphingobacteriales, Actinomycetales, Rhodospirillales, Rhizobiales, Pseudomonadales, Sphingomonadales and Bacteroidales varied among glaciers ($F_{4,64} \geq 4.207$, $P_{FDR} < 0.010$), with complex patterns of variation. The most abundant orders varied according to the glacier without showing any particular pattern, Iver was the glacier with the highest relative abundance of Burkholderiales, Morado the glacier with the highest relative abundance of Pseudomonadales and Perito Moreno had more Rhodospirillales (Figure 7.6).

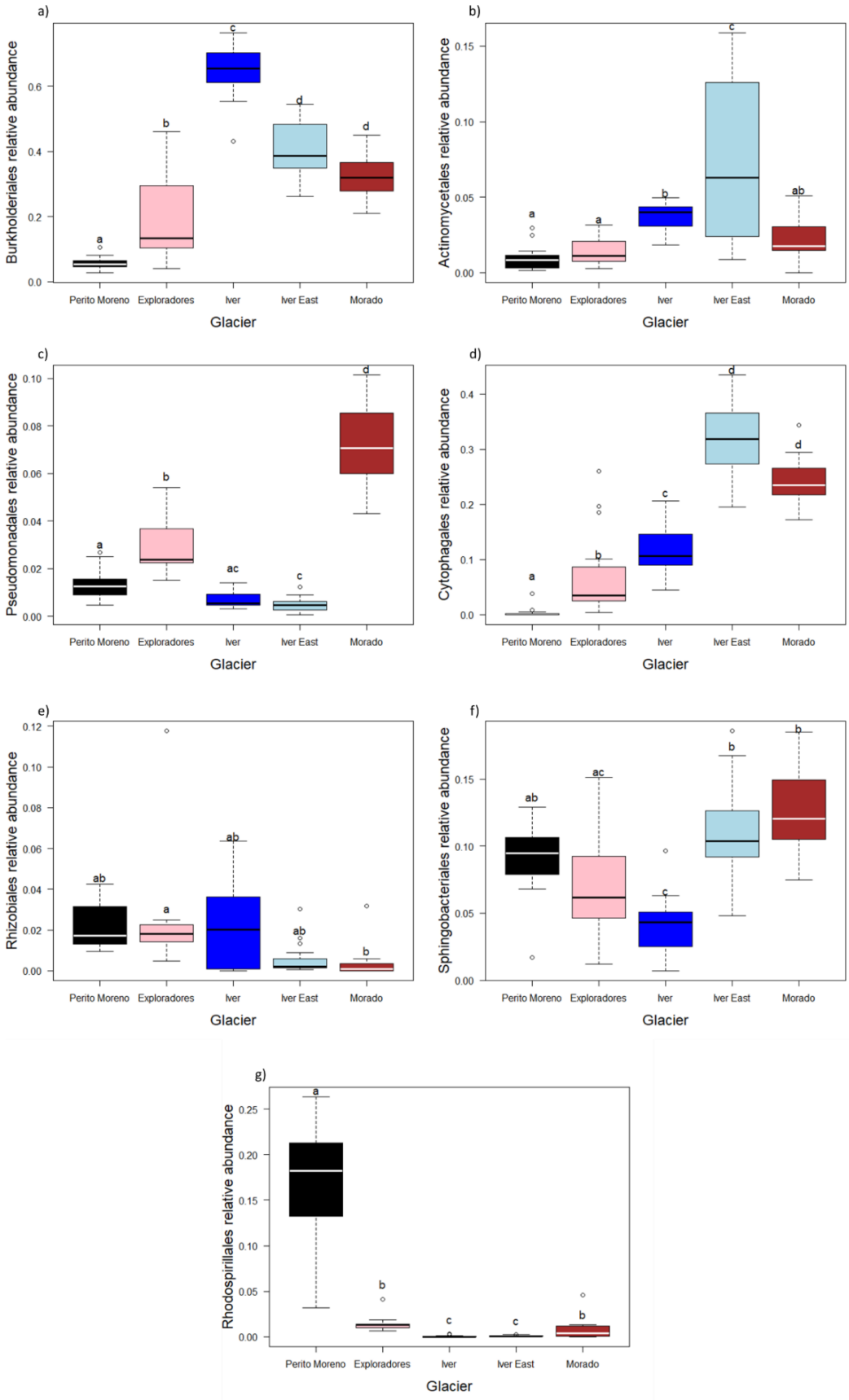

Figure 7.6 Boxplots of the relative abundances of Burkholderiales (a), Actinomycetales (b), Pseudomonadales (c), Cytophagales (d), Rhizobiales (e), Sphingobacteriales (f), Bacteroidales (g), Sphingomonadales (h) and Rhodospirillales (i) on the five glaciers where we collected cryoconite samples. The thick lines represent the median, boxes upper and lower limits the 25th and the 75th percentiles

respectively, whiskers the data that go beyond the 5th percentile (lower whisker) and the 75th percentile (upper whisker), dots represent the outliers and different letters indicate differences between the mean values of different groups

7.4. Discussion

In this study, we provide the first description of the bacterial communities of cryoconite holes from Andean glaciers, in particular from both small high elevation glaciers in the Santiago Metropolitan Region (Chile), and from the tongue of large Patagonian glaciers that reach low altitudes. Indeed, there is a big gap in our knowledge of these environments, that is the lack of data about microbial communities in supraglacial environments in this continent, that hosts about 30000 km^2 covered by ice corresponding to about 5% of the glacierized area of the whole planet outside the Antarctica (Pfeffer et al. 2014). Results showed that the large Patagonian glaciers (Exploradores and Perito Moreno) were the two glaciers with the highest oxygen concentrations, while Iver and East Iver had the lowest ones and Morado an intermediate value. This pattern seems related to the different altitude of the glaciers. Jacobsen et al. (2003) calculated that at 4000 m a. s. l. oxygen availability in water is one fifth of that at sea level also because the kinematic viscosity of water plays an important role and depends on the elevation. This result is consistent with $[O_2]$ values we found in our samples. Indeed, Exploradores and Perito Moreno are located in Patagonia at low altitude (< 200 m a.s.l.), while Iver and East Iver are the highest ones among those we investigated (samples were collected at about 4000 m a.s.l.) and Morado at an intermediate value (3400 m a.s.l.).

pH values seem to follow a different pattern: the highest values were recorded on Exploradores and Morado, while the lowest were recorded on the East Iver and Iver. However, all the mean pH values of the five glaciers were basic (between 8.57 and 10.47). Differences in pH among glaciers are not easy to explain and may be due to differences in lithology of the surrounding environments that act as source of cryoconite. We note, however, that we measured water pH rather than that of the cryoconite, because the first one was already reported to influence bacterial communities in cryoconite holes (Edwards et al. 2011; Ambrosini et al. 2017). However, water pH may also be partially affected by the metabolic activities of the same bacterial communities of cryoconite holes, so it is still unclear whether water pH affects cryoconite hole bacterial communities or in part also vice versa (Hu and Cai 2011).

The most abundant orders were (in decreasing order): Burkholderiales, Cytophagales, Sphingobacteriales, Actinomycetales, Pseudomonadales, Rhodospirillales, Rhizobiales and Sphingomonadales. These orders are typical of cryoconite holes and dominate bacterial communities in these environments in all the geographical areas investigated so far: Arctic (Cameron et al. 2012; Liu et al. 2017), Antarctica (Christner et al. 2003; Sommers et al. 2018), Europe

(Edwards et al. 2013; Pittino et al. 2018b), Asia (Segawa et al. 2014; Liu et al. 2017). Cryoconite holes of Andean glaciers seem therefore to host bacterial communities that are quite typical of these biodiversity hot spots on glacier environments worldwide.

Despite this general similarity of the dominant orders of bacterial communities at a global scale, a closer inspection at ASV level revealed that the glacier bacterial communities of the different glaciers differed to one another. In addition, the RDA biplot showed that the three small and high elevation glaciers clustered quite close to one another, whereas Perito Moreno and Exploradores were separate from one other and from the other glaciers. This pattern may be due to the altitude, geographic distance and ecological and environmental differences among glaciers. Indeed, bacterial communities in high elevation glaciers are exposed to high UV radiation and, therefore, to high oxidative stress (Orellana et al. 2018; Margesin et al. 2019) and different vegetation cover (Florinsky et al. 1996). Patagonian glaciers are 440 km to one another and c.ca 2000 km from the other glaciers we sampled, which, in turn, are less than 60 km to one another. In addition, it has been demonstrated that the main source of cryoconite bacterial communities is the local environment surrounding the glacier (Stibal et al. 2015; Franzetti et al. 2017a). The high elevation glaciers we sampled are small and above the three line, and generally in areas with few vegetation. In contrast, the Patagonian glaciers where we collected are huge and their tongues (where we collected the samples) is well below the tree line and therefore exposed to very different potential sources of microorganisms with respect of the other glaciers.

The RDA also showed that ΔpH and $\Delta[O_2]$ significantly explained the variability of bacterial communities of cryoconite holes. We stress that these variables represent the difference between pH and oxygen concentration of each cryoconite hole from the respective mean value of all the holes of that glacier. They therefore do not account for the difference in mean pH and oxygen concentration highlighted in the analyses discussed above, whose effect in this analysis is accounted for by the glacier factor, which accounts for any difference between glaciers. Thus, this analysis suggests that consistent bacterial communities vary consistently according to pH and oxygen concentration gradients present on each glacier, even if the different glaciers show on average different pH and oxygen concentration values. In other words, for example, an increase of two pH units seems to determine a similar variation in bacterial communities independently from the absolute pH value, at least in the range of variability of pH values recorded in this study. In addition, no effect of ΔpH on alpha diversity resulted from GLSs. Different studies already investigated the effect of both water and sediment pH on bacterial communities composition, proving that it

influences the community in river's sediment of different typology (i.e. glacier-fed streams, large rivers, riverine wetlands) (Shen et al. 2013; Wilhelm et al. 2013; Ligi et al. 2014; Liu et al. 2015). Nonetheless, so far the only evidence is the decrease of Acidobacteria at basic values of pH (Chu et al. 2011; Shen et al. 2013; Wilhelm et al. 2013; S. Liu et al. 2015), but in our case this effect is not evident since pH values of our samples varied from 7.25 to 12.71.

Cryoconite holes are mostly aerobic environments even in presence of an ice lid, thanks to the high O_2 solubility in cold environments, to the release of air bubbles from the ice that melts because of the presence of the dark sediment and to photosynthesis (Zdanowski et al. 2017a; Telling et al. 2012). On these glaciers, we observed on average lower oxygen concentrations in high altitude cryoconite holes probably because water oxygen depends on the equilibrium with the atmospheric oxygen. In addition, variation in oxygen concentration seems to play an important role in explaining bacterial community structures. An effect of oxygen concentration on cryconite hole bacterial communities was already reported in previous studies on Forni glacier (Italian Alps) (Franzetti et al. 2017b). Anyway, the mechanisms that link oxygen concentration and bacterial community structure are not easy to explain, because we measured oxygen concentration in water, while a study by Poniecka et al., (2018) demonstrated that oxygen concentration into the cryoconite at the bottom of the hole is different from that in the above melting water. Indeed, the water layer works as a barrier that limits oxygen diffusion from the atmosphere to the sediment, where anoxic conditions often develop (Poniecka et al. 2018). Consistently, presence of anaerobic bacteria in cryoconite holes has been reported (Zdanowski et al. 2017a) and can be explained by the formation of anoxic niches, particularly when the sediment is thick and the water layer is deep, or within the cryoconite grains (Poniecka et al. 2018). However, measuring oxygen content in the sediment was impractical in the present study. Anyway oxygen content in the liquid phase of cryoconite holes proved to affect bacterial communities inhabiting cryoconite, even if we know that this sediment is anoxic after few micrometers of depth (Poniecka et al. 2018). Nevertheless, it was already observed an effect of dissolved oxygen concentration on bacterial communities of the intertidal biofilm in estuarine waters (Guo et al. 2017), and that bacterial communities change along a vertical profile in river's sediment because of the switch from oxic to anoxic conditions (Huang et al. 2011). In marine sediment, it was observed that going deeper in the sediment, where dissolved oxygen decreased, diversity of nitrifying bacteria decreased probably because they are facultative aerobic and in the shallowest part they have mostly an aerobic metabolism, and also because of the deposition and the consequent decomposition and transformation of organic matter that provides more nutrients

availability (Tiquia et al. 2006). Unfortunately, in our case, the sediment depth is very limited (mostly < 0.5 cm) and it is not possible to obtain the vertical distribution of bacterial taxa along the vertical profile of the sediment, like in rivers and marine sediment where the effect of oxygen absence was visible after half centimetre of few centimetres (Tiquia et al. 2006; Huang et al. 2011). Anyway, this result proved that, even if mostly anoxic, the cryoconite layer microbial community is affected by the oxygen dissolved in water. Its direct affect likely acts on the intertidal bacterial biofilm, that represents a small fraction of the whole community, so probably the effect is appreciable at the whole community level because of the interactions that subsist between different *taxa,* and going deeper in the cryoconite layer we may see an indirect effect of dissolved oxygen.

The glaciers Iver and East Iver are the two geographically closest glaciers (only 56 km apart), and they were also the most similar ones in oxygen concentration and pH (Fig. 7.2a-b) as well as in alpha diversity values and in the structure of bacterial communites, as shown by the fact that they were close to one another in the RDA biplot. Interestingly, also the Morado glacier clustered close to them in the RDA biplot, even if it is c.ca 60 km from them. In contrast, the two Patagonian glaciers are very far on the same plot. These results, on the one side, support the hypothesis that the altitude and the geographic position plays an important role in defining bacterial community composition. On the other side, however, the three small glaciers in central Andes are also at similar elevation and they are surrounded by very similar environments (Ambrosini and Pittino, personal observation). Their similarity can therefore derive also from being exposed to the same general ecological conditions including high UV radiation and oxidative stress and probably to similar sources of bacterial communities. These results therefore highlight that correlative studies like the present ones can hardly disentangle the effects of geographical positions and ecological conditions on the structure of cryoconite hole bacterial communities, and further studies should be designed to add insight into this still open question.

Analyses on alpha diversity indicate that cryoconite holes on Exploradores glacier showed the highest richness and evenness. This may be because Exploradores is located in an area surrounded by a mixed broadleaf forests, whose canopy should host rich and diverse microbiome, which can act as a source of bacterial communities. The other Patagonian glacier, Perito Moreno, despite being below the treeline in the sampled area, is surrounded by a less diverse vegetation (woods dominated by southern beeches, *Nothofagus* ssp.), which can be a less diverse source of bacteria. Indeed, the analyses showed that the alpha diversity values of cryoconite holes of this glacier did not differ significantly from those of small glaciers in central Andes, which were above the treeline.

In addition, samples on the Exploradores were collected close to the glacier terminus and in an area with abundant supraglacial debris and frequented by tourists. In contrast, Perito Moreno samples were collected farther from the glacier border and in an area with less sparse supraglacial debris, visibly cleaner and not frequented by tourists. Therefore, the surrounding forest did not affect cryoconite bacterial communities as much as on the Exploradores glacier, where samples were collected in an area closer to the glacier terminus and to the surrounding forest. Of course, sampling a glacier in a more exhaustive way collecting samples from almost the whole area of the glacier would give a more complete overview of the communities present there, but for vast glaciers (like Perito Moreno and Exploradores) it would be very complicated.

It looks like the main differences may be due to the belonging to a high elevation rather than a low elevation glacier. Looking at the results about differences of the most abundant orders, Cytophagales, Burkholderiales and Actinomycetales are the three orders that show different abundances according to the belonging to low or high elevation glaciers (Fig. 7.7a-b-d). Burkholderiales are a quite heterogeneous order, and therefore it is difficult to understand their trend according to an ecological interpretation (Garrity et al. 2015). Cytophagales are gram negative bacteria (Reichenbach 2006), that are known to be less resistant to UV radiation than gram positive bacteria (Arrage et al. 1993), therefore it is unexpected their higher relative abundance in bacterial communities of Iver, Iver East and Morado glacier. The order Actinomycetales, on the other hand, are mostly Gram positive bacteria (Cummins et al. 1958), that is consistent with their higher relative abundance in high elevation glaciers since they are more resistant to high UV radiation (Arrage et al. 1993).

Bacterial communities of cryoconite holes show seasonal variation on temperate mountain glaciers (Pittino et al. 2018b), therefore it may be argued that the differences we observed in the community structures among glaciers may be due, at least partly, to differences in the stages of the seasonal ecological succession present on each glacier when we collected the samples. In other words, one may argue that the bacterial communities were identical on each glacier at the beginning of the melting season and changed seasonally according to identical ecological succession, but they appear different in our samples because we collected them at different stages. Despite we acknowledge that this process may contribute to the observed variability among glaciers, we consider this effect as minor, and we confidently suggest that the differences we observed depend mostly on spatial variation and on variation in the general ecological conditions of the glacier and of the surrounding environments (mostly altitude and vegetation). Indeed, even if microbial communities can change

along the ablation season, there always is a core community characteristic of one glacier. Furthermore the differences among glaciers we see in our samples' bacterial communities, are not ascribable to a temporal trend like the one described by Pittino et al. (2018b). Indeed, we do not see any difference in phototrophs and heterotrophs abundances, and Cyanobacteria had a relative abundance higher than 1 % only on the Exploradores glacier. While if there was a temporal trend, Cyanobacteria should have been more present on those glaciers where the ablation season started later, but this is not the case.

7.5. Conclusion

In summary we provide the first-ever description of the bacterial communities of cryoconite holes of glaciers in South America, which confirm that these environments are dominated by the same bacterial orders all over the world (Boetius et al. 2015; Liu et al. 2017; Christner et al. 2003; Franzetti et al. 2016). The dissolved oxygen concentration in the water seems to affect bacterial communities that are mostly anoxic. Water pH also influenced bacterial communities. Importantly, this study, is not based on one glacier only, and therefore, can also give some insights on the ecological features that drive the structure of cryoconite hole bacterial communities on different glaciers. The five glaciers we investigated are still a too small sample for thoroughly assess the ecological processes that control cryoconite hole bacterial communities, but this is still a much larger sample size than that on which the vast majority of the studies on cryoconite holes published so far was based and it can put the basis to further investigations aiming at understanding how different and, at the same time, how similar cryoconite holes bacterial communities are.

8. A HOLE IN THE NEMATOSPHERE: TARDIGRADES AND ROTIFERS DOMINATE THE CRYOCONITE HOLE ENVIRONMENT, WHEREAS NEMATODES ARE MISSING

The content of this chapter has been published in the following paper:
Zawierucha, K., D. L Porazinska, G. F. Ficetola, R. Ambrosini, G. Baccolo, J. Buda, J. L. Ceballos, M. Devetter, R. Dial, A. Franzetti, U. Fuglewicz, L. Gielly, E. Łokas, K. Janko, T. Novotna Jaromerska, A. Kościński, A. Kozłowska, M. Ono, I. Parnikoza, F. Pittino, E. Poniecka, P. Sommers, S. K. Schmidt, D. Shain, S. Sikorska, J. Uetake and N. Takeuchi 2020. "A Hole in the Nematosphere Tardigrades and Rotifersdominate the Cryoconite Hole Environment, Whereasnematodes Are Missing." *Journal of Zoology*.

ABSTRACT The worldwide distribution of microinvertebrates on glaciers, the coldest biome, is poorly known. Owing to their tolerance to hostile conditions, small size and dispersal abilities, nematodes, tardigrades and rotifers are considered cosmopolitan and together inhabit various ecosystems. In this study, we investigated their global distribution in cryoconite holes – a type of freshwater reservoir forming directly in the glacial ice that creates biodiversity hotspots on glaciers. We analysed cryoconite samples (using classical microscopic observations and environmental DNA metabarcoding) from 42 glaciers located around the world (the Arctic, Subarctic, Scandinavia, the Alps, the Caucasus, Siberia, Central Asia, Africa, South America and Antarctica), as well as using literature data. Samples from Antarctic, Karakoram and the Alps were analysed using next-generation sequencing (NGS) and classical observations under microscopes, while all other samples were analysed by microscope alone. Three general outcomes were found: (1) tardigrades and rotifers represented the most common invertebrates in cryoconite holes; (2) tardigrades and rotifers often coexisted together, with one or the other dominating, but the dominant taxon varied by region or by glacier; (3) nematodes – the most abundant, hyperdiverse and widespread metazoans on Earth, including in environments surrounding and seeding glacial surfaces – were consistently absent from cryoconite holes. Despite the general similarity of environmental conditions in cryoconite holes, the distribution of tardigrades and rotifers differed among glaciers, but not in any predictable way, suggesting that their distribution mostly depended on the random dispersal, extreme changes of supraglacial zone or competition. Although nematodes have been found in supraglacial habitats, cryoconite hole environments seem not to provide the necessary conditions for their growth and reproduction. Lack of physiological adaptations to permanently low temperatures (~0°C) and competition for different food resources in the cryoconite hole environment may explain the absence of nematodes in cryoconite holes.

8.1. Introduction

The spatial distribution of life forms has been studied for centuries (Aristotle, 350 B.C.E. ; Darwin 1859; MacArthur et al. 1967). However, historically, these biogeographical surveys have been limited to macroorganisms. The emergence of new optical and molecular tools now allows the pursuit of similar questions for microorganisms, including single-cell microbes and multicellular micrometazoans (Nkem et al. 2006; Nkem et al. 2006; Guil et al 2009; Velasco-Castrillón et al. 2014; Darcy et al. 2018; Velasco-Castrillón et al. 2018; Zawierucha et al. 2018b). Recent studies show that the distributions of micrometazoan taxa can be related to physiological and dispersal constraints (Nkem et al. 2006; Nkem et al. 2006; Dial et al. 2012; Shain et al. 2016; Zawierucha et al. 2018b; Zawierucha et al. 2019a), competition (Shaw et al. 2018), geographical barriers (Jørgensenand et al.

2007; Czechowski et al. 2016) and to ongoing shifts in environmental conditions (Andriuzzi et al. 2018). Since microinvertebrates (i.e. tardigrades, rotifers and nematodes) display similar size, dormancy and dispersal characteristics to unicellular organisms (Crowe et al. 1974; Sohlenius 1979; Ramazzotti 1983), they may be expected to show worldwide distribution that is similar to microbes (Fontaneto 2019). However, the long-standing expectation of cosmopolitan distributions for microbes – the 'Everything small is everywhere' hypothesis (Liu et al. 2017; Segawa et al. 2017; Fontaneto 2019) – has been refuted for some of microinvertebrates by contemporary inventories of species diversity that reveal examples of endemism (Faurby et al. 2012; Cesari et al. 2016; Fontaneto 2019). The evidence for endemism largely applies to higher precision taxonomic categories, like genus and species (Forró et al. 2008), although not always, while cosmopolitan distributions apply to more coarse categories, like phylum and class. However, not all taxa at low taxonomic resolution are cosmopolitan. The phylum Onychophora (velvet worms), for example, inhabits the tropics, while the microinvertebrate phylum Loricifera (brush heads) await confirmation of their distribution (Monge-Najera 1995; Kristensen 2002). A recent review (Fontaneto 2019) of the distribution of limno-terrestrial rotifers, tardigrades and nematodes found a wide array of geographical ranges from worldwide, to biogeographically restricted, to completely endemic. Unfortunately, typical studies of distribution patterns in microinvertebrates involve comparisons across very different habitats (e.g. soil, bryophytes or lichens, freshwater lakes, puddles) distributed across different biogeographical realms (Segers 2008; Kaczmarek et al. 2016; van den Hoogen et al. 2019). This lack of ecological focus can hamper biogeographical conclusions and generalizations. A worldwide analysis of microinvertebrates that focuses on a specific habitat characterized by a specific thermal regime and other environmental conditions is currently absent. Terrestrial ecosystems constitute 30% of Earth's surface. Although 10% of that is covered by glaciers and ice sheets, our knowledge about microinvertebrate diversity in these parts of the cryosphere is very incomplete. While polar terrestrial habitats have been the subject of many microinvertebrate diversity studies (Wu et al. 2011; Coulson et al. 2014; Iakovenko et al. 2015), glacial habitats have not been well characterized (Zawierucha et al. 2019; Kaczmarek et al. 2015). During the melt season, warm temperatures and solar radiation transform glacial snow into liquid water, thereby providing a suitable environment for psychrophilic organisms (Hodson et al. 2008; Cook et al. 2016b). Among habitats associated with glaciers, cryoconite holes – water-filled reservoirs (~ 20–50 cm deep) in the glacial ice (Wharton et al. 1985; Hodson et al. 2008; Stibal et al. 2010; Cook et al. 2016b) – are known glacier biodiversity hotspots. The formation of these meltwater holes results from sediments (soil,

rock fragments and mineral dust along with cryophilic organisms) that absorb solar radiation and so melt into the ice (Hodson et al. 2008; Takeuchi et al. 2010; Cook et al. 2016b; Uetake et al. 2016). Cryoconite holes are inhabited principally by bacteria (both heterotrophic and photoautotrophic), algae and microinvertebrates (e.g. tardigrades and rotifers) (Porazinska et al. 2004; Kaczmarek et al. 2015; Uetake et al. 2016; Zawierucha et al. 2018a; Zawierucha et al. 2018b). The photosynthetic rates and organic matter content in cryoconite holes are comparable to other nutrient-poor freshwater ecosystems, such as stony-bottom oligotrophic lakes (Säwström et al. 2002; Peters et al. 2005). Despite more than a century of research on cryoconite holes (von Drygalski 1897), the global biogeography of microinvertebrates – top consumers within cryoconite holes – remains poorly known; most studies generally focus on the worldwide distributions of bacteria and algae (Mueller et al. 2001; Liu et al. 2017; Segawa et al. 2017, 2018; Darcy et al. 2018).

8.2. Materials and methods

8.2.1. Original data

Cryoconite material was collected from cryoconite holes across a wide variety of glaciers differing in their (1) morphology (e.g. tidewater vs. valley), (2) thermal regime (i.e. polythermal vs. cold-base vs. temperate), (3) light and temperature [e.g. seasonal cycles (polar) vs. daily cycles (temperate)], and (4) elevation (i.e. terminating in the sea vs. high mountains up to 5200 m a.s.l.). A total of 42 glaciers (Fig. 8.1) were sampled in the Arctic [Greenland, Svalbard (Spitsbergen, Nordaustlandet)], the Subarctic (Alaska), Scandinavia (Norway, Sweden), the Alps, the Caucasus, Siberia, Central Asia (the Karakoram, Pamir, Tien and Quilian Shan, Himalaya), Africa (Tanzania), South America (Colombia, Chile), Maritime Antarctica (Anvers Island, King George Island) and Continental Antarctica (McMurdo Dry Valleys). We sampled a wide range of cryoconite hole depths to reflect the variability in niche stability. The deepest holes were >60 cm deep with relatively stable water bodies; the shallowest were to <5 cm deep and subject to flooding and freeze/thaw cycles. Details of sampled cryoconite holes are provided in Supporting Information Table S8.1. Information on cryoconite holes from other sites was added by reviewing the published literature (Supporting Information Figure S8.1; Supporting Information Table S8.1). Data on the area and depth of cryoconite holes in particular regions and glaciers are available in Zawierucha et al. (2018) for SW Greenland, Zawierucha et al. (2019) and Łokas et al. (2016) for Svalbard, Zawierucha et al. (2019) for the Alps, Takeuchi et al. (2000) for Himalaya, Buda et al. (2020) for Maritime Antarctica, and Porazinska et al. (2004) and Sommers et al. (2019) for Continental Antarctic.

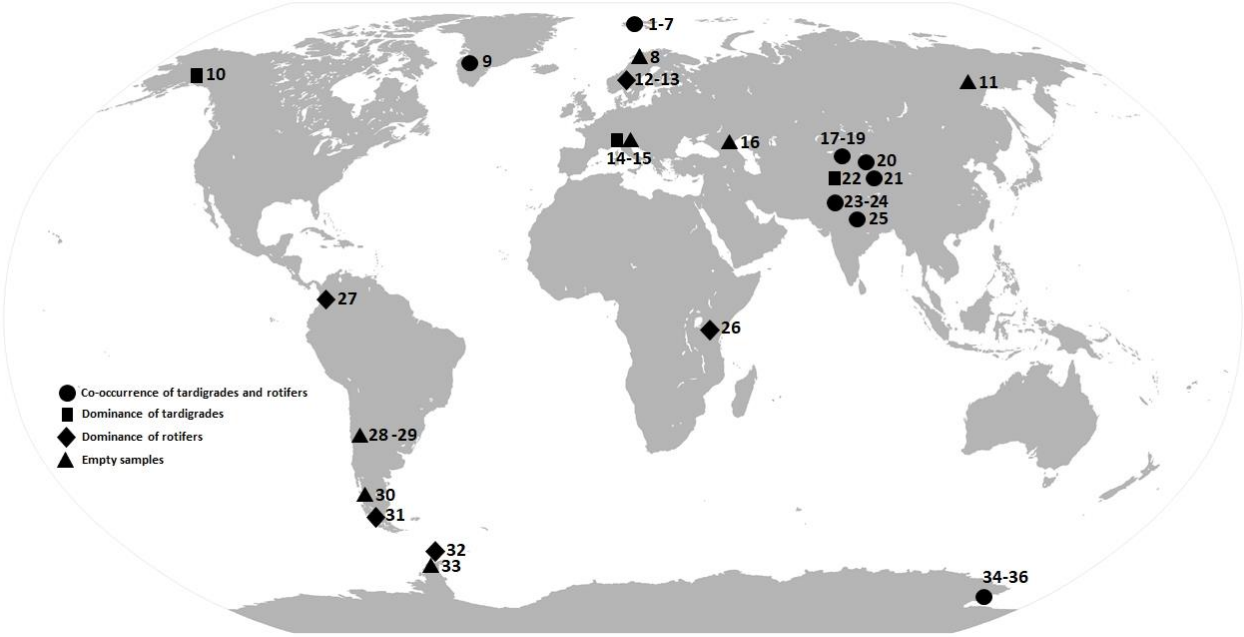

Figure 8.1 List of sampled glaciers (original data). Svalbard: Nordaustlandet: 1 – Area of Goosbukta, 2 – Dunerbreen, 3 – Nordre Franklinbreen, Spitsbergen: 4. Midre Lovenbreen, 5 – Svenbreen, 6 – Ebbabreen, 7 – Nordenski€oldbreen, 8 – Longyearbreen, 9 – Larsbreen, 10 – Werenskioldbreen, 11 – Hansbreen, Scandinavia: 12 – Storglaci€aren (Sweden), 13 – Svartisen, 14 – Okstindbreen (Norway), Greenland: 15 – Dark zone of Greenland Ice Sheet, Alaska: 16 – Gulkana Glacier, Siberia: 17 – Suntarhyata no. 31, Scandinavia: 18 – Middalsbreen, 19 – Blaisen (Norway), Alps: 20 – Morteratsch, 21 – Forni, Caucasus: 22 – Gergeti, Pamir Mountains: 23 – Lenin Glacier, Tien Shan: 24– Urumqi No.1, 25 – Grigoriev Ice Cap, 26 – Miaoergou, Quilian Shan: – 27 - Qiyi, Karakoram: 28 – Baltoro, Himalaya: 29 – Kang Yatse, 30 – Chamser Kangri, 31 – Yala, Kilimanjaro: 32 – Kersten, Andes: 33– Colombia (La Conojeras), 34 – Iver, 35 – El Morado, 36 – Exploradores, 37 – Tyndall, Maritime Antarctic: 38 – Marr Ice Piedmont, 39 – Blancmange, Continental Antarctic: 40 – Canada, 41 – Taylor, 42 – Commonwealth.

The presence of microinvertebrates was evaluated using two complementary approaches; a classical approach based on microscopy (dissecting, inverted or both) and environmental DNA (eDNA) metabarcoding. Sampled cryoconite holes varied in shape and size across regions, across glaciers, and across the same glacier (Fig. 8.2). For microscopy, we collected sediment samples from the bottom of each hole by two methods determined by whether the holes were open or frozen. Sediments from open holes were sampled with independent sterile disposable Pasteur pipettes or scoops and placed directly in vials. zip bags or jars. Sediments from frozen holes were retrieved from a 10-cm-diameter core and placed into sterile plastic bags. All sediment samples were either immediately frozen or preserved in 70% or 96% ethylic alcohol, then transported to home laboratories at the Adam Mickiewicz University in Poznan (Poland); Cardiff University (UK); Chiba University (Japan); University of Colorado Boulder (USA); or Charles University and Biology Centre AS CR (Czech Republic). At least 0.3–1.5 mL (in few cases for samples from Longyearbreen, Hansbreen and Marr) up to 10 mL of preserved cryoconite material was scanned from each sample for the presence of invertebrates on Petri dishes scored with perpendicular lines (for facilitating observations). Frozen cores collected from glaciers located in McMurdo Dry Valleys were melted and subset as described below in the Crary Lab in McMurdo, Antarctica, where microinvertebrates

were extracted from 20 g of melted sediment subsamples using modified White Trays for 24 h (Porazinska et al. 2018) and immediately counted. In addition to microscopy counts, samples for eDNA metabarcoding analysis were collected from Forni Glacier in the Alps, Baltoro Glacier in the Pakistan Karakoram and McMurdo Dry Valleys glaciers in Antarctica (Taylor, Canada and Commonwealth glaciers). We performed metabarcoding analyses at two laboratories: University of Milan and University of Colorado. For the University of Milan metabarcoding analysis, samples from Forni (18 samples on August 28th, and 19 samples on September 25th) and the Baltoro Glacier (19 samples from June 28th to July 9th) were collected in 2013. Cryoconite was deposited with a laboratory spoon (sterilized with alcohol) into 50 mL plastic tubes. Total DNA was extracted from 0.7 g of cryoconite using the FastDNA Spin for Soil kit (MP Biomedicals, Solon, OH, USA) according to the manufacturer's instructions. The extracted DNA was amplified in quadruplicates with the primers Euka02, which amplifies a ~123 bp fragment of 18S rDNA (Guardiola et al. 2015; Taberlet et al. 2018). The primer pair amplifies all eukaryotes at the family/order level (Guardiola et al. 2015; Taberlet et al. 2018). The amplification success was checked using Capillary electrophoresis (QIAxcel System; Qiagen, Venlo, Netherlands). Extraction and PCR controls were also included (Parducci et al. 2017). Sequencing was performed using Illumina MiSeq V2 platform (2 9 250 bp chemistry) at Fasteris, Geneva, Switzerland. DNA sequences were filtered (i.e. removing sequences: with low quality score; missing both primers or exact tag sequences; containing ambiguous nucleotides, shorter than 7 bp; or occurring only once in the dataset) and chimerachecked using the OBITOOLS software (Boyer et al. 2016). For the Forni + Baltoro metabarcoding experiment, 2.39 million reads were retained after the bioinformatic filtering steps and assigned to the samples (average number of reads per sample: 28 821). Potential contaminants – identified as those with highest occurrence in negative control samples – were removed. Sequences were retained in the final dataset only if their taxonomic assignment score was >80% and their occurrence was higher than 100 reads across the whole dataset. Pansu et al. (2015) provide a complete description of the filtering steps. Sequences were assigned taxonomy using the ecotag program and EMBL database (Boyer et al. 2016).

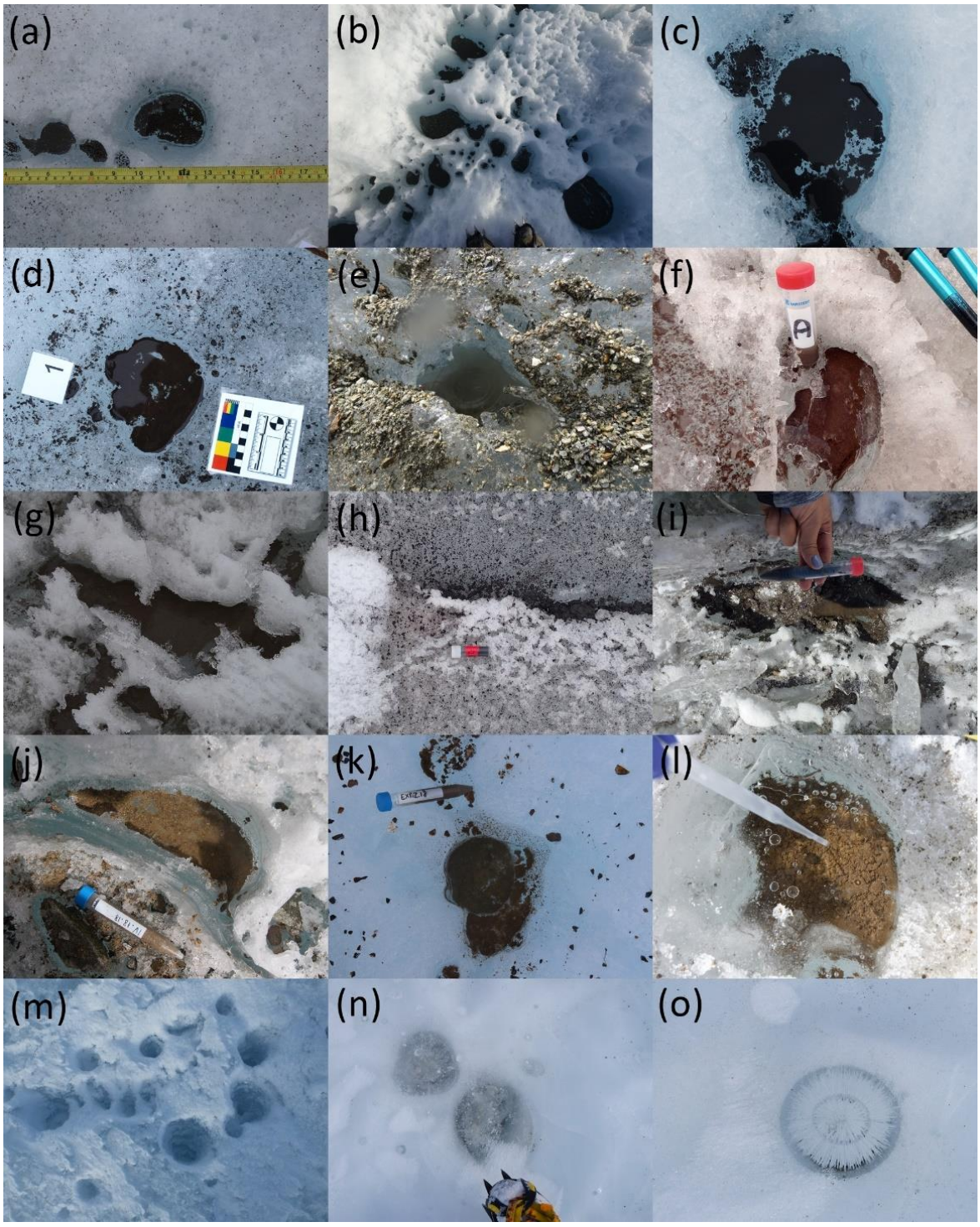

Figure 8.2 Cryoconite holes on glaciers around the world. (a) Svalbard, Longyearbreen, August 2016, (b) south-west Greenland, September 2015, (c) Norway, Blaisen, August 2018, photographs for all K. Zawierucha, (d) Alps, Forni, July 2017, photograph J. Buda, (e) Caucasus, Chaalati, July 2014, photograph K. Zawierucha (f) Caucasus, Gergeti, September 2019, photograph A. Koscinski, (g) Himalaya, Kang Yatse, August 2017, photograph M. Devetter (h) Colombia, La Conejeras, 2015, photograph Jun Uetake, (i) Kilimanjaro, Kersten, September 2019, photograph U. Fuglewicz, (j) Andes, Iver, 2018, photograph R. Ambrosini, (k) Andes, Exploradores, 2018, photograph R. Ambrosini, (l) Maritime Antarctica, Ecology, January 2017, photograph T. Budzik, (m) Maritime Antarctica, Marr Ice Piedmont, February 2018, photograph I. Parnikoza, (n, o) Continental Antarctica, Canada, December 2017, photograph P. Sommers.

For the University of Colorado metabarcoding analysis, 90 samples were collected from frozen cryoconite holes on three glaciers in the McMurdo Dry Valleys, Antarctica using a SIPRE corer between 7 and 17 November 2016. Sampling details are described in Darcy et al. (2018) and

Sommers et al. (2019). The cores were stored in sterile Whirl-Pak_ bags (Nasco, Fort Atkison, WI, USA) at -20°C for up to 1 month. In the Crary Laboratory at McMurdo Station, the sediment portion was separated from the rest of the core and washed with deionized water to melt the outer layer and remove potential cross-contamination from the drill, then placed in separate acid-washed high-density polyethylene beakers covered with aluminium foil and melted at 4°C for 12–24 h. Final melting took place at room temperature when necessary immediately prior to homogenizing, subsampling and filtering. DNA was extracted from c. 0.3 g cryoconite using a PowerSoil DNA Isolation Kit (MoBio Inc., Carlsbad, CA, USA). Tubes were frozen at _20°C until the DNA was extracted, up to 1 month later, following the manufacturer's protocol. Extracted genomic DNA was amplified in triplicate using 18S (1391f-EukBr primers, Caporaso et al. 2012; Amaral-Zettler et al. 2009) SSU ribosomal gene markers. Amplified DNA was pooled and normalized to equimolar concentrations using SequalPrep Normalization Kit (Invitrogen Corp., Carlsbad, CA, USA) and sequenced using the Illumina MiSeq V2 (2 9 250 bp chemistry) at the BioFrontiers Sequencing Core Facility at the University of Colorado at Boulder. Sequences have been deposited in the NCBI SRA database under project PRJNA480849. At the Colorado laboratory, we used QIIME v1.9.1 (Caporaso et al. 2010) to de-multiplex and quality filter the raw reads, and VSEARCH (Rognes et al. 2016) to join paired-end reads. The reads were clustered into operational taxonomic units (OTUs) at 97% similarity using UCLUST (Edgar 2010). Taxonomy was assigned using QIIME's parallel_assign_ taxonomy_blast.py script with a hand-curated version of SILVA 128 Ref NR99 database (Quast et al. 2013). Based on this classification, bacterial OTUs were removed from the eukaryotic OTU table. Singletons were discarded, as were any OTUs that made up at least 1% of the extraction blank sequences and were at least 1% of the samples, which were considered likely lab contaminants. A total of 2.36 million high-quality joined reads remained after this processing of reads from the 90 cryoconite hole samples, along with an additional 10 samples from stream microbial mats surrounding Canada Glacier in the Taylor Valley, Antarctica, included as a positive control for the presence of nematodes (see Supporting Information Figure S8.2).

8.2.2. Previously published distribution data

Published literature about cryoconite holes was identified using Scopus, Web of Science, Google Scholar and ResearchGate search engines with the following keywords: biota, cryoconite, cryoconite hole, ecosystems, glacier, glacial, animal, invertebrates, insects, Tardigrada, Rotifera, Nematoda, biodiversity and metazoans. All papers reporting microinvertebrate in cryoconite holes were included in the present study (Supporting Information Table S8.2), with an important exception. We

excluded from our literature search studies based solely on metagenomic DNA analysis that report the presence of sequences matching Nematoda alongside sequences of organisms such as members of Chordata, marine Echinodermata and tropical Onychophora (e.g. Edwards et al. 2013). The reporting of these taxa that are certainly not part of the active cryoconite community makes it impossible without microscopic confirmation to know whether nematode sequences also originated from individuals living outside the cryoconite holes. Cases where microinvertebrates are known by the authors as present in samples but unreported in original papers are also listed in Supporting Information Table S8.2 (e.g. rotifers in Takeuchi et al. 2004 and Zawierucha et al. 2016). As specific papers often focused a single taxon from cryoconite hole samples, omissions of other taxa from the paper were not taken as conclusive evidence of their absence from those samples. Although survey methods differed among studies (e.g. microscopy vs. molecular gene markers), we included publications from a variety of survey methods to better understand the biogeography of microinvertebrates in cryoconite holes.

8.3. Results

8.3.1. Original data

We investigated material from 42 glaciers globally (Fig. S8.1 and S8.7, Supporting Information Table S8.1). Using microscopy, we found that cryoconite holes were dominated mostly by tardigrades and rotifers (Figs 8.3, 8.4 and 8.7); unexpectedly, nematodes were never observed (Fig. 8.7). We found considerable differences among glaciers in taxonomic dominance. In the Arctic (Svalbard, Greenland) and Continental Antarctic, cryoconite holes were co-dominated by tardigrades and rotifers regardless of glacier thermal regimes or type (polythermal, cold base, valley, tidewater), or latitude or altitude in Svalbard and southwest Greenland. Cryoconite holes on Norway's Bl_aisen were dominated by rotifers, although a few tardigrades were also present in both 2018 and 2019. Cryoconite holes on Forni in the Alps during 2019 showed the opposite pattern with nearly exclusive presence of tardigrades and only a few rotifers. Dominance of tardigrades was similarly observed on Gulkana on Alaska sampled in 2015 and 2019. In contrast, cryoconite holes on the Alpine Morteratsch in 2017 and 2018 supported neither tardigrades nor rotifers. Similarly, cryoconite material from Gergeti in the Caucasus, Suntarhyata no. 31 in Siberia, Storglaciaren in Sweden and Marr in the Maritime Antarctic, all appeared to be free of any animals in cryoconite holes. Out of five sampled glaciers in Colombia and Chile, rotifers were found on only two (La Conejeras and Tyndall), whereas no tardigrades have been reported in South America. No tardigrades, but many rotifers, were also detected from Kersten in Tanzania and Ecology on King George Island (South

Shetland Islands). Out of the 480 molecular operational taxonomic units (OTUs) recovered from eDNA analysis on Forni and Baltoro samples, the majority were assigned to protists (36%) and fungi (31%) (Fig. 8.5, Supporting Information Figure S8.3, Supporting Information Table S8.3). Almost two per cent (1.8%) of MOTUs belonged to metazoan taxa from three phyla: Arthropoda, Rotifera and Tardigrada (Supporting Information Table S8.3). None of the detected MOTUs were assigned to Nematoda. Comparison of sequences belonging to invertebrates from Forni, Baltoro and Continental Antarctic glaciers shows a clear dominance of tardigrades on Forni and Baltoro (with minority of arthropods and rotifers) and coexistence of rotifers and tardigrades (with dominance of the former) on Antarctic glaciers (Fig. 8.5).

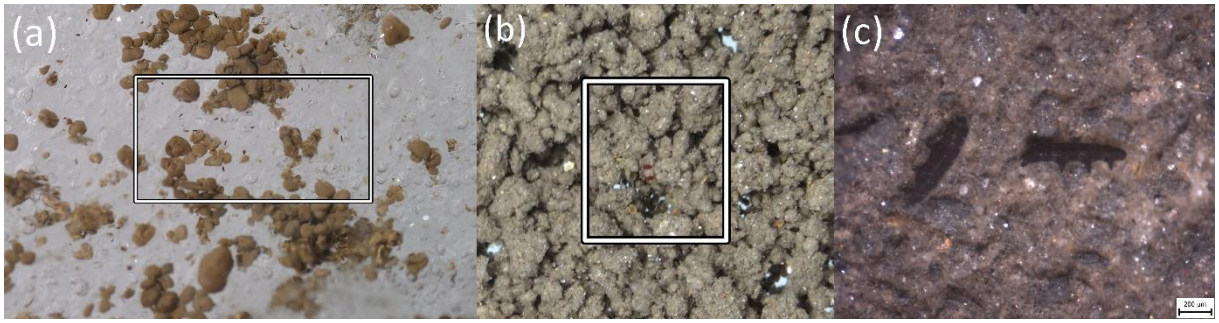

Figure 8.3 Tardigrada. (a) Cryoconicus kaczmareki Zawierucha et al., 2018 from Urumqi Glacier (Central Asia) – dark small dots in the frame, and (b, c) Cryobiotus klebelsbergi (Mihelcic 1959) from Forni Glacier (picture b – small brownish dots on cryoconite granules in the frame).

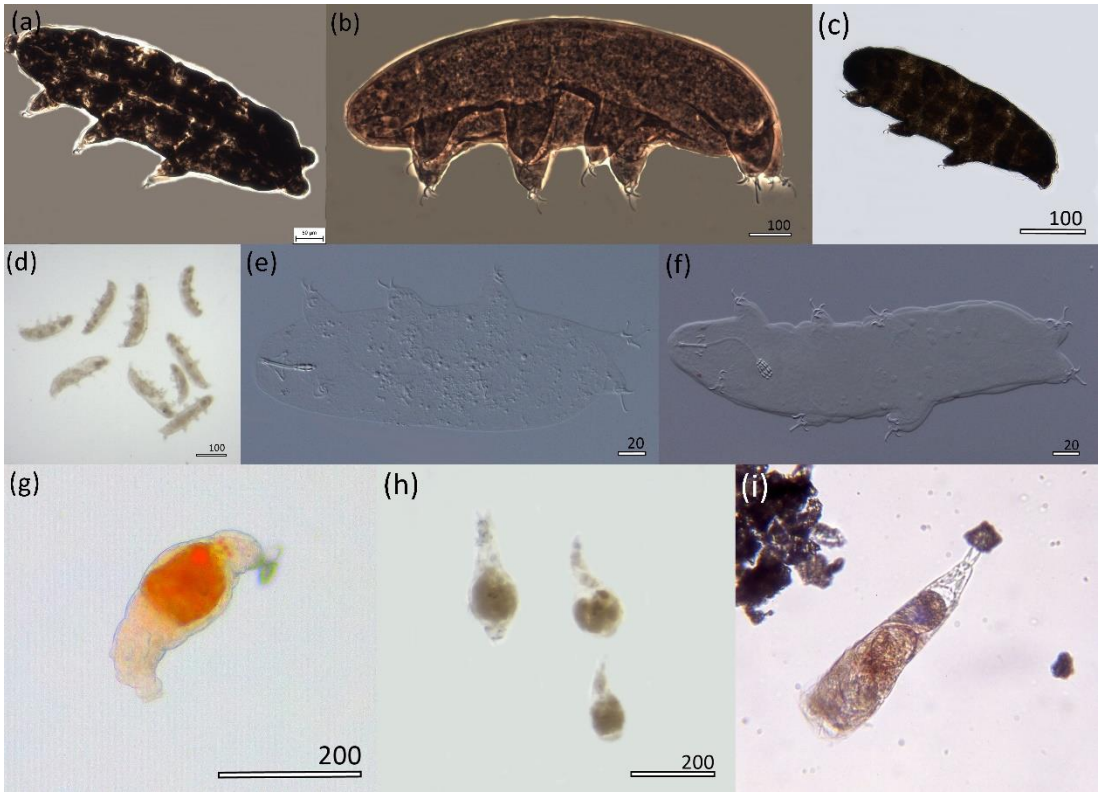

Figure 8.4 Tardigrada (a–f) and Rotifera (g–i). (a) Cryobiotus klebelsbergi (PCM – phase contrast microscopy), (b) Cryoconicus kaczmareki (PCM), (c) eutardigrade from Gulkana glacier (DIC – differential interference contrast microscopy), (d) tardigrades from Svalbard (Longyearbreen) BFM –(bright-field microscopy), (e) Hypsibius cf. dujardini from Svalbard (DIC), (f) Pilatobius sp. from

Greenland (DIC), (g) bdelloid rotifer from Svalbard, (h) bdelloid rotifers from Greenland (preserved in alcohol) and (i) bdelloid rotifer from Tyndall Glacier (BFM – g, h, i).

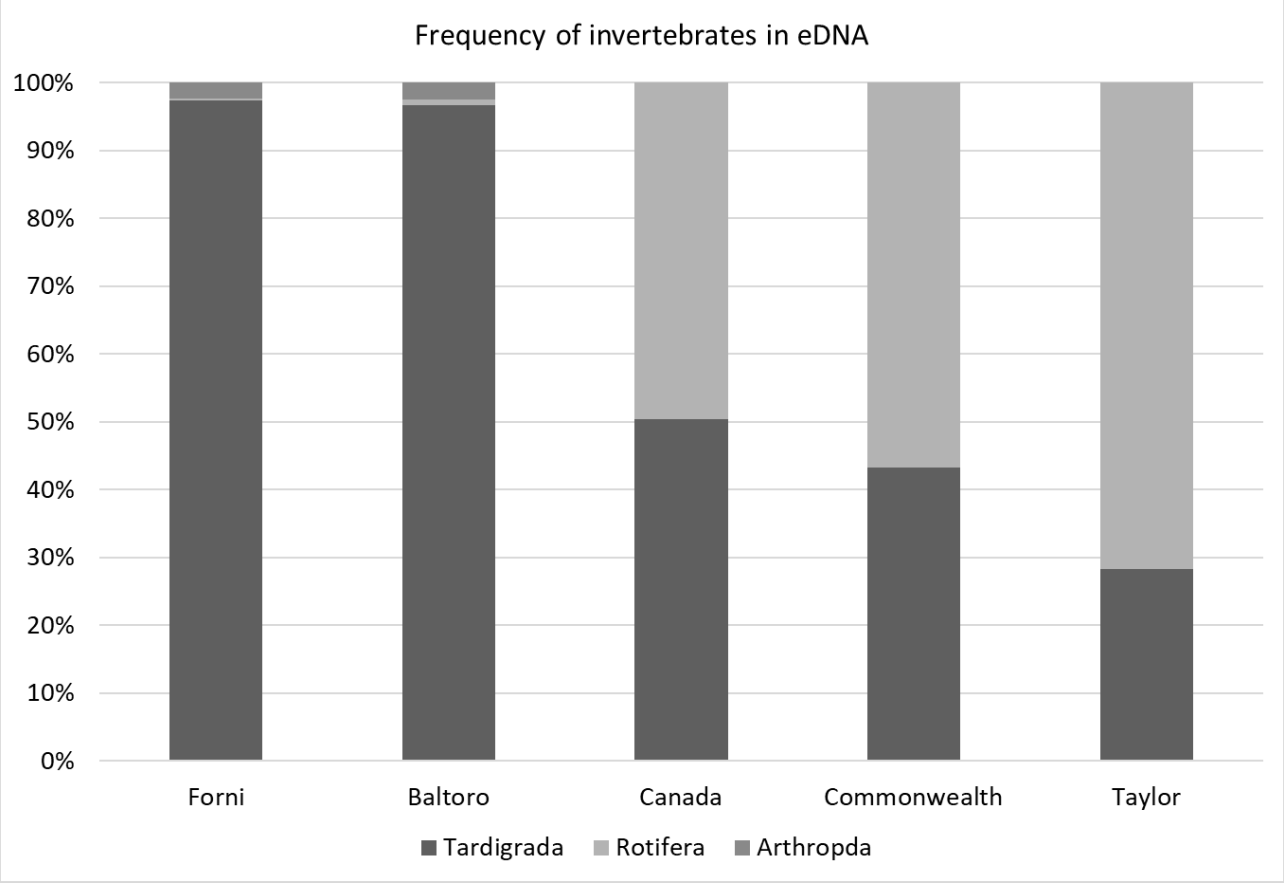

Figure 8.5 Frequencies of invertebrate sequences detected in eDNA using fragment of 18S rDNA from cryoconite holes on Forni (Alps), Baltoro (Karakoram) and Antarctic glaciers (Canada, Commonwealth and Taylor).

8.3.2. Previously published distribution data

Our review of 46 publications in which 41 localities (Supporting Information Figure S8.1, Supporting Information Table S8.2) were investigated, confirmed that cryoconite holes around the world are mostly inhabited by either tardigrades, rotifers or both (Supporting Information Table S8.2). In some cases, cryoconite holes are inhabited by other animal groups such as Diptera in New Zealand (Odell et al. 1956; Boothroyd et al. 1999) and the eastern Himalaya, Gangla Karchung La (R. Dial, pers. obs.), Plecoptera in the Andes, Copepoda in the Himalaya, or Acari in the Maritime Antarctica (Buda et al. 2020) (Fig. 8.6). However, these animals have narrow geographical distributions and mostly represent endemic species (Kohshima 1985; Kohshima 1984). Some studies focused on the description or redescription of new microinvertebrate taxa (Mihelcic 1959; Ramazzotti 1968; Dastych 2004, 1993, 2019; Kikuchi 1994; Dastych, Kraus, and Thaler 2003; Zawierucha et al. 2018b). Others aimed to describe cryoconite communities, and authors discussed all invertebrates found in cryoconite holes (De Smet et al. 1994; Grongaard et al. 1999; Porazinska et al. 2004; Vonnahme et al. 2016; Zawierucha et al. 2018b; Lutz et al. 2019; Zawierucha et al. 2019; Zawierucha et al. 2019a;

Zawierucha et al. 2019b; Buda et al. 2020). In some papers, animals were mentioned as an ecological variable; in others, the focus was on faunistic reports (Dastych 1985; Dabert et al. 2015; Łokas et al. 2016). Both old (von Drygalski 1897; Dastych 1985; De Smet et al. 1994) and recent literature (Zawierucha et al. 2018a; Zawierucha 2019; Zawierucha et al. 2019a; Zawierucha et al. 2019b) indicate that Arctic cryoconite holes are inhabited by tardigrades and rotifers without any visible general pattern at the global scale (Supporting Information Figure S8.1, Supporting Information Table S8.2). In the Alps, Zawierucha et al. (2019) and Dastych et al. (2003) found cryoconite holes inhabited by tardigrades with only little or no rotifer presence, over multiple summers. However, tardigrades and rotifers were found earlier by Steinböck (1936) on other glaciers in the Alps (albeit without quantitative data). Analysis of material from Chalaati and Adishi glaciers in the Caucasus showed that neither tardigrades nor rotifers were present (Makowska et al. 2016).The literature suggests that Central Asian glaciers are inhabited mostly by tardigrades; however, some of papers from this area were purely taxonomic (Supporting Information Table S8.2) and offered no descriptions of entire invertebrate assemblages. In the Maritime Antarctica, cryoconite holes on the Ecology Glacier (King George Island, South Shetland Islands) were inhabited by rotifers, but no tardigrades nor nematodes were found (Zawierucha et al. 2019a). Microscopy-based and eDNA surveys of cryoconite holes on Continental Antarctic glaciers showed the presence of both tardigrades and rotifers (Porazinska et al. 2004; Sommers et al. 2018; Lutz et al. 2019). Porazinska et al. (2004) sampled five Dry Valley glaciers and found relatively equitable rotifer:tardigrade ratios in the wetter east but very high rotifer:tardigrade ratios in the drier west. Both groups were least abundant on the glaciers receiving the least snow. In cryoconite holes in Marie Byrd Land, Continental Antarctica, only rotifers were reported (Broady 1989), whereas in cryoconite holes in Queen Maud Land both rotifer and tardigrade DNA were found together three times and separately five times (tardigrades once, rotifers four times) in ice covered cryoconite holes (Lutz et al. 2019). Of the 5014 97% similarity OTUs recovered from the cryoconite hole sediments in Continental Antarctica (Canada, Taylor, and Commonwealth glaciers), 14% were assigned to Rotifera and Tardigrada, and these OTUs accounted for 30– 40% of the reads in the samples (Sommers et al. 2019). Two studies from Antarctica detected nematodes in cryoconite holes. One used classical microscopy (Mueller et al. 2001), while the other used eDNA analysis (Christner et al. 2003). Other studies from the same glaciers reported no nematodes in cryoconite holes (Porazinska et al. 2004; Sommers et al. 2019). While Sommers et al. (2018) reported the presence of nematode sequences using eDNA, these samples did not have microscopy associated with them and later, more extensive

sampling of cryoconite holes from the same glaciers including microscopy (Sommers et al. 2019) failed to find nematodes (Fig. 8.7). Also Murakami et al. (2018) detected nematodes in the faecal metagenomes of Plecoptera Andiperla willinki which also might be found in cryoconite holes. However, the presence of nematodes in faeces does not offer conclusive evidence of their presence in cryoconite holes because Plecoptera could find their preys directly on the glacier surface.

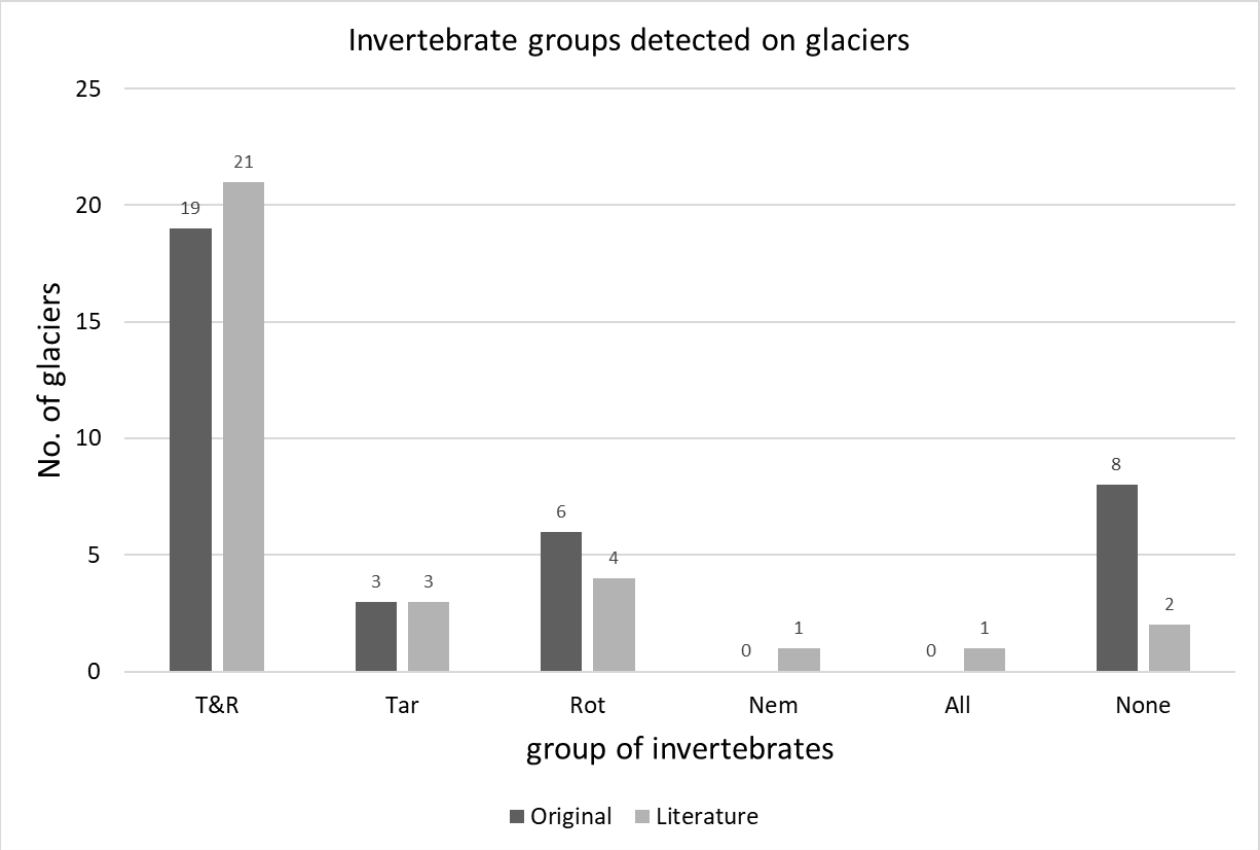

Figure 8.7 Invertebrate groups (Tardigrada, Rotifera, Nematoda) detected in cryoconite holes on glaciers (original and literature data, Fig. 1, Supporting Information Figure S1, Supporting Information Tables S1 and S2). T&R: Tardigrada and Rotifera; Tar: Tardigrada; Rot: Rotifera; Nem: Nematoda; All: all three (Tardigrada, Rotifera and Nematoda); and None: absence of these groups from cryoconite holes of studied glaciers. In literature data, taxonomic papers focused only on particular groups were removed from this comparison. For selection criteria of literature records, see Supporting Information Table S1. In consulting original data, we considered dominance by a group when cryoconite was exclusively dominated by one group with few or no coexisting specimens of another.

8.4. Discussion

8.4.1. Dominant taxa

In this study, we used both original data (Fig. 8.1, Supporting Information Table S8.1) and published records (Supporting Information Figure S8.1, Supporting Information Table S8.2) to show that the microinvertebrate communities in cryoconite holes from >70 locations consist of mostly rotifers, tardigrades or both, but, with the exception of two studies in Antarctica (one microscopy-based and one genetic), never include nematodes. Although nematodes have been detected in glacial mosses (Coulson and Midgley 2012), on the glacial ice (Shain pers. obs.), in sediments overlying glacial surface (Azzoni et al. 2015, Porazinska pers. obs.), in sediments of glacial streams in Antarctica

(Sommers and Porazinska pers. obs.) or even in glacial Plecoptera faeces (Murakami et al. 2018), cryoconite hole environments seem not to provide the necessary conditions for growth and reproduction of nematodes. Due to their physiological adaptations to survive low temperatures, freezing, high UV and osmotic stress (Altiero et al. 2011; Guidetti et al. 2012; Jönsson et al. 2017; Zawierucha et al. 2019; Zawierucha et al. 2018b), dominance in cryoconite holes by tardigrades and rotifers is not surprising. Despite the similarity of conditions across cryoconite holes (e.g. low temperatures, periodic freezing), the composition of animal phyla varied within regions and even within glaciers. On Blaisen (Norway), Forni (the Alps), Ecology (Maritime Antarctic) and Kersten (Africa) glaciers, cryoconite holes were dominated by either rotifers or tardigrades. For example, in Norway (Blaisen, Midtdalsbreen), we observed the numerical dominance of rotifers over tardigrades. Regrettably, detailed records of the cryoconite hole community structure on the same glaciers in previous studies were missing; consequently, it was impossible to infer which group dominated in the past (Soemme 1996). In the Alps, on Forni Glacier, there was almost excusive dominance by tardigrades over multiple seasons (Zawierucha et al. 2019, present study). The same pattern was observed on Rotmoosferner in the Alps by Dastych et al. (2003), who reported few rotifers among thousands of tardigrades in cryoconite material. Arctic and Continental Antarctic cryoconite holes hosted both rotifers and tardigrades, while no invertebrates were detected in cryoconite holes on Morteratsch Glacier in the Alps (sampled during two seasons) or on Adishi, Chalaati and Gergeti glaciers in the Caucasus. Tardigrades were undetected in cryoconite holes in South America, whereas rotifers were found on one Colombian and one Chilean glacier. Taking into account the high dispersal abilities of both terrestrial and glacier invertebrates (Jørgensen et al. 2007; Ptatscheck et al. 2018; Zawierucha et al. 2018b; Fontaneto 2019; Zawierucha et al. 2019), we hypothesize that the nature of glaciers (thermal regime, type, altitude, latitude) is not the driving factor of microinvertebrates distribution patterns. Instead, local climatic or environmental factors and infrequent extreme weather events (e.g. strong rains (Zawierucha 2019b)) might be more important. More intense and standardized (using the same techniques and combining molecular and microscopy approaches) sampling, long-term monitoring and manipulative field experiments could resolve these questions.

Figure 6.8 Animals known from glaciers that are occasionally found in cryoconite holes, yet inhabit other glacier niches: (a) Annelida (glacier ice worms, Mesenchytraeus solifugus) from an Alaskan glacier and known to be abundant in the englacial zone of the maritime glaciers. Polish grosz as a scale. (b) Collembola from a glacier in the Alps and known to be widespread and abundant on ice and snow surfaces, particularly under stones and in weathering crust (Fjellberg 2010; Krzysztof Zawierucha, Buda, Azzoni, et al. 2019).

8.4.2. Other animals in cryoconite holes

Zawierucha et al. (2015) postulated that cryoconite holes should be inhabited by many groups of micrometazoans, including crustaceans and gastrotrichs, as found in other polar or high-alpine freshwater bodies (Janiec 1996; Hinden et al. 2005; Kolicka 2016). The habitat provides abundant organic matter as a food source and relatively low levels of competition. However, our global survey did not corroborate this hypothesis. We found evidence of only a few other animals being found in some cryoconite holes. The Northern and Southern Patagonian Icefields were inhabited by arthropods such as Plecoptera Andiperla willinki (Aubert 1956) and Collembola. In addition, some Himalayan cryoconite holes were inhabited by copepods (Copepoda) and chironomids (Insecta) (Koshima et al. 1984; Takeuchi et al. 2000; Dial pers. obs.). All of these examples are mostly endemic glacierobligate invertebrates, which feed and reproduce in very narrow geographical areas (one mountain range, ice field or glacier). Recently, Zawierucha et al. (2019) and Buda et al. (2020) reported presence of various instars of mite (Nanorchestes nivalis) in cryoconite holes on Ecology Glacier in the Maritime Antarctic during two seasons and showed that the mites on this glacier can be persistent residents of the cryoconite holes. Platyhelminthes have been reported by DNA in Continental Antarctica (Sommers et al. 2018); however, confirmation of their affinity with cryoconite holes requires microscopic analysis. Glacier ice worms (Mesenchytraues solifugus) – the only known glacier-obligate annelid, were reported in cryoconite holes in the Pacific Northwest of North America (Goodman 1971); however, despite the habitat suitability formed by stable thermal

structure of cryoconite microenvironments (e.g. Gulkana Glacier), they were absent from the material analysed in this study. Collembola have been found in the bottom of cryoconite holes in the Alps and the Caucasus (Supporting Information Table S8.2), but benthic ecosystems are not their typical habitat and they have probably been passively dispersed to the holes by water, as observed in the Alps by Zawierucha et al. (2019) (Fig. 8.8, Supporting Information Figure S8.4). Springtails have also been reported in weathering crust on glaciers but not in the holes bottom (Fjellberg 2010). Although arthropods sequences were detected in environmental DNA, their absence (complete or active specimens) from microscope observations suggests the detected DNA sequences were likely due to wind-blown material rather than active components of communities on Baltoro and Forni glaciers. In samples from Forni and Baltoro glaciers, remains of insects (wings, legs, heads), but not living individuals, suggest insects are primarily allochthonous (confirmed in this study and (Zawierucha et al. (2019)). Recently, Dastych (2019) found along with tardigrades and rotifers, several small fragments of arthropod cuticles, a dead small aphid and a beetle of the genus Bembidion in cryoconite holes on the Gihccejiekna Glacier in northern Norway. All of those arthropods were not typical cryoconite hole dwelling invertebrates but accidental faunal elements. Although Edwards et al. (2013) reported DNA sequences from many animal taxa from cryoconite holes in the Alps including marine Echinodermata, and tropical Onychophora, without a confirmation with microscopy the actual presence of live individuals which belongs to these groups is seriously doubtful. The disparity in richness of animals between cryoconite holes and other freshwater environments remains poorly understood and is one of the most striking scientific questions in studies on the distribution of invertebrates in the cryosphere.

8.4.3. Cryoconite holes as suitable habitats for cryophilic invertebrates

In some respects, cryoconite holes are not as extreme as often described (Maccario et al. 2015). Liquid water makes them oases of life in a frozen landscape, supporting abundant and diverse microbial communities and potentially providing sufficient food resources (Stibal et al. 2010; Cook et al. 2016b; Zawierucha et al. 2018a; Buda et al. 2020). Even though cryoconite holes are harsh environments in many respects, they provide an adequate food resources for various invertebrates. Cryoconite holes are considered biodiversity hotspots for cryophilic species of algae, cyanobacteria, heterotrophic bacteria, amoebas, fungi and ciliates (Takeuchi 2002; Hodson et al. 2008; Cook et al. 2016b; Pittino et al. 2018b; Sommers et al. 2018, 2019; Zawierucha et al. 2018a; Buda et al. 2020; Stibal et al. 2020). The biodiversity of photoautotrophs includes many opportunistic and glacier specific taxa which may attain the biomass of 0.998 µg dm^3 and the photosynthetic rates from 0.63

C L^{-1} h^{-1} to 156.99 µg C L^{-1} h^{-1} (Säwström et al. 2002; Buda et al. 2020). Metagenomic studies showed that copy number of 16S rRNA gene (a proxy for bacterial concentration) ranges between regions, but is generally very high (105– 107 copies g^{-1} on Adishi in the Caucasus, 108–109 copies g^{-1} on the Greenland ice sheet (Makowska et al. 2016; Stibal et al. 2015)). Cryoconite holes generate and store organic matter on glaciers (Stibal et al. 2010), for example up to 18% in Greenland (Gerdel et al. 1960), 16% in the Caucasus (Łokas et al. 2018), 14% in Svalbard (Łokas et al. 2016), 13% in Central Asia (Takeuchi 2002) and 7% in Maritime Antarctic (Buda et al. 2020). Moreover, apart from autochthonous production, cryoconite holes food webs on a small glacier receive allochthonous wind-blown organic input from fragments of mosses, leaves, insects and others windborne materials (Stibal et al. 2007; Dastych 2019; Zawierucha et al. 2019). Primary production in holes is comparable to other oligotrophic water pools in Arctic regions (Säwström et al. 2002; Michelutti et al. 2005), and the bacterial number is similar to other freshwater ecosystems (Bomberg et al. 2019). Values of organic matter in cryoconite holes surpass the organic matter content in oligotrophic lakes (c. 10% in stony-bottom lakes) that support diverse meiofaunal groups (Peters et al. 2005). Tardigrades in cryoconite holes are mostly herbivores and microbivores, while rotifers are filter feeders. Thus, microbivorous and omnivorous (even carnivorous) nematodes should find adequate resources for growth and reproduction. However, it is possible that specific food sources required by invertebrates such as nematodes are missing from these environments. As cryoconite holes are the most productive and species-rich habitats on glacial surfaces, more factors than only trophic resources must prevent nematodes from occupying this aquatic habitat. More holistic and thorough approaches to studying cryoconite communities are therefore necessary to describe their ecological networks and limiting factors.

8.4.4. Limiting factors

This study spanned a worldwide collection of glaciers from a broad variety of environmental conditions, from cold-polar, to wet-temperate, to dry, high-altitude glaciers. We therefore suggest that factors other than biogeographical region, climate, latitude and light cycles explain the presence of tardigrades and rotifers and the absence of nematodes in cryoconite holes. The harsh physical conditions of glacial environments may reduce the faunal diversity in cryoconite holes. Surrounded by ice instead of soil and rock, cryoconite holes remain near freezing even during summer days and freeze solid over winter. For instance, the temperature in cryoconite holes on Arctic glaciers in summer averages 0.08°C with a maximum of 0.22°C, (Zawierucha et al. 2019b). In comparison, shallow polar ponds or lakes may reach 10°C (Sheath 1986; Schneider et al. 2016) and

consequently can be inhabited, not only by tardigrades and rotifers, but by a diversity of freshwater fauna (Coulson et al. 2014; Kolicka 2016). The permanently low temperatures in cryoconite holes may suppress basic life activities, including reproduction, hatching and foraging. In addition to low temperatures, cryoconite holes are dynamic environments and characterized by periodic freezing, removal of sediments from the bottom, ablation and/or collapse during extreme weather events (e.g. rain and föhn winds) (Mueller et al. 2001; Fountain et al. 2004a; Zawierucha 2019b). Consequently, animals can be exposed to intense UV radiation directly on the surface or simply be flushed out into the proglacial field. Most importantly, not even all tardigrades are able to enter anhydrobiosis (Bertolani et al. 2004; Nelson et al. 2019), which might be a crucial mechanism to survival on the ice surface during the drying of removed sediment (Zawierucha et al. 2019). Even though cryoconite tardigrades are able to survive desiccation, their recovery success is lower in comparison with other limno-terrestrial species (e.g. Bertolani et al. 2004; Nelson et al. 2019). Nevertheless, their anhydrobiotic state may favour dispersal (Zawierucha et al. 2019). Indeed, desiccation enables their passive dispersal by wind (Nkem et al. 2006) and successful colonization of new habitats on other glaciers. In contrast, limited anhydrobiosis may lead to endemism. For example, Himalayan copepods and chironomids, and New Zealand chironomids, Patagonian Plecoptera (Odell et al. 1956; Takeuchi et al. 2000; Takeuchi et al. 2004; Murakami et al. 2018) are restricted to mountain glaciers in relatively small areas. Larger body size and a lack of anhydrobiosis abilities probably limit other microinvertebrates in terms of passive aerial dispersal and successful establishment on glaciers, specifically in cryoconite holes. The low concentration of available phosphorous or nitrogen in water to support primary producer populations could furthermore limit the diversity of invertebrates in cryoconite holes. Such a hypothesis related to freshwater ecosystems was previously suggested by Hart et al. (1990) and demonstrated
in experimental freshwater mesocosms (Urabe et al. 1997; Michiels et al. 2005). The negative influence of phosphorous limitation on zooplankton and benthic grazers is well known in freshwater systems (Hart et al. 1990; Urabe et al. 1997). Glaciers are very likely ecosystems where organisms are limited by dissolved phosphorous availability (Holland et al. 2019), which is a crucial biogenic element. This, in turn, may limit the presence of other taxa in cryoconite holes, but to our knowledge nematodes are not more sensitive to the resulting effects on the food web of phosphorous limitation than are other microinvertebrates. Anthropogenic contaminants might constitute another factor potentially reducing animal diversity, albeit perhaps a minor role (Lorimore et al. 1998; Bezchlebová et al. 2007; Hussain et al. 2009). Worldwide, contamination of supraglacial

ecosystems by pollutants includes black carbon, POPs (persistent organic pollutants), radionuclides and heavy metals (Langford et al. 2014; Baccolo et al. 2017; Ferrario 2017a; Łokas et al. 2018). For example, on Adishi Glacier in the Caucasus, unexpected high concentrations of radionuclides have been detected, and a concomitant absence of animals (except for few collembolans) has been observed (Łokas et al. 2018). A similar observation was made on Morteratsch in the Alps (Baccolo et al. 2017). However, radionuclide contamination has only been investigated for one glacier in the Caucasus and a few in the Alps, suggesting that this topic deserves further investigation. Moreover, in Continental Antarctica, contamination of the cryosphere is low (Khan et al. 2017), but nematodes are absent from cryoconite holes there as well. Thus, contaminants alone cannot explain nematode absence.

8.4.5. Absence of nematodes

Generally, tardigrades, rotifers and nematodes all enjoy large geographical ranges across a multitude of habitats. The three phyla often coexist in both terrestrial and aquatic (freshwater and marine) ecosystems (Schwarz et al. 1993; Janiec 1996; Albuquerque et al. 2007). Nematodes are considered one of the most hyperdiverse taxa on Earth and the most abundant multicellular organisms (Hodda 2007; van den Hoogen et al. 2019). Besides taxonomic diversity, they also present a variety of feeding habits, including algal, bacterial and fungal feeders, omnivores, predators and parasites. Although many species are well adapted to survive desiccation, they are aquatic organisms needing at least a film of water for movement, feeding, growth and reproduction to occur (Wharton 1986). Consequently, cryoconite holes, due to the presence of liquid water and potential food sources, appear to be suitable habitats for nematodes. Though nematodes are one of the most resilient invertebrate groups, able to survive under many unfavourable conditions (Treonis et al. 2005; Nkem et al. 2006; Hodda 2007; Wharton et al. 2015), it seems odd that the conditions of cryoconite holes appear unsuitable for their growth. Since nematodes were absent from all the cryoconite holes we investigated, we suspect that evidence of nematodes in cryoconite by Mueller et al. (2001), Christner et al. (2003) and Sommers et al. (2018) may be allochthonous. In support of this interpretation, (Nkem et al. 2006) reported mostly dead nematodes in air traps on Antarctic glaciers. Similarly, sediments on the surface of Antarctic glaciers often support abundant populations of bacterial-feeding nematode Plectus sp. (Porazinska, pers. obs.), but never inside cryoconite holes. Christner et al. (2003) used a small subset of samples from Porazinska et al. (2004), where a large amount of sediments were processed for counts without detecting a single nematode. Similarly, nematode DNA presence reported by Sommers et al. (2018) was not supported by

microscope counts from subsequently collected samples from the same glaciers. Even though nematodes are well adapted to tolerate cold in terrestrial habitats, permanently low temperatures in cryoconite holes may limit their reproduction or limit their ability to compete for food with rotifers and tardigrades. For instance, nematodes survive freezing and desiccation in Antarctic soils through dormancy but reproduce when water and temperature conditions are favourable (Wharton 2003). The presence of nematodes overlying glaciers in supraglacial debris (Azzoni et al. 2015) and in glacier mice (Coulson et al. 2012) indicates the presence of these favourable conditions in these habitats. Overhoff et al. (1993) found that temperatures of 10°C were required for growth of Scottnema lindsayae, the most dominant Antarctic nematodes adapted to cold soils – a temperature unlikely achieved in ice-bound cryoconite holes. This hypothesis is corroborated by previous observations of Zawierucha et al. (2019)who did not find nematodes in cryoconite holes on Forni Glacier, but did find them in sediments from a proglacial lake. Similarly high Arctic tundra tardigrades (different than Arctic cryoconite hole species (Zawierucha et al., under review)) reproduce faster in higher temperatures (Stec pers. comm.). It could be that – as with the North American ice worm, an obligate glacierdwelling organism that cannot survive below about -7°C (Dial et al. 2016) – the flexible cuticle of a vermiform morphology cannot survive the cold damage of being immersed in nearly pure frozen water, something that tardigrades and rotifers in cryoconite holes can withstand. This hypothesis would seem amenable to experimentation. As nematodes are among the most numerous benthic animals in oligotrophic lakes (Ristau et al. 2011; Majdi et al. 2015), low levels of nutrients do not appear to determine their presence/absence. To explain the absence of nematodes from the otherwise apparently suitable habitat offered by cryoconite holes, we hypothesize the existence of some other biological or ecological barriers. Cyanobacteria are reported to be inhibitory towards some nematode species: for example, in permanent cyanobacteria mats or blooms in freshwater ecosystems (Munoz et al. 2006; Nascimento et al. 2009). However, this hypothesis fails for some environments, as demonstrated by Jungblut et al. (2012) who showed that nematodes are found in Arctic and Antarctic cyanobacterial mats (see also Supporting Information Figure S8.2). Another barrier may be rapid changes in oxygen. While cryoconite holes are considered ultraoligotrophic environments, rapid development of anoxic zones in the sediment has been observed by Poniecka et al. (2018). Quick shifts from oxic to anoxic conditions may be a potential barrier for survival and reproduction, enabling the existence of only some taxa that can respond equally rapidly. Finally, microbial assemblages in cryoconite holes are distinct from those in soil or benthic environments (e.g. Franzetti et al. 2017a), such that crucial

components of the nematode diet are missing. Thus, competition and absence of necessary resources may prevent nematode occupancy of cryoconite holes. Undoubtedly, further studies will be necessary to confirm the absence of nematodes from cryoconite holes and other englacial and subglacial habitats characterized by temperatures that are almost constantly near-freezing. Subglacial ecosystems, considered for years by the scientific community to be sterile, have now gained scientific attention by biologists and bio-geochemists, with many studies currently addressing their diversity and productivity (Yde et al. 2011; Achberger et al. 2017; Dubnick et al. 2017; Zdanowski et al. 2017b). We cannot exclude the hypothesis that some specialized nematodes inhabit both subglacial ecosystems and englacial channels thermally similar to cryoconite holes. For example, Northern American glaciers host oligochaetes inhabiting the englacial zone (Dial et al. 2012; Dial et al. 2016). Similarly, deeper layers of weathering crust are inhabited by rotifers on Icelandic glaciers (Shain et al. 2016) and also nematodes on New Zealand (Shain pers. obs.).

8.5. Conclusion

The microinvertebrate communities of cryoconite holes worldwide appear to be dominated by Tardigrada and Rotifera. In some locations, tardigrades dominated (e.g. Gulkana on Alaska, Forni in the Alps); in others, rotifers (e.g. Ecology in maritime Antarctic, Blaisen in Norway). We found, however, that tardigrades and rotifers mostly co-dominated in communities. In contrast, nematodes were absent in material originated from cryoconite holes from 42 glaciers. This absence may be related to a combination of factors including consistently low temperatures, periodic freezing, UV irradiation, nutrient limitation, low solute content, high levels of contamination in cryoconite worldwide (often higher than in other benthic ecosystems) and competition for food. The most likely explanations include the effects of permanently low temperatures in cryoconite holes, which may limit nematode reproduction, or their ability to compete for specific food with glacial rotifers and tardigrades. This observation lays the groundwork for comparisons that can shed light on the limits to multicellular life in the cryosphere.

8.6. Acknowledgements

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8.7. Data availability statement

The data that support the findings of this study are openly available in Supplementary material and under NCBI SRA database under project PRJNA480849.

8.8. Supplementary material

Table S8.1 List of samples analysed in this study. Caption: Typ. gl. – type of glacier, Ther. reg. – thermal regime, Light fluct. – light fluctuation, Biog. – biogeographic realm, Ts. – type of sampling (1 - sampling from single cryoconite holes, 2 - sampling from many cryoconite holes (pooling)), N – number of samples, Alt. – altitude, Pres. – type of preservation, Nem. – Nematoda, Tar. – Tardigrada, Rot. – Rotifera, Coll. – collector, Inv. – investigator (person who analysed samples). Abbreviation in table: tidwat. – tidewater, vall. – valley, pied. – piedmont, polyt. – polythermal, cold. – cold-base, temp. – temperate, alc. – alcohol, freez. – freezing, form. – formaladehyde, fresh – sample were analysed just after collection, Coll. and Inv.: RA (R. Ambrosini), AS (A. Anesio), GB (G. Baccolo), JB (J. Buda), JC (J. Cook), MD (M. Devetter), UF (U. Fuglewicz), KJ (K. Janko), TNJ (T. Novotna Jaromerska), AK (A. Kościński), AK (A. Kozłowska), MO (M. Ono), IP (I. Parnikoza), ŁP (Ł. Pawłowski), MP (M. Pokorny), DLP (DL. Porazinska), SS (S. Sikorska), PS (P. Sommers), NO (N. Takeuchi), JU (J. Uetake), KZ (K. Zawierucha).

Region	Glacier	Typ. gl.	Ther. reg.	Light fluct.	Biog.	GPS	Ts.	N	Alt. (approx.)	date	Pres.	Nem.	Tar.	Rot.	Coll.	Inv.
Svalbard	Area of Gooskbukta	outlet	polyt	season	Pal	80°13' N, 19°51' E	1	10	N/A	17.07.2018	freez	-	+	+	MD	MD
Svalbard	Nordre Franklinbreen	tidwat	polyt	season	Pal	80°04' N, 19°27' E	1	10	N/A	18.07.2018	frezz	_	+	+	MD	MD
Svalbard	Dunérbreen	valley	polyt	season	Pal	79°49' N, 16°51' E	1	10	N/A	22.07.2018	freez	-	+	+	MD	MD

Svalbard	Midtre Lovénbreen	vall	polyt	season	Pal	78°53'N 11°58' E	1	2	N/A	18.07.2019	freez	-	+	+	RA	KZ, JB
Svalbard	Nordenskiöld	tidwat	polyt	season	Pal	78°40' N, 17°08' E	2	2	300	7.9.2016	freez	-	+	+	TJ	KZ, TJ
Svalbard	Sven	vall	polyt	season	Pal	78°43' N, 16°16' E	2	4	430	7.8.2016	freez	-	+	+	TJ	KZ, TJ
Svalbard	Ebba	vall	polyt	season	Pal	78°41' N, 16°49'	2	14	430	7.8.2016	alc,	-	+	+	TJ	KZ, TJ
Alps	Forni	vall	temp	daily	Pal	46°23' N, 10°35' E	2	10	2650	Summer, 2019	freez	-	+	-	KZ	SS,AK, TJ
Tienshan	Miaregou Glacier	vall	temp	daily	Pal	43°08' N, 94°11' E	1	20	4330	8.2003	freez	-	+	+	NT	NT
Tienshan	Urumqi No.1 Glacier	vall	temp	daily	Pal	43°06' N, 86°48' E	1	40	3825	8.2017	freez	-	+	+	NT	NT
The Caucasus	Gergeti	vall	temp	daily	Pal	42°40' N, 44°32' E	2	3	3400 - 3600	25.09.2019 r.	alc	-	-	-	AK	KZ, JB, MO
Tienshan	Grigoriev ice cap	Ice cap	temp	daily	Pal	41°58' N, 77°55' E	1	30	4250	8.2007	freez	-	+	+	NT	NT
Pamir-Alay	Lenin Glacier	vall	temp	daily	Pal	39°24' N, 72°51' E	1	30	4350 - 4400	8.2016	freez	-	+	+	NT	NT
Qilian Mts.	Qiyi Glacier	vall	temp	daily	Pal	39°14' N, 97°45' E	1	30	4830	9.2005	freez	-	+	+	NT	NT
Karakorum	Baltoro	vall	temp	daily	Indo	35°41' N, 76°38' E	1	3	5200	6, 7.2013.	alc	-	+	-	RA	KZ
India	Kang Yatse	vall	temp	daily	Indo	33°45' N, 77°36' E	1	4	5620	8.2017	alc	-	+	+	MD	MD, KZ
India	Chamser Kangri	vall	temp	daily	Indo	32°59' N, 78°26' E	1	3	5800	9.2017	dried	-	+	+	MD	MD, KZ
Himalayas	Yala	vall	temp	daily	Indo	28°14' N, 85°36' E	1	10	5250	8.2008	form	-	+	+	NT	NT
Africa	Kersten (Kilimanjaro)	n/a	n/a	daily	Etiop	3°04' S, 37°21' E	1	3	5850	09.2019	alc	-	-	+	UF	KZ, JB

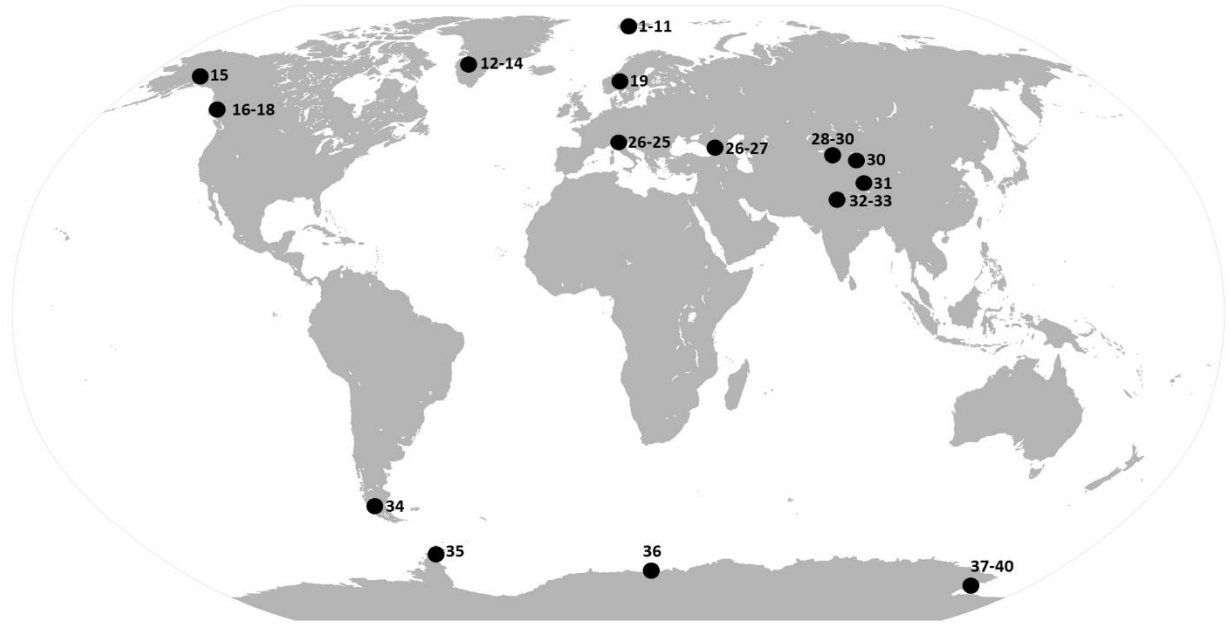

Figure S8.1 List of previously studied glaciers in terms of invertebrates. For details see Table S1. Svalbard: 1 – Sörbreen, 2 – Tryggvebreen, 3 – Buchanbreen, 4 - Midre Lovénbreen, 5 – Waldemarbreen, 6 – Horbybreen, 7 – Ebbabreen, 8 – Nordenskioldbreen, 9 – Longyearbreen, 10 – Hansbreen, 11 – Hyrnebreen, Greenland: 12 – Thule area, 13 – Disko Island and western coast, 14 – Edge of Greenland Ice Sheet, Alaska: 15 – Gulkana, Canada: 16 – White Glacier, 17 – Casement, 18 – Manatee, Scandinavia: 19: - Finse, the Alps: 20 – Sulztalerferner, 21 – Niederjochferner, 22 – Marzellferner, 23 – Langtalerferner, 24 – Ramolferner, 25 – Forni, the Caucasus: 26 – Chalaati, 27 – Adishi, Tien Shan: 28 – Urumqi No.1, 29 – Grigoriev Ice Cap, 30 – Miaoergou, Quilian Shan – 31 - Qiyi, Himalaya: 32 – Yala, 33 – Nare Glacier, the Andes: 34 – Tyndall, Maritime Antarctic: 35 – Ecology Glacier, King George Island, South Shetland Islands, Continental Antractic: 36 – Queen Maud Land, 37 – Edward VII Penisula, 38 – Canada, 39 – Commonwealth, 40 – Howard.

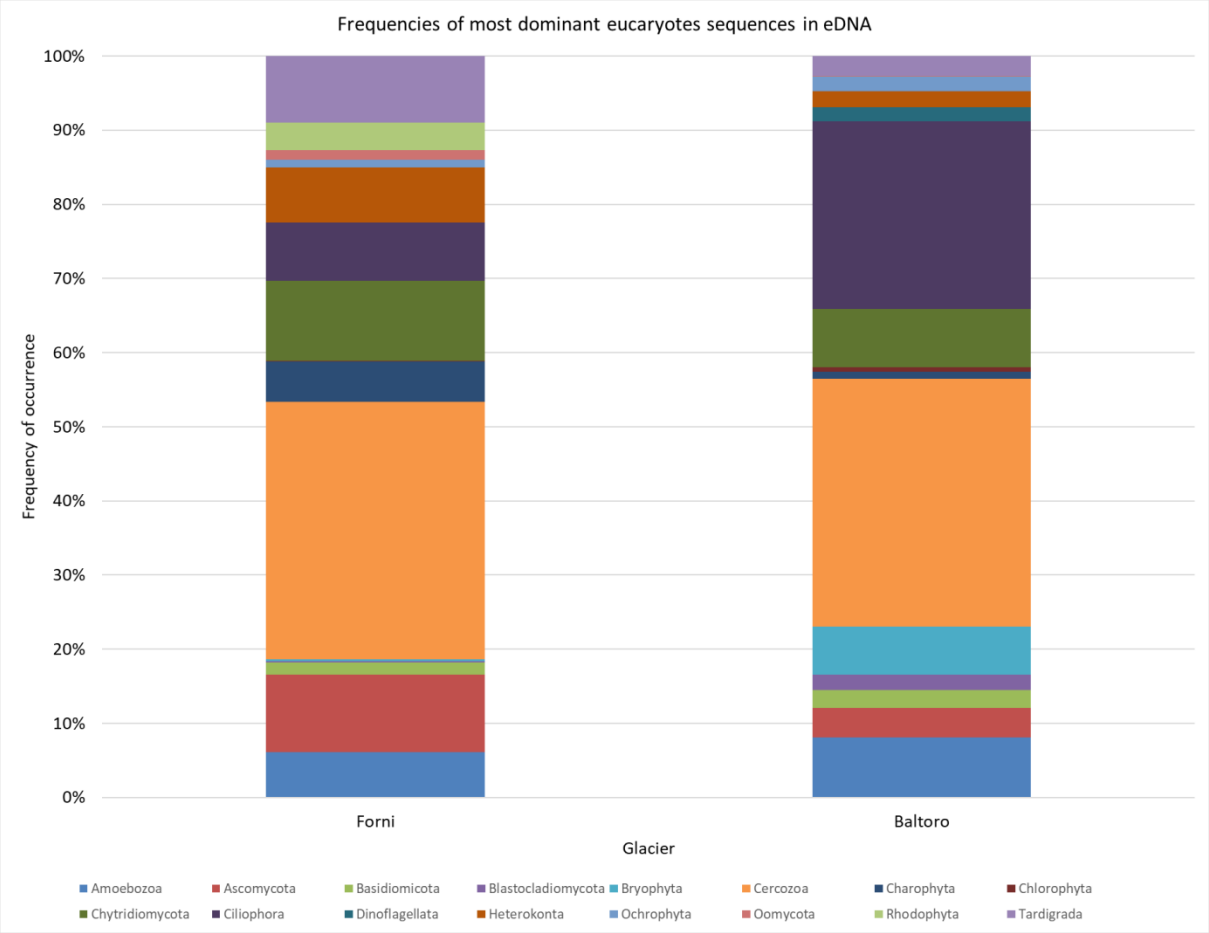

Figure S8.2 The most dominant eukaryotic taxa (constituting more than 1% of all sequences) detected in eDNA using 18S rDNA fragment from Baltoro in Karakoram and Forni in the Alps.

*Table S.8.2 Literature records of invertebrates in cryoconite holes. * - records of nematodes in cryoconite holes (both come from McMurdo Dry Valleys, Canada Glacier), 1 – classical microscope observations, 2 – eDNA analysis, # - pure taxonomic/faunistic reports/papers, ^ - new species descriptions or faunistic reports without notes on other faunal elements*

Biogeographic realm	Region	Locality	Animals				References
Arctic	**Svalbard**		**Nematoda**	**Rotifera**	**Tardigrada**	**Other animals**	
		Sörbreen, Tryggvebreen	-	+	+		*Dastych 1985[1#]*
		Hyrnebreen	-	+	+		*De Smet and Van Rompu 1994[1]*
		Midre Lovénbreen	-	+	-		*Säwström et al. 2002[2]*
		Nordenskiöldbreen, Ebbabreen, Hørbyebreen	-	+	+		*Vonnahme et al. 2015[1], Zawierucha et al. 2016b[1]*
		Hansbreen	-	+	+		*Zawierucha et al. 2016a[1#], b[1], Łokas et al. 2016[1]*
		Buchanbreen, Waldemarbreen	-	+	+		*Zawierucha et al. 2016a[1#]*,
		Longyearbreen	-	+	+		*Zawierucha et al. 2019b[1], c[1]*
	Greenland						
		Unidentified	-	+	+		*Drygalski 1897[1]*

		Unidentified	-	-	+		*Jensen 1928*[1]
		Thule area	-	+	+		*Gerdel & Drouet 1960*[1]
		Western coast	-	+	+		*Grøngaard et al. 1999*[1]
			-	-	+		*Séméria 2003*[1#]
		South-West	-	+	+		*Zawierucha et al. 2018a*[1]
	Canada	White	-	-	+		*Mueller et al. 2001*[1]
Subarctic		Casement	-	-	-	Oligochaeta	Goodman 1971[1]
		Manatee	-	-	-	Oligochaeta	McIntyre 1984[1]
	Scandynavia (Norway)	Finse	-	-	+		*Sømme 1996*[1]
Temperate	**the Alps**	Sulztalerferner, Niederjochferner, Marzellferner	-	+	+		*Wittrock 1885*[1], *Steinböck 1936*[1], *Mihelčič 1959*[1]
		Niederjochferner, Marzellferner	-	+	+		*Dastych et. al. 2003*[1], *Kraus 1977*[1], *Mihelčič 1963*[1]
		Langtalerferner, Ramolferner	-	-	+		*Dabert et al. 2015*[1]

		Forni	-	-	+		*Zawierucha et al. 2019a*[1]
	The Caucasus	Adishi	-	-	-	Collembola	*Makowska et al. 2016*[1]
		Chaalati	-	-	-		*Makowska et al. 2016*[1]
	Tien Shan	Ürümqi No.1, Miaoergou, Grigoriev Ice Cap	-	-	+		*Zawierucha 2018b*[1]
	Quilian Shan	Qiyi	-	-	+		*Zawierucha 2018b*[1]
	The Himalaya	Yala	-	+	+	Insecta:Chironomidae, Crustacea: Copepoda	*Kohshima 1984*[1], *Kikuchi 1994*[1], *Takeuchi et al. 2000*[1]
		Nare Glacier	-	-	+		*Ramazzotti 1968*[1], *Dastych 2004a*[1], *b*[1]
South America	**the Andes**	Tyndal	-	+	-	Collembola, Plecoptera	*Takeuchi and Kohshima 2004*[1]
Antarctica	**Maritime Antarctic**	Ecology Glacier	-	+	-	Mites	*Zawierucha et al. 2019b*[1], *Buda et al. 2019*[1]
Antarctica	**Continental Antarctic**	Queen Maud Land	-	+	+		*Lutz et al. 2019*[2]
		Edward VII Peninsula, Marie Byrd Land	-	+	-		*Broady 1989*[1]
		McMurdo Dry Valleys, Canada Glacier	+	+	+		*Mueller et al. 2001**[1], *Christner et al. 2003**[1]; *Sommers et al. 2018; 2019*[1,2]

	McMurdo Dry Valleys: Canada, Commonwealth, Howard	-	+	+	Plathelminthes	*Porazińska et al. 2004*

References Table S8.2

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Table S8.3 MOTUs (molecular operational taxonomic units) recovered from eDNA analysis on Forni and Baltoro samples

Kingdom	N MOTUs	N reads
Animals	8	80 830
Plants	29	204 529
Chromista	28	94 476
Protista	172	766 586
Fungi	151	541 540
Unassigned	92	183 972

9. GLACIER ALGAE FOSTER ICE-ALBEDO FEEDBACK IN THE EUROPEAN ALPS

The content of this chapter has been published in the following paper:
Mauro, B Di, R Garzonio, G Baccolo, A Franzetti, F Pittino, B Leoni, and D Remias. 2020. "Glacier Algae Foster Ice-Albedo Feedback in the European Alps." *Scientific Reports*, 1–9. https://doi.org/10.1038/s41598-020-61762-0.

ABSTRACT The melting of glaciers and ice sheets is nowadays considered a symbol of climate change. Many complex mechanisms are involved in the melting of ice, and, among these processes, surface darkening due to organic material on bare ice has recently received attention from the scientific community. The presence of microbes on glaciers has been shown to decrease the albedo of ice and promote melting. Despite several studies from the Himalaya, Greenland, Andes, and Alaska, no quantitative studies have yet been conducted in the European Alps. In this paper, we made use of DNA sequencing, microscopy and field spectroscopy to describe the nature of glacier algae found at a glacier (Vadret da Morteratsch) of the European Alps and to evaluate their effect on the ice-albedo feedback. Among different algal species identified in the samples, we found a remarkable abundance of *Ancylonema nordenskioeldii*, a species that has never previously been quantitatively documented in the Alps and that dominates algal blooms on the Greenland Ice Sheet. Our results show that, at the end of the ablation season, the concentration of *Ancylonema nordenskioeldii* on the glacier surface is higher than that of other algal species (i.e. *Mesotaenium berggrenii*). Using field spectroscopy data, we identified a significant correlation between a reflectance ratio (750 nm/650 nm) and the algae concentration. This reflectance ratio could be useful for future mapping of glacier algae from remote sensing data exploiting band 6 (740 nm) and band 4 (665 nm) of the MultiSpectral Instrument (MSI) on board Sentinel-2 satellite. Here we show that the biological darkening of glaciers (i.e. the bioalbedo feedback) is also occurring in the European Alps, and thus it is a global process that must be taken into account when considering the positive feedback mechanisms related to glacier melting.

9.1. Introduction

Glaciers and ice sheets are not lifeless (Anesio et al. 2012). It has been demonstrated that several species of microorganisms, algae and small arthropods find their optimal environment on melting ice and snow. These organisms are also able to shape their environment, through a feedback cycle that involves the albedo. In fact, since these organisms are darker than snow and ice, their presence increases the light absorption and promotes the melting of underlying snow or ice (Nordenskiöld 1883; Takeuchi et al. 2001; Di Mauro et al. 2017). On the surface of glaciers, such extremophiles often aggregate with inorganic material (i.e. mineral dust) to form cryoconite (Bøggild et al. 2010; Cook et al. 2016b). Cryoconite is a dark sediment (Cook et al. 2016b; Fountain et al. 2004b) that increases the absorption of visible radiation

(Di Mauro et al. 2017; Takeuchi 2009), and promotes the formation of characteristic cryoconite holes (Takeuchi 2002) which are globally recognized as an hot spot of biodiversity on ice (Stibal et al. 2012a). Besides living organisms, cryoconite is known to concentrate pollutants such as contaminants and radionuclides (Tieber et al. 2009; Baccolo et al. 2017). This may represent a problem in the future, with a possible secondary release of these substances to the environment

(Ferrario et al. 2017; Owens et al. 2019). Cryoconite accumulated in cryoconite holes has a limited impact on glacier and ice sheet surface mass balance. Instead, the presence of distributed organic material on the margin of ice sheets recently motivated the formalization of the concept of "bioalbedo feedback" (Cook et al. 2017; Cook et al. 2017; Kohshima et al. 1993). This feedback is associated with the spatial distribution of organic material that increases the absorption of light, promotes the phase transition of ice, produces a film of meltwater on snow- and ice-fields, and allows the growth of the algal population (Williamson et al. 2018; Williamson et al. 2019). The decrease of ice albedo has important consequences on glacier mass balance (Oerlemans et al. 2009; Naegeli et al. 2017) and represents an active field of research (Naegeli et al. 2019; Fugazza et al. 2019). Recent literature focused on the impact of algae on snow and ice in the Greenland Ice Sheet (Stibal et al. 2017; S. Wang et al. 2018; Ryan et al. 2018), Iceland (Lutz et al. 2015), Norway (Lutz et al. 2016), Himalaya (Takeuchi et al. 2010; Kohshima et al. 1993), Alaska (Ganey et al. 2017; Takeuchi et al. 2006), Sierra Nevada (Painter et al. 2001), Andes (Takeuchi et al. 2004) and Antarctica (Huovinen et al. 2018). These studies highlighted the presence of different species of algae on snow and ice, and their role in the albedo decrease. While recent literature focusing on the southwest margin of the Greenland Ice Sheet identified a strong impact of glacier algae on the optical properties of ice (Williamson et al. 2018; Stibal et al. 2017; Ryan et al. 2018; Tedstone et al. 2017; Cook et al. 2020), this phenomenon remains largely unexplored in the European Alps. In the Alps, most of the studies deal with the characterization of communities living in melting snow (Remias et al. 2005). In contrast, studies of glacier surfaces (i.e. bare ice after snow melting) are sparse. Recently, the glacier alga *Mesotaenium berggrenii* was described in Austrian glaciers (Remias et al. 2012a; Remias et al. 2009). Kol (1938) first reports about the filamentous alga *Ancylonema nordenskioeldii* in a glacier close to the Mont Blanc. The latter species is well known from polar icefields (Williamson et al. 2018; Stibal et al. 2017; Remias et al. 2012b), but its presence in Europe was questionable (Anesio et al. 2017). The impact of glacier algae on ice albedo can be studied through spectroscopy data collected both in the field (Stibal et al. 2017) and from aerial (Ryan et al. 2018) and satellite sensors (Wang et al. 2018). While field spectroscopy data are fundamental for assessing the local impact of glacier algae on the optical properties of ice (Stibal et al. 2017), remotely sensed data can provide a synoptic view of the phenomenon. In particular, the launch of new satellite missions such as Sentinel-2 and Sentinel-3, from the European Space Agency (ESA) Copernicus program, has created new opportunities for the study of the cryosphere from space (Malenovský et al. 2012; Kokhanovsky et al. 2019). The spatial, spectral and temporal resolution of

these missions allows the monitoring of changes in both alpine and polar glaciers. Sentinel-2 is particularly suited for mapping spatial and temporal variability of the cryosphere at fine scale (Paul et al. 2016), while Sentinel-3 allows a broader perspective (Kokhanovsky et al. 2019). Some studies have already exploited these data for mapping algae distribution in Maritime Antarctica (Huovinen 2018) and Southwest Greenland (Wang et al. 2018; Cook et al. 2020). The objectives of this paper are to identify the algae living on the surface of an Alpine glacier, and to determine their impact on the optical properties of ice. We addressed these objectives with data collected during a survey at the Vadret da Morteratsch Glacier in the Swiss Alps. This glacier has been the focus of numerous studies in recent decades (Di Mauro et al. 2017; Oerlemans, Giesen et al. 2009; Oerlemans et al. 2002; Zekollari et al. 2018). In particular, Oerlemans et al. (2009) identified a decreasing trend in the albedo of bare ice and attributed it to the accumulation of dust from lateral moraines. More recently, Di Mauro et al. (2017) demonstrated that the presence of high concentrations of elemental and organic carbon may have contributed to the albedo decrease on this glacier. Here, we report the identification of glacier algae through DNA sequencing of samples collected on the Morteratsch Glacier at the end of the ablation season on September 2016. Furthermore, we measured the impact of glacier algae on the optical properties of ice using near surface reflectance measurements collected with a field spectrometer, and we identified a reflectance ratio that was correlated with algae density. The spectral measurements were then resampled at the same spectral resolution as the ESA MultiSpectral Instrument (MSI) onboard Sentinel-2 to evaluate the potential of global satellite mission observations for mapping the spatial and temporal distribution of algae in the alpine environment.

9.2. Materials and methods

9.2.1. Field spectroscopy and sampling

On September 13th 2016, a campaign was conducted on the ablation zone of the Vadret da Morteratsch Glacier (46°24’34”N, 9°55’54”E), an alpine valley glacier located in the Bernina Massif (4049 m a.s.l, Raethian Alps, Switzerland-Italy). Morteratsch is a large glacier (area ~7.5 km2) in the Bernina range, with an altitudinal range of ~2000 m. The glacier snout is located at 2100 m a.s.l. The glacier is characterized by a continental climate, with ablation seasons that can last up to three months during warm summers. This glacier has been extensively studied in recent years (Di Mauro et al. 2017; Oerlemans et al. 2009; Paul et al. 2016; Zekolari et al. 2018). Morteratsch Glacier has been rapidly retreating(Oerlemans et al. 2009), and it represents the perfect test bed for studying the impact of glacier algae in the European Alps. During the campaign, field spectroscopy data were

measured with a HandHeld Analytical Spectral Devices (ASD) Field Spec (spectral range = 325–1075 nm, spectral sampling interval = 1 nm). The hemispherical conical reflectance factor (HCRF) was calculated by normalizing the reflected radiance with the incident radiance measured from a calibrated Spectralon© panel. Each acquisition was the average value of 15 spectra. A levelled bare optical fiber (field of view = 25°) was used to collect data at 80 cm from the ice surface (footprint diameter = 35 cm). All measurements were collected around midday for minimizing the effect of the changing solar illumination. Further details on the methodology can be found in previous papers (Di Mauro et al. 2017; Di Mauro et al. 2015). Spectral reflectance was acquired at 18 sampling points distributed on bare ice in the ablation zone of the glacier. For each sampling point, we collected surface material in correspondence of the spectrometer field of view. Since a strong variability of ice properties can be found on the ablation area of Alpine glaciers, the sampling methodology represents a complexity in spectroscopy of ice. For this reason, we paid much attention in sampling ice exactly from the area measured by the ASD field spectrometer. From reflectance data, we calculated all possible spectral ratios and we created a series (n = 423801) of linear regression models between different spectral indexes and the algal density. This variable selection analysis has been already used for other type of impurities (Di Mauro et al. 2015), and it is useful for identifying hot spot of correlation in spectral data. We resampled (i.e. averaged) the reflectance measured with the ASD spectrometer to the spectral resolution of Sentinel-2. This sensor features a high spatial resolution (up to 10 m in the visible spectrum) (Drusch et al. 2012) and it is promising for mapping glacier algae distribution from space (Huovinen et al. 2018). Furthermore, we calculated the continuum removal (between 655 and 700 nm) at the Chlorophyll-a absorption feature located at 680 nm (Painter et al. 2001). The continuum removal quantifies the absorption features at specific wavelengths, normalizing the reflectance spectra to a common baseline (Clark et al. 1984). This is achieved by approximating the continuum between local spectral maxima through straight-line segments: a value of 1 is assigned to the local maxima, and a value between 0 and 1 is obtained in correspondence of the absorption features. The continuum removal calculated at 680 nm was then directly compared with algal concentration through linear regression analysis.

9.2.2. Cell counting

Eighteen samples of ice were placed inside 50 ml conical bottomed phials. These were kept in freezing condition and transported to Milano-Bicocca University, where they were preserved at −30 °C. For the laboratory analysis, the samples were completely thawed at room temperature. The algal classification by light microscopy and count were performed on fresh material. Identification keys

(Huber-Pestalozzi 1972) and literature (Stibal et al. 2017; Remias et al. 2005; Remias et al. 2009; Remias 2012; Hoham et al. 2020) were used to identify the algae. The organisms were counted by inverted microscopy (400× magnification) and a camera, pictures were analyzed using AxioVision software, density and biovolume were estimated as reported in literature (Marti et al. 2016). In order to characterize the size of the identified organisms, we measured the length and width of at least 50 cells for each species (Rott, 1981). Using a Whatman GF/C (1.2 µm) glass fiber filter we also quantified the total inorganic sediments contained in samples collected on the Morteratsch Glacier.

9.2.3. DNA extraction and sequencing

Ice was melted and centrifugated at 12000 × g for 2 minutes and the supernatant was discharged. The pellet was resuspended in 978 µL of Sodium Phosphate Buffer and 122 µL of MT Buffer of the FastDNA Spin for Soil kit (MP Biomedicals, Solon, OH) and the DNA was then extracted according to the manufacturer's instructions. Samples volume varied from 9 to 24 mL of melted ice. Further samples were collected from the bottom of nine cryoconite holes found on the Morteratsch Glacier. The DNA composition of these samples was analyzed and compared with samples from surface ice DNA extraction from the cryoconite holes samples was performed with the same kit, as for surface ice samples, from 0.7 g of sample, according to the manufacturer's protocol. The V4-V5 hypervariable region of the 18S rRNA gene was amplified using the eukaryotic primers 528F and 706 R (Cheung et al. 2010). A first DNA amplification was performed for each sample to evaluate its quality on the original ad on the 1:10 dilution to identify inhibition or insufficient sample. The regions were sequenced with MiSeq Illumina (Illumina, Inc., San Diego, CA) with a 2 × 250 bp paired-end protocol and Operational Taxonomic Units (OTUs) were defined with an aggregative clustering of sequences with 99% of sequence identity for the 18S rRNA gene fragment. To prepare the libraries for sequencing, a PCR was performed on the samples with GoTaq® Green Master Mix (Promega Corporation, Madison, WI) and 1 µM of each primer, for a final volume of 50 × 2 µL each. Illumina adapters (6 bp) were added at 5' end. The cycling conditions for the 18S rRNA fragment were: initial denaturation at 95 °C for 4 min; 30 cycles at 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s and a final extension at 72 °C for 5 min. Then amplicons were purified with (Wizard® SV Gel and PCR Clean-up System, Promega Corporation, Madison, WI) and quantified with Qubit® (Life Technologies, Carlsbad, CA, USA). Library preparation with the addition of standard Nextera indexes (Illumina, Inc., San Diego, CA, USA) and sequencing were carried out at Parco Tecnologico Padano (Lodi, Italy).

9.2.4. DNA sequence processing and statistical analyses

The obtained reads were demultiplexed according to the indexes. Forward and reverse reads were merged only if without mismatches. Taxonomic assignation of the 18S rRNA sequences was performed by BLAST (Basic Local Alignment Search Tool) comparison against the SILVA 132 SSURef Nr 99 database (Quast et al. 2013) assigning the sequences to the "best hit". The ten most abundant OTUs were assigned by BLAST (Altschul et al. 1990). *A. nordenskioeldii* was the most predominant alga in all the samples, but conventional clustering methods sometimes were not able to distinguish between *A. nordenskioeldii* and *Mesotaenium* species (Lutz et al. 2018). Thus, the oligotyping pipeline (Eren et al. 2013) was used on the *A. nordenskioeldii* sequences. Singletons (OTUs present once in one sample only) were removed from the analyses because their inclusion could inflate variance explained by multivariate analysis (Legendre et al. 2012). Analyses were performed with R 3.4.2 (R Core Team, 2014), with the VEGAN, BIODIVERISTYR, MULTTEST, MULTCOMP packages. We normalized algal OTUs abundance to 100 to compare sequences of different samples and Hellinger-transformed them. Redundancy Analyses (RDA) were performed to see which variables significantly explained the communities variation, correcting P-values for multiple testing according to the false discovery rate (FDR) procedure (Benjamini et al. 2001).

9.3. Results and discussion

9.3.1. Population densities

Among the algal species identified on the ablation zone of the Vadret da Morteratsch by light microscopy (Fig. 9.1g), *Ancylonema nordenskioeldii, Mesotaenium berggrenii* and *Sanguina nivaloides* were the most representative. We point out the rediscovery of *A. nordenskioeldii* (Fig. 9.1a–c) in the European Alps, with a mean density of 2.4×10^4 cells ml^{-1}. *A. nordenskioeldii* features a different size (cell length = 30.7 ± 5.7 µm and cell width = 12.7 µm ± 1.0 µm) from those of the polar regions, whose cells have smaller lengths (Remias et al. 2012b; Uetake et al. 2010), and from the Chilean ones, which feature larger lengths (Takeuchi et al. 2004). *M. berggrenii* was present with two distinct non-filamentous varieties (Fig. 9.1d,e): the *alaskana* variety (Rossini et al. 2018), easily distinguishable by a single chloroplast characterizing cells after division, was the most widespread species on the glacier with average density of 6.7×10^4 cells ml^{-1}. For both glacier algae only vegetative cells were present, and no zygotes were observed. Red spherical cysts resembling *S. nivaloides* (Fig. 9.1f) were found in 6 out of 18 samples, with an average density of 767 cells ml^{-1}. The average algal cell density found in this work (i.e. 20.3×10^4 cells ml^{-1}) is comparable with those found in South-West Greenland (Stibal et al. 2017; Yallop et al. 2012). Thus, we might expect that

the algae density observed on Vadret da Morteratsch Glacier will have an effect on optical properties similar to that already observed on polar glaciers (Stibal et al. 2017; Cook et al. 2020).

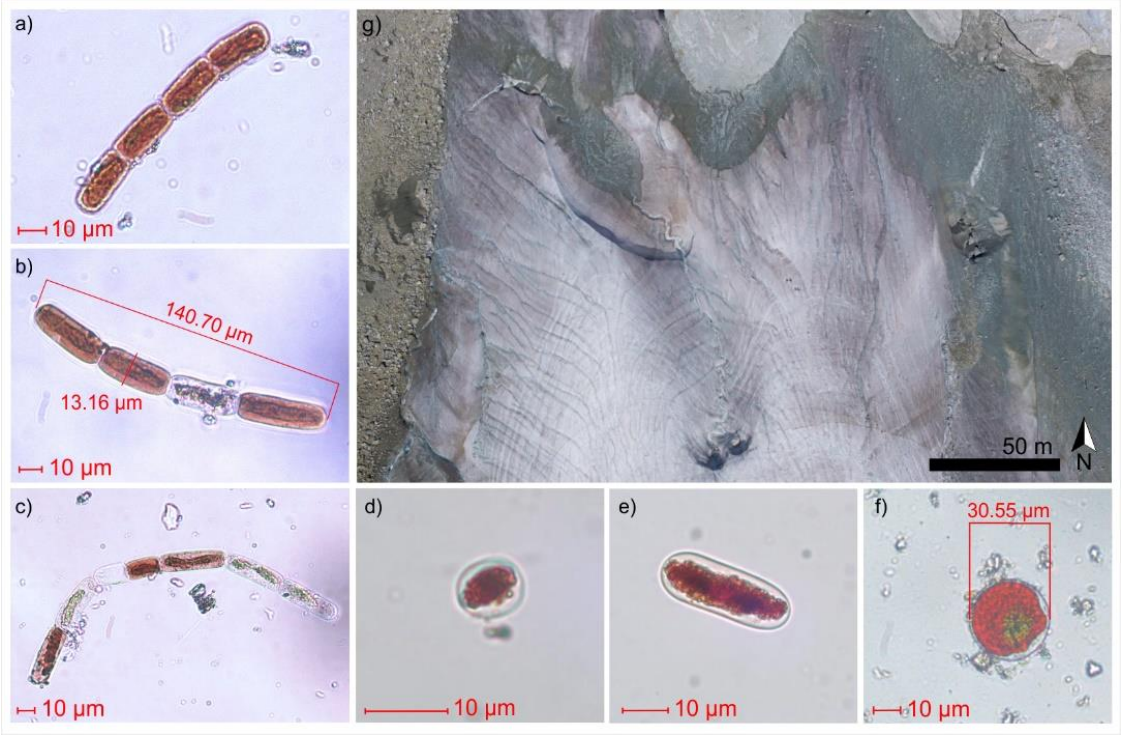

Figure 9.1 (a–f) Light micrographs of algae found at the glacier. (a–c) Filaments of Ancylonema nordenskioeldii. (d,e) unicellular Mesotaenium berggrenii. (f) red cyst of putative Sanguina nivaloides (g) Aerial view of the Morteratsch glacier tongue acquired in September 2016 from an Unmanned Aerial Vehicle (UAV) (Rossini et al. 2018).

9.3.2. DNA sequencing

The full classification and the relative abundance of algal Operational Taxonomic Units (OTUs) are reported in Table S1. Results show that algae were relatively more abundant in surface ice than in cryoconite samples (Fig. 9.2a). Moreover, the composition of glacier algal communities was different from those in cryoconite holes. Particularly, algal communities in ice were dominated by *Ancylonema nordenskioeldii* with an average abundance of 70%, whereas in cryoconite holes members of Trebouxiophyceae were the dominant taxa (Fig. 9.2b). Redundancy analysis (RDA) showed that algal community structure varied significantly according to the type of sample (cryoconite holes or ice surface) (Table S9.2). Indeed, the RDA plot (Fig. 9.2c) shows that samples cluster according to the supraglacial habitats. Figure 9.2c also shows that the cryoconite and ice samples are distributed along two parallel directions. This suggests that a factor not investigated in this study (e. g. irradiation, pH, total organic carbon, nutrients concentration) may describe part of the unexplained variance of the algal community (Holland et al. 2019). Given the complexity of the supraglacial environment, even the relatively low explained variance (i.e. 36.8%) can be considered satisfactory (Møller et al. 2002). We remark that RDA analysis was conducted using only the variable "supraglacial habitats" (i.e. ice surface or cryoconite hole). Generalized linear models (GLMs)

performed on the three most abundant algae showed that the abundance of *Ancylonema nordenskioeldii*, *Mesotaenium berggrenii var. alaskana* and the Unclassified Trebouxiophyceae changed according to the type of sample. In particular, *Ancylonema nordenskioeldii* (Fig. S9.2) and *Mesotaenium berggrenii var. alaskana* (Fig. S9.2) were more abundant in surface ice than in cryoconite holes ($F_{1,26} > 7.08$; $P_{FDR} < 0.006$), the opposite occurred for the algae belonging to the Trebouxiophyceae (Fig. S9.3) ($F_{1,26} = 21.66$; $P_{FDR} < 0.006$). The taxonomic affiliation of the most abundant OTUs is reported in Table S9.3. The results show that most of the eukaryotes living on bare ice are algae, while in cryoconite holes the eukaryotic community is more heterogeneous. One possible explanation could be that glacier algae are well adapted to the surface ice environment and thus able to develop blooms during the melting period. For example, algae such as *Ancylonema nordenskioeldii* and *Mesotaenium berggrenii var. alaskana* produce dark phenolic pigments to protect them from the high solar radiation (Stibal et al. 2012a). This is consistent with the fact that these species are more abundant on the bare ice than Trebouxiophyceae which lack these secondary pigments (Leliaert et al. 2012; Lutz et al. 2018). The dark secondary pigmentation of *Ancylonema nordenskioeldii* and *Mesotaenium berggrenii var. alaskana* may explain the variation in ice reflectance (Uetake et al. 2010), which will be discussed in the next section.

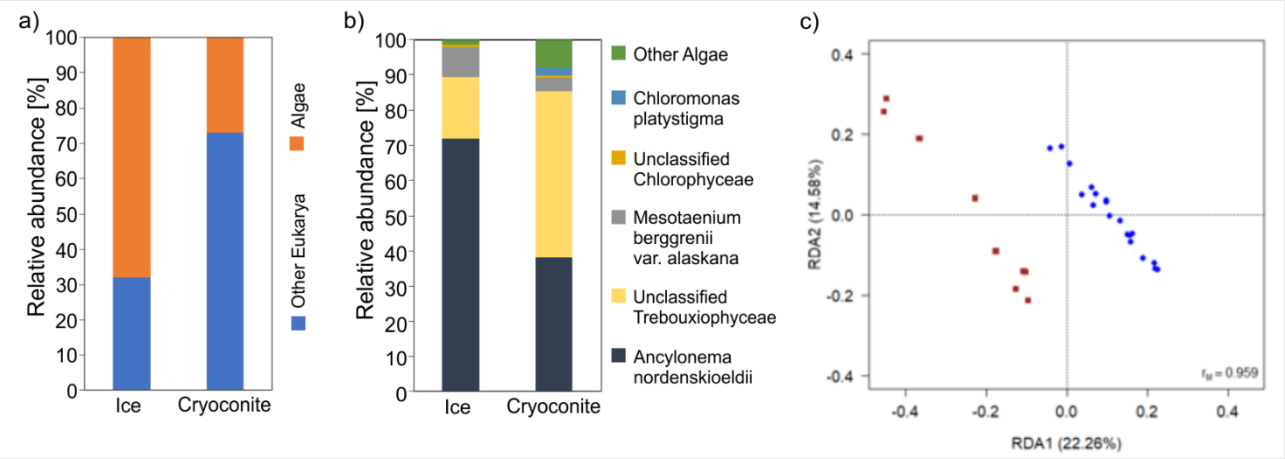

Figure 9.2 (a) Relative operational taxonomic units (OTUs) abundance of algae and other Eukarya in both ice surface and cryoconite holes samples. (b) Relative abundance of the algal OTUs grouped in taxa (taxa whose abundance was lower than 1% were grouped in "Other Algae") (c) Biplot from RDA on Hellinger-transformed algal OTU abundance. Each point represents one sample. The analysis includes ice surface samples (blue dots) and cryoconite hole samples (brown squares).

9.3.3. Impact of glacier algae on the optical properties of ice

The presence of algae on bare ice causes a decrease of the reflectance at wavelengths shorter than 750 nm because of the absorptions by several intracellular pigments (e.g. Chlorophyll-a, Chlorophyll-b, photosynthetic carotenoids, photoprotective secondary carotenoids, phenols etc.) (Cook et al. 2017; Dauchet et al. 2015) (Fig. 9.3a). As previously showed for Greenland (Stibal et al. 2017), different absorption features can be recognized in the spectra of bare ice containing algae.

In particular, an absorption located at 680 nm is usually linked to the presence of Chlorophyll-a. This feature was observed also in the Alaska's Harding Icefield (Ganey et al. 2017; Takeuchi et al. 2006) and in the Yosemite National Park (Painter et al. 2001). The specific photosynthetic absorption of algae in the visible wavelengths has been exploited for their estimation from satellite remote sensing data (Wang et al. 2018; Ganey et al. 2017; Takeuchi et al. 2006). In Fig. 9.3a we show the reflectance spectra of surface ice samples with varying algal abundance. The total concentration of algae spanned from 0.2×10^5 cell/mL (sample ID: SP16) to 2.9×10^5 cell/mL (sample ID: SP20). Photographs of the area measured with the field spectrometer and then sampled for the analysis are showed in Fig. 9.3b. A variable selection approach was developed to identify the reflectance ratio index that correlated the most with algae concentration. A hot spot of correlation between spectral ratios and concentration of algae was found at wavelengths between 600 and 700 nm. In particular, the red-edge ratio between reflectance at 750 nm and 650 nm resulted in the highest correlation coefficient (R2~0.6). We compared our correlation hot spot with other indices proposed by Takeuchi *et al*.(2006) (T06 in Fig. 9.4a) and Wang *et al*.(2018) (W18 in Fig. 9.4a). The correlation coefficient for these two indices was respectively 0.4 and 0.5. However, these indices were developed for tracking variations in algal abundance from satellite platforms, thus using sensors characterized by a different spectral resolution. In particular, W18 index was calculated using the relatively high spectral resolution of the Ocean and Land Colour Instrument (OLCI) sensor on board the Sentinel-3 platform. Despite the relatively high correlation found between W18 and algal density, the use of Sentinel-3 for mapping the algae spatial and temporal distribution in alpine areas is hampered by its too coarse spatial resolution (300 m pixel size). Conversely, the use of the Sentinel-2 satellite characterized by a high spatial resolution (10 to 20 m pixel size) has proved successful for studying alpine glaciers (Paul et al. 2016; Naegeli et al. 2017). Reflectance measurements collected with the field spectrometer were resampled (i.e. averaged) using the bandwidth of the MSI on board the Sentinel-2 platform. The reflectance ratio that best correlated with the algal density (R2 = 0.53, p-value = 0.001) was the one corresponding to Sentinel-2 band 6 (centered at 740 nm) and band 4 (centered at 665 nm) (Fig. 9.4b). No significant relation was found between the 740/665 nm ratio and the inorganic sediments found in surface ice (R2 = 0.07, p-value >0.05). This result fosters the use of this reflectance ratio for mapping glacier algae. Reflectance ratios of wavelengths across the red edge position are established methods for mapping autotrophic life from satellite data (Filella et al. 1994). We here propose the use of the spectral ratio between the reflectance at 740 nm and 665 nm as a useful tool for mapping the presence of algae on ice

using remote sensing data collected by different platforms (e.g. satellite sensors, airborne sensors and unmanned aerial vehicles). The relatively weak correlation found between the reflectance ratio and the algae concentration (Fig. 9.4b) may be explained by other variables related to glacier ice that are not considered in this paper, such as the presence of mineral dust, soot, melt water, grain size, ice density etc. In particular, the reflectance ratio proposed in this paper shows a higher scattering for algal densities greater than 3 × 105 cell/mL. This represents a source of uncertainty in using the reflectance ratio to estimate algal abundance over wider areas using remote sensing. Further research is needed to validate the application of this method at alpine or polar scales. We exploited an additional approach to evaluate the correlation between the reflectance spectra and the algal abundance, i.e. the continuum removal applied on the Chlorophyll-a absorption feature at 680 nm. We found a significant correlation (R2 = 0.52, p-value = 0.001) between the continuum removal and the concentration of algae in surface ice samples. The absorption feature of Chlorophyll-a due to algae is very narrow (Fig. 9.3a) and can be resolved only from hyperspectral data. Thus, this index can be proposed for mapping algae from high spectral resolution unmanned aerial systems (Garzonio et al. 2017) and hyperspectral satellite data (Labate et al. 2009; Di Mauro et al. 2017). In contrast, the reflectance ratio that we propose in this paper requires less sophisticated measurements to be calculated. While for the Greenland Ice Sheet, it has been demonstrated that the biological darkening of ice is more important than the inorganic one , this may not hold true for nonpolar glaciers, where the availability of mineral impurities from the proglacial area and from melting ice can induce a stronger albedo feedback. The decoupling of the impact of organic and inorganic particles on the optical properties of ice is still unresolved. In this context, the integration of hyperspectral imaging data with radiative transfer modeling could be a promising tool for studying the bioalbedo feedback at different scales.

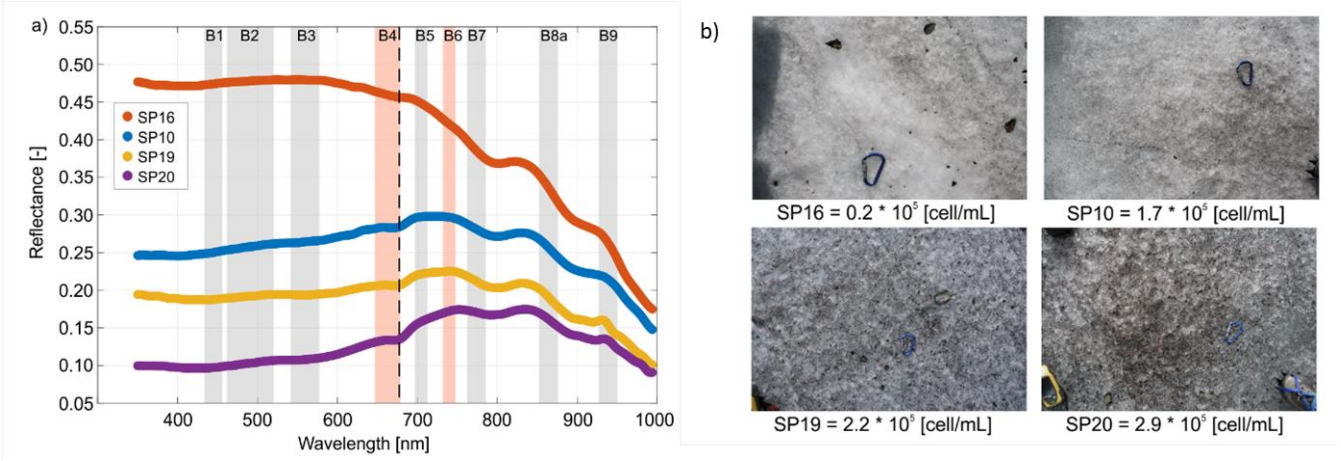

Figure 9.3 (a) Spectral reflectance of bare ice containing different algal densities. The shaded areas represent Sentinel-2 spectral bands (B1–B9). Red areas are the Sentinel-2 bands (B4 and B6) used for calculating the reflectance ratio here proposed. Dotted vertical line

indicates the position of the Chlorophyll-a absorption feature at 680 nm. (b) Images of the four sampling sites represented in (a). Carabiner (length = 7 cm) for scale.

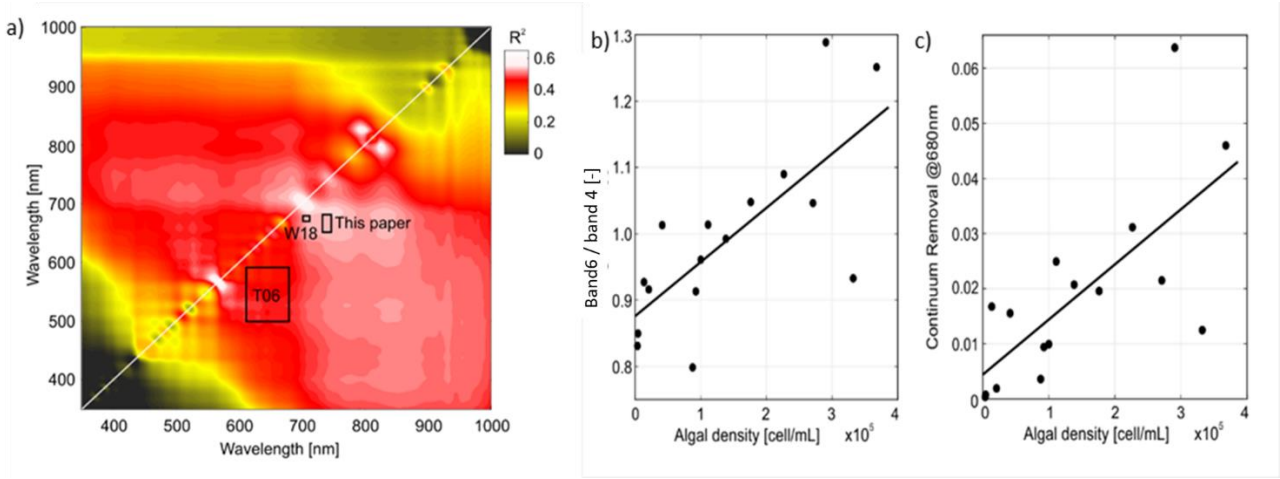

Figure 9.4 (a) Correlation matrix of the coefficient of determination R2 created from all possible reflectance ratios using the ASD field spectrometer. Rectangles represents the wavelengths used in previous studies (Takeuchi et al.30: T06, and Wang et al.25: W18). (b) Linear regression (R2 = 0.53, p-value = 0.001) between the reflectance ratio calculated resampling ASD reflectance on Sentinel 2 bands 4 and 6 and the concentration of algae [cell/mL]. (c) Linear regression (R2 = 0.52, p-value = 0.001) between the continuum removal at 680 nm and the concentration of algae [cell/mL].

9.4. Conclusions

In this paper, we have reported the occurrence of glacier algae at a glacier (Vadret da Morteratsch, Switzerland) of the European Alps. In our samples, we found an average algal concentration of 14.2 × 104 cells ml^{-1}, which is comparable with sites on the Greenland Ice sheet. The characterization of the communities of both cryoconite holes and surface ice samples showed that cryoconite holes host a more diverse eukaryotic community including heterotrophs, while bare ice is mostly dominated by the autotrophic algae. This may be due to the algae's capability in colonizing such a harsh and virtually competition-free environment, while cryoconite holes provide more shelter against abiotic stress. In particular, we documented the presence of *Ancylonema nordenskioeldii*, a glacier algal species that has never previously been quantitatively documented in the Alps, and that is known to dominate algal blooms in polar regions. We also report the effect of this bloom on the optical properties of bare ice during the ablation season. Typical absorption features of photosynthetic pigments were detected in reflectance spectra. The reflectance ratio between 740 nm and 665 nm was the index that best correlated with algal abundance and not with inorganic sediments. This index appears promising for the estimation of the abundance of algae on glaciers from remotely sensed data such as those from the ESA Sentinel-2 satellite mission. However, for a wider application of the proposed approach, further validation datasets are needed. The presence of algae on glacier ice increases the absorption of solar radiation, fostering the ice-albedo feedback during the melting season. Our dataset represents the first direct evidence of the impact of glacier algae on the optical properties of ice in the European Alps, and it is intended to pave the way for

future studies on the bioalbedo feedback in the Alps. The identification of all the players involved in ice darkening is a fundamental task for understanding surface glacier melt, and for predicting the response of Alpine glaciers to future climate change.

9.5. Acknowledgements

T. Julitta (JB Hyperspectral Devices), G. Tagliabue (UNIMIB) and V. Bottacin are acknowledged for the help during field campaign and data processing. A. Gargan (UNIMIB) helped with cell counting. Samples were stored at the EuroCold facility (University of Milano-Bicocca, UNIMIB). DR acknowledges the Austrian Science Fund (FWF) project P29959-B29. This study was also partly funded by Project MIUR – Dipartimenti di Eccellenza 2018–2022. The authors would like to thank the editor and three anonymous reviewers who considerably helped to improve the quality of this manuscript.

9.6. Author contributions

B.D.M. conceived the idea of the paper, organized the field campaign, analyzed the data and wrote the manuscript with contributions from all the other authors. R.G. analyzed spectroscopy data. G.B. participated to the field campaign and helped in data interpretation. A.F. and F.P. analyzed DNA data and helped in their interpretation. B.L. analyzed algal cells at the microscope. D.R., R.C. and M.R. supervised the research.

9.7. Supplementary material

Table S1 can be found at https://doi.org/10.1038/s41598-020-61762-0

Table S9.2 RDA of Hellinger-transformed algal OTU abundance on type of sample based on both cryoconite and ice samples

Variable	df	Variance	F	P
Type	1	0.0491	16.75	0.001
Residuals	26	0.0762		

$F_{1,26}$=16.75, P=0.001, Adjusted-R^2=0.37

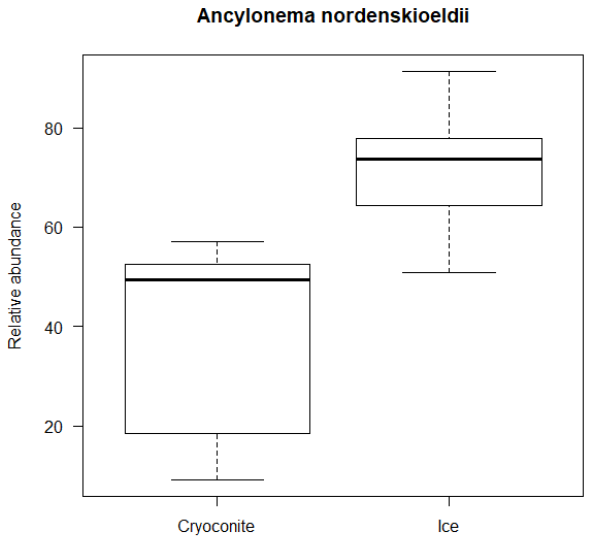

Figure S9.1 Boxplots that represent A. nordenskioeldii variation between ice and cryoconite samples.

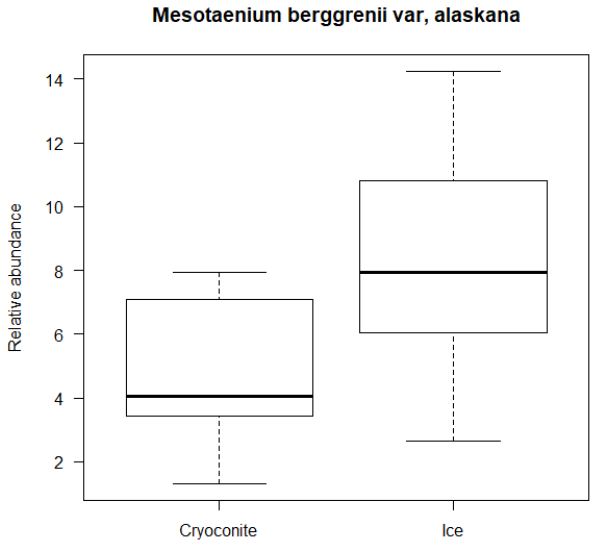

Figure S9.2 Boxplots that represent M. berggrenii var. alaskana variation between ice and cryoconite samples.

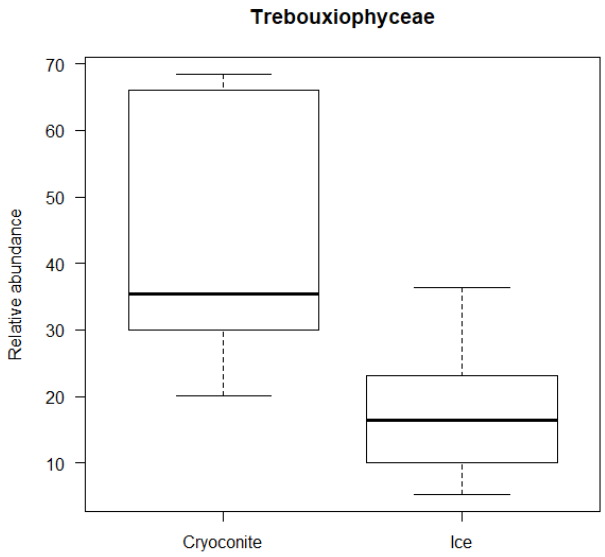

Figure S9.3 Boxplots that represent algae belonging to the class Trebouxiophyceae variation between ice and cryoconite samples.

Table S9.3 NCBI BLAST table of the 10 most abundant algal OTUs

OTU	Description	E value	Per. Ident	Accession	comment (habitat)
OTU_1	Ancylonema nordenskioeldii strain CCCryo BS_0001-2000 18S ribosomal RNA gene, partial sequence	0.0	99.42%	AF514397.2	glacier alga
OTU_2	Trebouxiophyceae sp. KMY-2018 SR1-B small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	0.0	99.42%	MK262787.1	cryoconite hole alga
OTU_1067	Trebouxiophyceae sp. KMY-2018 SR1-B small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	5,00E-162	96.24%	MK262787.1	cryoconite hole alga
OTU_24	Uncultured alga gene for 18S ribosomal RNA, partial sequence, clone: Otu003	0.0	99.71%	LC371418.1	*Chloromonas miwae* (snow)
OTU_25	Chloromonas platystigma strain CCCryo 020-99 18S ribosomal RNA gene, partial sequence	0.0	99.71%	AF514401.1	*Chloromonas muramotoi* (snow)
OTU_4039	Ancylonema nordenskioeldii strain CCCryo BS_0001-2000 18S ribosomal RNA gene, partial sequence	3,00E-160	96.21%	AF514397.2	glacier alga
OTU_43	Chloromonas nivalis subsp. tatrae voucher LP01 18S small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence	0.0	100.00 %	KY499614.1	*Chloromonas nivalis* (snow)
OTU_477	Mesotaenium berggrenii var. alaskana strain CCCryo BS_0002-2009 18S ribosomal RNA gene, partial sequence	2,00E-180	99.13%	JF430424.1	glacier alga
OTU_498	Mesotaenium berggrenii var. alaskana strain CCCryo BS_0002-2009 18S ribosomal RNA gene, partial sequence	2,00E-180	99.13%	JF430424.1	glacier alga
OTU_59	Mesotaenium berggrenii var. alaskana strain CCCryo BS_0002-2009 18S ribosomal RNA gene, partial sequence	0.0	99.71%	JF430424.	glacier alga

10. POST-DEPOSITIONAL BIODEGRADATION PROCESSES OF POLLUTANTS ON GLACIER SURFACES

The content of this chapter has been published in the following paper:
Pittino, Francesca, Roberto Ambrosini, Roberto Azzoni, Guglielmina Diolaiuti, Sara Villa, Isabella Gandolfi, and Andrea Franzetti. 2018. "Post-Depositional Biodegradation Processes of Pollutants on Glacier Surfaces." Condensed Matter 3 (3): 24. https://doi.org/10.3390/condmat3030024.

ABSTRACT Glaciers are important fresh-water reservoirs for our planet. Although they are often located at high elevations or in remote areas, glacial ecosystems are not pristine, as many pollutants can undergo long-range atmospheric transport and be deposited on glacier surface, where they can be stored for long periods of time, and then be released into the down-valley ecosystems. Understanding the dynamics of these pollutants in glaciers is therefore important for assessing their environmental fate. To this aim, it is important to study cryoconite holes, small ponds filled with water and with a layer of sediment, the cryoconite, at the bottom, which occur on the surface of most glaciers. Indeed, these environments are hotspots of biodiversity on glacier surface as they host metabolically active bacterial communities that include generalist taxa able to degrade pollutants. In this work, we aim to review the studies that have already investigated pollutant (e.g., chlorpyrifos and polychlorinated-biphenyls (PCBs)) degradation in cryoconite holes and other supraglacial environmental matrices. These studies have revealed that bacteria play a significant role in pollutant degradation in these habitats and can be positively selected in contaminated environments. We will also provide indication for future research in this field.

10.1. Introduction

For more than 40 years, we have known that inorganic and organic pollutants are present in cold remote areas, such as polar and mountain regions, far from their emission sources. Early studies were conducted in Arctic regions and reported high concentrations of radionuclides in layers corresponding to nuclear test periods (Jaworowski et al. 1975) and the presence of chlorinated compounds in Arctic and Antarctic regions (Sladesn et al. 1966; Ottar 1981). The organic contaminants in these remote areas are referred to as persistent organic pollutants (POPs), which consist of several groups of chemicals with similar structures and physical-chemical properties that are extensively used worldwide in agriculture (pesticides), industrial and health applications. Recent studies have revealed that alpine environments are affected by the presence of POPs (Blais et al. 1998; Kang et al. 2009; Hodson 2014). These chemicals are ubiquitous, show long-range transport potential, and many of them are hydrophobic. In addition, they bio-accumulate in organisms via respiration, dermal contact, and through diet (Mangano et al. 2017). Some classes of contaminants comprised in the POPs are polychlorinated-biphenyls (PCBs), -dioxins (PCDDs), -furans (PCDFs), polybrominated-diphenyl ethers (PBDEs), -biphenyls (PBBs), perfluorinated compounds (PFCs) and other halogenated hydrocarbons, used as pesticides (Bilcke 2002). PCBs are one of the classes of

compounds that have been studied in the context of pollutant biodegradation on glaciers (Weiland-Bräuer et al. 2017). They are toxic chemicals that the chemical treaty is trying to abolish by 2025, because of their persistence and difficult degradation. Their use is very heterogeneous (from the industry to agriculture) (Borja et al. 2005). PCB biodegradation can occur either through anaerobic reductive dechlorination (mostly in soil and sediment) or through aerobic oxidative degradation (preferably in water) and a wide range of different bacterial genera are able to perform these degradative pathways (Borja et al. 2005; Abraham et al. 2002). PCB degradation rate, measured in lab experiments, varies according to the number of the chlorine atoms, slowing down as the number of Cl atoms increases. This is the reason why the range of the degradation rate varied from approximately 90% to approximately 20% (Cai et al. 2018). Atmospheric medium- and long-range transport has been identified as a major source of semivolatile and persistent organic contaminants in polar and alpine ecosystems. Cold areas act as condensers (Calamari et al. 1991), interfering with the atmospheric transport and global cycling of semi volatile organic compounds (SVOCs) (Carrera et al. 2001; Villa et al. 2003; Ferrario et al. 2017b; Arellano et al. 2015; Villa et al. 2014), thus promoting the scavenging of these molecules from the atmosphere (Grannas et al. 2013). Indeed, PCB concentrations in snow increase with altitude, especially for more-volatile, less-chlorinated di- and tri-chlorobiphenyl congeners (Blais et al. 1998). High altitude environments may also be more prone than polar ones to contamination by those compounds that are not suitable for long latitudinal transport because mountain ranges can be relatively close to source sites of contaminants (Kallenborn 2006). A relevant fraction of the contaminants reaching cold areas is deposited on glaciers, where pollutants undergo post-depositional processes of partitioning among different environmental matrices (e.g., snow, ice, water, interstitial atmospheric gases and supraglacial sediments) and alteration processes. Post-depositional alteration consists of both physical-chemical processes, such as photodegradation, hydrolysis, revolatilization (Grannas et al. 2007; Herbert et al. 2006), and biological ones, particularly biodegradation (Hodson 2014). The balance of these partition and alteration processes defines the amount of pollutants that can either enter the food-chain or be released into the environment by meltwater, which, in turn, can impact the downstream environments (Morselli et al. 2014; Bizzotto et al. 2009). The current and foreseen increase of glacier retreat and melting will therefore likely lead to an increase of the release of pollutants from glaciers. Indeed, it has been already reported that high retreat episodes of glaciers correspond to high fluxes of pollutants in the melting water (Schmid et al. 2011). A recent model showed that the glacier melting due to global warming will lead to an earlier and more concentrated

release of pollutants (e.g., PCBs, DDT, PCDD/Fs) stored in glacier bodies, than if the climate were stable (Bogdal et al. 2010). Among post-depositional alteration processes that lead to the net reduction of the contaminant mass on the glacier, the biodegradation of organic molecules by microorganisms has been rather overlooked so far (Hodson 2014). However, it is well known that microorganisms inhabit glaciers and have the metabolic abilities to degrade complex organic compounds even at low temperatures (Margesin 2007). Among the glacial environments, supraglacial ones are the most biodiverse, and host rich bacterial communities (Boetius et al. 2015). In these environments, the cryoconite, a wind-borne fine debris deposited on glacier surfaces, represents a potential sink for organic and inorganic pollutants because of its high content of organic matter (Cook et al. 2016b). When heated by solar radiation, the cryoconite can promote ice melting and form small ponds filled by meltwater, called cryoconite holes, which are considered the most microbiologically active supraglacial habitats. Indeed, different studies report bacterial abundance in cryoconite in the order of 108–109 cells_g^{-1} (Stibal et al. 2015; Ferrario et al. 2017), similar to these in other type of soils (e.g., agricultural soils, marsh or mountain soils) (Henry et al. 2006). Cryoconite holes therefore represent ideal environments to investigate the processes that determine pollutant accumulation and degradation on glaciers (Cook et al. 2016b). In this work, we review the current literature about the accumulation of contaminants in the cryoconite and other supraglacial matrices and the microbiological processes affecting their fate, and we provide suggestions and direction for future research.

10.2. Accumulation of Pollutants and Microbiological Response in Cryoconite

Cryoconite is a granular sediment found on glacier surfaces. Owing to its dark color, cryoconite absorbs solar radiation and promotes the formation of quasi-cylindrical holes, called cryoconite holes, through differential ablation rates compared to the surrounding ice (Cook et al. 2016b). Cryoconite is composed of both inorganic and organic material. The former is mainly composed by mineral fragments, often dominated by phyllosilicate, tectosilicate and quartz with differences due to the geological source of the debris (Cook et al. 2016b); the latter is composed of a wide variety of living and dead microscopic organisms, the products of their autotrophic activity, and allochthonous organic material of wind-borne particles (Grzesiak et al. 2015). Cryoconite in holes has higher concentration of organic matter and inorganic nutrients (nitrogen, phosphorus...) than the surrounding ice and supraglacial sediments (Franzetti et al. 2017a; Bagshaw et al. 2013). This characteristic leads, on the one hand, to the incorporation of hydrophobic compounds such as POPs into the cryoconite organic matter (Hodson 2014) and, on the other hand, it promotes the presence

of biodiverse and active microbial communities (Franzetti et al. 2016), which can biodegrade the contaminants. In the last five years, some studies investigated the origin and the accumulation of pollutants in cryoconite and their post-depositional fate, including the biodegradation processes. Polycyclic Aromatic Hydrocarbons (PAHs) are among these pollutants. They are found in the environment as consequence of combustion or processing of hydrocarbon fuels. Their biodegradation can occur in different environments thanks to bacteria, fungi or algae and the efficiency depends on the number of benzene rings (Haritash et al. 2009). PAHs are known to be better biodegraded in water, but Kuppusamy and colleagues showed new consortia of bacteria able to perform PAH degradation in soil with good performances (Kuppusamy et al. 2016). Li and colleagues identified 15 PAHs containing 3–7 rings in 61 cryoconite samples collected from seven glaciers on the Tibetan Plateau (TP) (Dong, Qin, et al. 2016). The average concentration of total PAHs in cryoconite samples was in the range of 6.67–3906.66 ngg^{-1} dry weight. The highest average total PAH concentration was found in the southeastern TP, followed by the northern TP. The central TP contained the lowest number of PAHs. Moreover, correlation analysis showed that total organic carbon (TOC) and grain size were only minor determinants of the accumulation of PAHs in cryoconite of the TP. Factor analysis and diagnostic ratios indicated that PAHs were produced mainly from the incomplete combustion of coal, fossil fuels and biomasses. The exhaust gas of locomotives also contributed to the accumulation of PAHs on glaciers. Toxicity Equivalent Quantity (TEQ) of cryoconite was calculated for all the glaciers and results showed that cryoconite had a low biological risk regarding PAHs in all the investigated glaciers, except for YL Snow Mountain, which is a touristic area where shuttle vehicles are widely used and five-seven ring PAHs accounted for more than half of total PAHs. Overall results showed that long-range atmospheric transport was the main source of PAHs deposited on glaciers. Similarly, Dong and colleagues reported that the higher ratio of anthropogenic particles in the southern TP is likely caused by atmospheric pollutant transport from southern Asia, whereas cryoconite in the northern locations of the TP, containing higher dust and salt particle ratio, is influenced by the large deserts in central Asia (Dong et al. 2016b). Therefore, the transport and deposition of cryoconite is significant for understanding the regional atmospheric environment and circulation. A large amount of material such as biological particles, NaCl, and mixed cation sulfate particles, was also found in the cryoconite, implying that, in addition to dust and black carbon, many types of light absorbing impurities combined could influence the glacier albedo change and enhance ice melting in the mountain glaciers of the TP. The anthropogenic impact on cryoconite was also studied in the Alps and Arctic where high concentrations of

radionuclides were found in two independent studies (Łokas et al. 2016; Baccolo et al. 2017). Both studies attributed the origin of these contaminants to long-range transport and anthropogenic activities. The extremely high concentration of metal and radioactive compounds was explained by the capability of extracellular substances excreted by microorganisms to bind these compounds and remove them from the meltwater. A recent study (Łokas et al. 2016) investigated anthropogenic radionuclides in cryoconite from the Adishi glacier (Georgia) and found activity concentrations varying from 0.37 0.04 $BqKg^{-1}$ for 238Pu (238Pu activity concentrations in the first centimeter of soil in an undisturbed area in Korea was in the range of 0.006–0.062 Bq Kg^{-1} (Kim et al. 1998)) to a maximum of 4940 ± 610 for ^{137}Cs (^{137}Cs activity concentration detected after Fukushima nuclear accident at soil depth of 0.5–1 cm was 5610 ± 40.8 Bq Kg^{-1} (Kato et al. 2012)). Interactions between radionuclides and bacteria can be very different: among them there are, noteworthy, biotransformation, bioprecipitation and also biosorption, depending on the characteristics of the radionuclides (Shukla et al. 2017). These compounds can be naturally present in the environment or due to human activity (e.g., nuclear weapons, uranium mining and milling, commercial fuel reprocessing) (Hu et al. 2010). Due to the limited knowledge about glacial biology, how these high amounts of radionuclides and heavy metals affect biota and how these contaminants are transferred along food web remain open questions to be addressed in the future investigations. To the best of our knowledge, only six studies characterized the microbial communities and their metabolisms associated to the contamination, besides addressing the presence of pollutants in cryoconite and other supraglacial environments The metabolic potential of the culturable fraction of cryoconite bacteria toward pollutants was pioneeringly investigated by Margesin in 2002. In this study they estimated the abundance of bacteria able to grow on natural and anthropogenic recalcitrant substrates (Margesin et al. 2002). Results showed that cold-adapted bacteria were able to degrade carbohydrates (starch), fats (tributyrin), mineral oil hydrocarbons (diesel oil) and PAHs as sole carbon sources. (Tab. 10.1, Fig. 10.1).

Table 10.1 Summary of the published studies addressing both the presence of pollutants and the microbial processes related to them in cryoconite and other supraglacial environmental matrices.

Geographic Area	Contaminants	Matrices	Reference
Italian Alps	Chlorpyrifos (CPF)	cryoconite	Ferrario et al., 2017
Antarctica	Synthetic oil	surface ice and sediments	Jarula et al., 2009

Geographic Area	Contaminants	Matrices	Reference
Greenland	2,4-Dichlorophenoxyacetic Acid	surface ice	Stibal et al., 2012
Svalbard	Mercury	snow (above soil)	Larose et al., 2013
Austrian Alps	PCBs	cryoconite	Weiland-Bräuer et al., 2017
Greenland	PAHs, PCBs, mercury, lead	cryoconite	Hauptmann et al., 2017

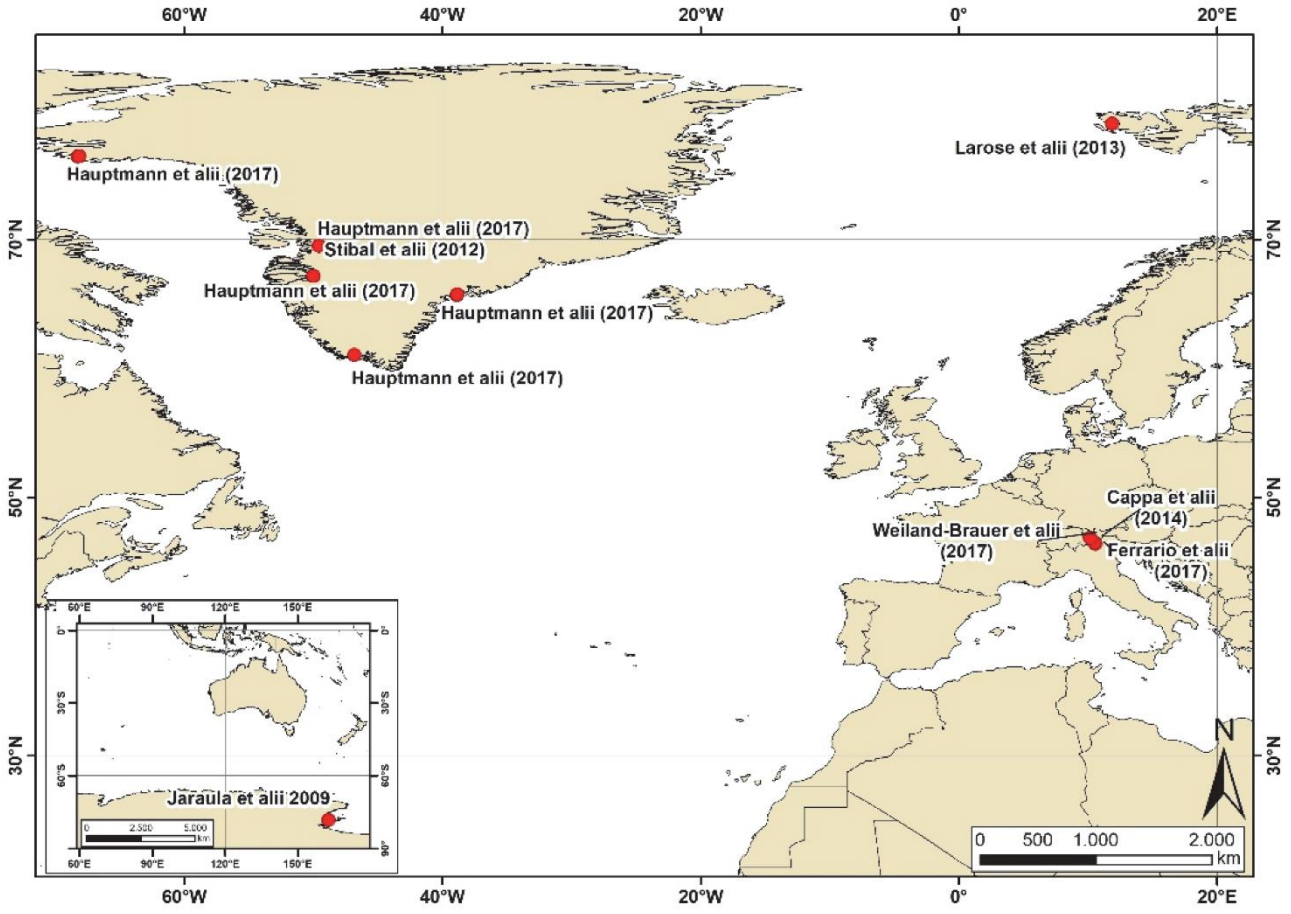

Figure 10.1 Geographic location of the studies addressing both the presence of pollutants and the microbial processes related to them in cryoconite and other supraglacial environmental matrices.

Larose and colleagues (Larose et al. 2013) studied the impact of mercury, whose concentration is increasing in the Arctic food web, on snow microbial communities. Despite the investigated snowpack was not supraglacial, but consisted of seasonal snow accumulated above the soil, the study provides important insights into the response of cold adapted communities to inorganic contaminants. Mercury can be subjected to long-range atmospheric transport and can persist in the air for at maximum one year: enough to reach also the most remote area of the planet (Morel et al.

1998). Hg can be found in different forms: elemental Hg (Hg0 (aq)) that is volatile but not reactive, mercuric species (Hg(II)) and organic mercury (e.g., methyl-mercury, that is bioaccumulative) (Morel et al. 1998). Bacteria can play an important role reducing the soluble form Hg(II) to the precipitating Hg(0) and it has been demonstrated they can perform this reaction with better results at Hg(II) concentrations lower than 6 mg/L in water (von Canstein et al. 2002). In the study by Larose et al. (Larose et al. 2013) both forms of mercury resulted to influence the structure of the snow microbial communities. Indeed, the microbiological analyses revealed the presence of mercury-resistant taxa in Hg contaminated snow and, at molecular level, the abundance of the merA gene, which confers resistance to the inorganic form of Hg, was positively correlated with Hg concentration. merA codes for the mercuric reductase enzyme which catalyzes the reduction from the water soluble Hg(II) to the volatile Hg(0) form. The presence of this metabolic activity might also explain the observed reemission in the atmosphere of the Hg entrapped in the snow (Møller et al. 2011). Further studies are needed regarding mercury in glacial ecosystems, especially regarding Me–Hg that can be bioaccumulated along the trophic chain (Morel et al. 1998). Indeed Larose et al., found a negative correlation between bioavailable Hg and Me–Hg indicating a probable Hg biotic methylation (Larose et al. 2013); in fact, bacteria can both cleave the bond or be responsible of methylation especially in anaerobic conditions (Morel et al. 1998; Colombo et al. 2013). Hg concentration in mountain firn core was found to be in the range of 2–35 ng/L^{-1} (Wang et al. 2008), and in freshwater from uncontaminated sites was in the range of 1–20 ng·L (Morel et al. 1998), but a study by Moller and colleagues found in snow/brine concentrations of 70–80 ng L^{-1} (Møller et al. 2011). The effect of organic molecule pollution was addressed in a work by Cappa and colleagues (Cappa et al. 2014), who investigated the impact of a summer ski resort on the structure of bacterial communities on a glacier surface. Their findings revealed that the concentrations of PAHs and PCBs were significantly higher close to the ski resort than in areas impacted only by long-range transport. They also observed that the presence of pollutants can favour the selection of bacterial strains able to metabolize them. In another study, after that an unexpected synthetic oil spilling happened in Taylor Valley (Antarctica) because of a helicopter crash, biodegradation was detected preferentially in sediment rather than in water and fluid-filled bubbles of an ice core (Jaraula et al. 2009). These first evidences that organic pollution can select specific degrading microbial populations, thus promoting the biodegradation of the same contaminants on glaciers, were further confirmed by subsequent studies, which reported the actual biological removal under field conditions and used molecular cultivation-independent methods to better characterize the microbial communities. For instance,

the study by Weiland-Brauer and colleagues found high concentrations of eighteen different congeners of PCBs, 16 PAHs and 29 different organochlorine pesticides in the cryoconite of an Alpine glacier (Weiland-Bräuer et al. 2017). In this study, microcosms containing cryoconite were set up with the addition of PCBs to determine the bacteria responsible of PCB degradation. The results showed that different genera, among which the most abundant were Pseudomonas, Shigella, Polaromonas, Variovorax, Janthinobacterium, Subtercola, and Chitinophaga, were able to degrade PCBs. On a molecular basis, the ability to aerobically biodegrade PCBs was identified in the presence of the gene bphA coding for the enzyme biphenyl dioxygenase, which was found in both metagenomic DNA and in the genome of the bacterial isolates.
The microcosm approach was also used in two other studies with the aims of simulating glacier surface environments and estimating the biodegradation rates of pesticides (Ferrario et al. 2017; Stibal et al. 2012b). In the first study, the ability of supraglacial bacteria to degrade the pesticide 2,4-dichlorophenoxyacetic acid (2,4-D) was investigated and, although no taxa related to known 2,4-D degraders were found, the authors succeeded in estimating the biodegradation rate in simulated field conditions. There are no data about the presence of this pesticide in/on glaciers, and not surprisingly, the rates of 2,4-D mineralization were lower than those observed in temperate environments. This work first demonstrated that anthropic contamination on glaciers can be attenuated by the local natural microbial community. Ferrario and colleagues (Ferrario et al. 2017; Ferrario et al. 2017a) detected high concentrations (2–3 mg kg−1) of the pesticide chlorpyrifos (CPF) in the cryoconite and meltwater of an Alpine glacier, while in agriculture and termiticidal initial soils (i.e., in the application area) its concentrations vary from 10 to 1000 mg per kg of soil respectively (Murray et al. 2001). The authors estimated, by in situ microcosms, that the rate of pollutant removal due to biodegradation was much higher than those of hydrolysis and photodegradation, leading to a CPF half-life in cryoconite ranging from 35 to 65 days (Figure 10.2). In other environments CPF half-life was reported to vary from 6 days (in aquatic aerobic conditions) to 128 days (in soil/slurry anaerobic conditions) (Tiwari and Guha 2014), showing that in cryoconite the biodegradation rate is not different from others even if there are harsh conditions in this microhabitat. A whole shotgun metagenomics analysis of the bacteria occurring in the cryoconite of the same glacier allowed the genome reconstruction of a bacterial population that could be responsible of the pesticide biodegradation (Ferrario et al. 2017). These putative CPF-degrading bacteria resulted to be photoheterotrophic Burkholderiales, thus strengthening previous findings on the metabolic versatility and the importance of Betaproteobacteria in the heterotrophic

metabolisms of cold environments (Franzetti et al. 2016; Darcy et al. 2011; Caliz and Casamayor 2014).

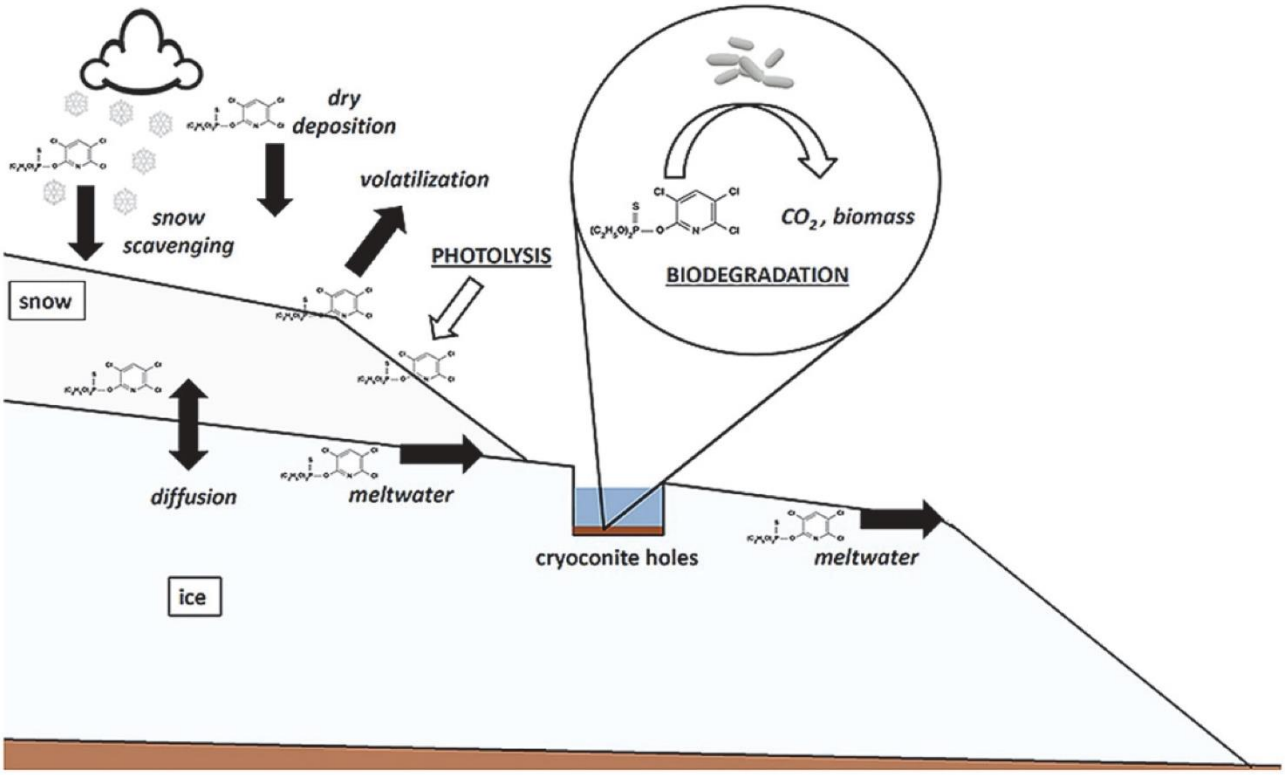

Figure 10.2 Processes affecting the fate of chlorpyrifos on the glacier surface. Biodegradation in cryoconite is highlighted. Reproduced with permission from Ferrario et al., 2017

A metagenomics approach was used also in a recent work addressing the metabolic potential of microbial communities in cryoconite collected on the Greenland ice sheet. Results disclosed the potential for resistance to and degradation of contaminants, including polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), and the heavy metals mercury and lead. The presence of genes for contaminant resistance and degradation was spatially variable but present in all samples across the ice sheet (Hauptmann et al. 2017).

10.3. Conclusions and future perspectives

This short review of the studies that have addressed the biodegradation of pollutants on the glacier surface reveals how this field has been investigated only very recently, and that there is therefore a wide potential for future studies. New studies should investigate the biodegradation potential of glacier microbes for further pollutants that are known to occur on glaciers (e.g., terbuthylazine (Ferrario et al. 2017a)). The review has also highlighted the importance of using microcosms to investigate degradation rates. A well-designed experimental setup of microcosms under different conditions (e.g., light and dark conditions, sterilized and non-sterilized cryoconite, etc., see (Ferrario et al. 2017a) for an example) can indeed allow for the assessing of the relative contribution of

different processes to pollutant degradation. We also stress the importance of carrying out microcosm studies in situ, whenever this is logistically feasible, in order to be as close as possible to natural conditions.

Another important feature emerging from this review is the potential of metagenomic studies to improve our understanding of the metabolic processes that allow glacier bacteria to degrade pollutants. Indeed, the identification of the microbial populations involved in these processes was critical in most studies. With the decrease of costs of sequencing and the availability of new bioinformatic tools that will ease these studies, we hope for further investigations of pollutant degradation on glaciers combining microcosms and metagenomics.

We also hope that further studies will fill the large geographical gaps in these investigations, for instance in South America. Finally, there is also a strong need for models of the environmental fate of pollutants in cold areas, which incorporate also biological processes, as they will be pivotal to properly forecast the future dynamics of contaminants released by glaciers in future warmer climatic conditions.

10.4. Acknowledgments

The authors acknowledge the valuable contribution of all former researchers and students involved in the studies of microbiological processes in glaciers. Among them, they particularly thank Claudio Smiraglia, Ilario Tagliaferri and Claudia Ferrario.

11. FIRST EVIDENCE OF MICROPLASTIC CONTAMINATION IN THE SUPRAGLACIAL DEBRIS OF AN ALPINE GLACIER

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Ambrosini, Roberto, Roberto Azzoni, Francesca Pittino, Guglielmina Diolaiuti, Andrea Franzetti, and Marco Parolini. 2019. "First Evidence of Microplastic Contamination in the Supraglacial Debris of an Alpine Glacier" Environmental Pollution 253: 297–301. https://doi.org/10.1016/j.envpol.2019.07.005.

ABSTRACT Contamination by plastic debris has been documented in most regions of the world, but their occurrence in high mountain areas has not been investigated to date. Here we present the first report of the occurrence and amount of microplastic in any terrestrial glacier environment. In the supraglacial debris of the Forni Glacier (Italian Alps), we observed the occurrence of (mean ± standard error) 74.4 ± 28.3 items kg^{-1} of sediment (dry weight). This amount is within the range of variability of microplastic contamination observed in marine and coastal sediments in Europe. Most plastic items were made by polyesters, followed by polyamide, polyethylene and polypropylene. We estimated that the whole ablation area of Forni Glacier should host 131-162 million plastic items. Microplastic can be released directly into high elevation areas by human activities in the mountain or be transported by wind to high altitude. The occurrence of microplastic on Forni Glacier may be due to the gathering of debris coming from the large accumulation area into the relatively smaller ablation area of the glacier, as a consequence of its flow and melting.

11.1. Introduction

Accumulation of plastic polymer (hereafter plastic) items is one of the most ubiquitous and long-lasting changes to the earth surface that human activities have produced since mass production of plastic commenced in the 1950s (Barnes et al. 2009; Thompson et al. 2004). Concerns have been raised in recent years on the potential impacts of plastic on ecosystems. Plastic is persistent, it can easily accumulate in the environment and enter the trophic chain, and its impacts on ecosystems have already been demonstrated (Cole et al. 2011; Wright et al. 2013; Barnes et al. 2009). In recent years, particular attention has been focused on microplastics, plastic items smaller than 5 mm. They can be specifically produced to be used in diverse personal care products and in different industrial applications (i.e., primary microplastics), or can be generated by the break-down of macroplastics (secondary microplastics) (Eerkes-Medrano et al. 2015). Surveys on marine and freshwater ecosystems have been established to monitor the temporal trends in the amount of plastic items. Oceanic campaigns have estimated the amount of macro- and microplastics afloat in the sea, and the amount transported to sea by rivers from terrestrial ecosystems (Eriksen et al. 2014). Moreover, recent studies have demonstrated that microplastics can be transported to virtually any part of the globe, also in the so-called remote areas. Indeed, plastic fragments have been found in deep sea, Southern Oceans, Arctic and Antarctica (Hamid et al. 2018), as well as in sub-alpine lake sediments (Imhof et al. 2013), in pelagic water and shoreline debris from high-mountain lakes (Free et al. 2014;

Zhang et al. 2016), and floodplain soils in Alpine valleys (Scheurer et al. 2018). In addition, recent evidence of atmospheric microplastic deposition in a remote, pristine mountain area of the French Pyrenees suggests that microplastics can reach and affect remote, sparsely inhabited areas far from emission sources through atmospheric transport (Allen et al. 2019). However, to the best of our knowledge, no published study has documented the occurrence and the amount of plastic items on glaciers so far.

Glaciers are accumulation sites for aerially transported debris and pollutants as glacier ice form through the transformation of accumulated snowfalls, which are particularly efficient in scavenging contaminants, including small debris, from the atmosphere (e.g. Lei et al. 2004; Lovett et al. 1990). Temperate glaciers then flow down valley, and the ablation area of valley glaciers thus concentrates the debris and the contaminants collected over the much vaster accumulation area of the glacier (Nakawo et al. 1986). For this reason, supraglacial debris is particularly rich in air-transported pollutants (Cook et al. 2016b; Pittino et al. 2018a), including radionuclides (Baccolo et al. 2017; Łokas et al. 2016). We thus hypothesized that similar processes may act also for microplastics, and we present here the first evidence of the occurrence and the first estimate of the amount of microplastic in the supraglacial debris of a large valley glacier in the Italian Alps.

11.2. Materials and methods

11.2.1. Study area

This study was carried out on the ablation tongue of Forni Glacier, one of the widest Italian glaciers, with a total surface area of 11.34 km^2 and an ablation area of 0.59 km^2 (Azzoni et al. 2018). This glacier is located in the Ortles-Cevedale group (Stelvio National Park, Central Italian Alps) and is rather close to the highly urbanized areas. For instance, the town of Sondrio (~21,000 inhabitants) is 58 km from the Glacier, and those of Brescia (~197,000 inhabitants), Bergamo (~120,000 inhabitants), Verona (~257,000 inhabitants), and Vicenza (~111,000 inhabitants), in the highly urbanized Po Plain area of Northern Italy, are between 100 and 120 km from the Glacier. Forni Glacier is also very popular with mountaineers and hikers.

11.2.2. Sample collection

On 20 and 24 July 2018, we collected two cryoconite samples and four samples of sparse and fine (< 2 mm) supraglacial debris from the ablation area of Forni Glacier at about 2580 m a.s.l. (Fig. 11.1 and Tab. S11.1). Cryoconite is a fine supraglacial deposit of mostly aeolian origin that, whenever thinner than a critical thickness (Mihalcea et al. 2008) and heated by solar radiation, promotes the melt of the underlaying ice forming small ponds. These ‘cryoconite holes’ are considered the most

biologically active environments on glaciers (Cook et al. 2016b). Sparse and fine debris is similar in grain size to cryoconite, but does not form ponds because it is widely spread on the ice surface and its main effect is ice darkening. To avoid contamination, samples were collected in glass jars with a gardening metal shovel or a metal laboratory spoon. Jars and sampling tools were accurately cleaned with acetone before sampling. We also took care to avoid contamination by plastic fibres releasing from the clothes and shoes of the operator who collected samples by wearing a 100% cotton surgical gown and 100% wooden clogs (Fig. S11.1). Only the operator entered the area where we collected samples, while the other members of the team remained > 10 m apart for avoiding sample contamination.

11.2.3. Microplastic floating and identification

Extraction of microplastic items from supraglacial and cryoconite debris samples was performed according to the method first developed by Thomspon and co-authors (Thompson et al. 2004), and adapted by Klein and co-authors (Klein et al. 2015) to river sediments. All the glassware and stainless forceps used during extraction procedure were previously washed with ultrapure filtered water to avoid potential laboratory contamination. The extraction was performed under a laminar-flow hood. The synthetic polymers were separated from glacial sediments by density separation using a saturated sodium chloride solution (density = 1.2 g cm^{-3}; 365 g L^{-1}). About 50 g of sediments (dry weight; range 50.87 - 53.52 g; see Tab. S11.1) were mixed with 300 mL of the NaCl solution, previously filtered on glass fibre filters (Ø = 0.45 µm¸ Whatman GF/A 47 mm, GE Healthcare Europe GmbH), and stirred for 15 min. The sediment particles were allowed to settle overnight. Then, the solution containing the floating particles was transferred by using a 50 mL glass volumetric pipette to a glass separation flask, where it was filtered on glass fibre filters by using a water-jet pump. The saturated NaCl solution was continuously added to the separation flask. At the end of the filtration process, the flask was rinsed three times with 20 mL of ultrapure filtered water to recover potential plastic particles adherent to the glass flask. Natural organic debris was removed by filters through an overnight treatment with a hydrogen peroxide (H_2O_2) solution (15%). The excess of H_2O_2 solution was then rinsed twice with ultrapure filtered water, vacuum-filtrated through glass fibre filters, rinsed again, and dried under a laminar-flow hood for 48 h. During all steps of sample preparation, glassware and filtration flask were covered with tinfoil to prevent contamination by plastic items. A blank sample was performed before and after the extraction from cryoconite and fine and sparse debris samples to check for any laboratory contamination. Saturated salt solution (300 mL) was stirred for 15 min, settled overnight and then filtered on glass fibre filters as described above. Filters

were then digested with H_2O_2 (15%) overnight and, after rinsing with ultrapure filtered water, dried under a laminar-flow hood for 48 h. All the obtained filters were examined under a binocular microscope (Leica DM750; 20x – 40x of magnitude) and plastic items were classified by their shape into fragments or fibres. The size of plastic items observed on the filters was measured with the image processing package Fiji freeware software (Schindelin et al. 2012). Visual examination of all the particles was carefully performed to exclude natural debris (e.g. insect exuviae) and/or mineral components. These residual materials were removed with stainless steel forceps. All the plastic items collected on filters were chemically characterized using the Fourier Transform Infrared Microscope System (µFTIR; Survey IR Microspectroscopy Accessory coupled with Nicolet iS5 FTIR Spectrometer, Thermo Fisher Scientific; detection limit ~100 µm) to confirm their plastic nature and to identify polymer typologies. Analysis was performed in attenuated total reflection (ATR) mode operating on single reflection mode in the range between 600 and 3,800 cm^{-1} and with 16 scans per analysis.

11.2.4. Estimate of the amount of debris

The quantification of supraglacial debris was performed using image classification technique from high-resolution airborne orthophotos (pixel size 0.5 m × 0.5 m) of the glacier surface (Paul et al., 2004). The area covered by scattered and sparse debris was estimated with a maximum likelihood classification in ESRI ArcMap software (ESRI, Redlands, California) excluding areas covered by moraines (Azzoni et al. 2018) (Fig. 10.1). This procedure is a supervised classification method of the spectral signature of high-resolution images of the glacier (ESRI 2011). First, the user manually classifies a sample of pixels as bare ice or debris based on his or her experience and *a priori* knowledge of the Forni Glacier. Then, the software calculates statistics from the spectral signature of these pixels and creates a parametric signature for bare ice and one for debris. Finally, it classifies each pixel in the image as bare ice or debris based on these spectral statistics (ESRI 2011). For increasing the reliability of the classification, both the manual classification of pixels and the following automatic characterization of the whole image were repeated ten times and the minimum and maximum value of the extent of the area covered by debris were considered. The total volume of debris was then calculated assuming a mean debris thickness of 0.5 cm (Azzoni, unpublished data) and its total weight was finally calculated using debris density values (from 1,600 kg m^{-3} to 2,000 kg m^{-3}) obtained following Ponce (1989) on the basis of supraglacial debris size distribution (Azzoni et al. 2016).

11.3. Results and discussion

We found 2-7 plastic fragments per sample that correspond to 74.4 ± 28.3 SE items kg^{-1} of sediment (dry weight) on average, with no difference between cryoconite (70.5 ± 32.9 items kg^{-1}) and sparse and fine supraglacial debris (78.3 ± 30.2 items kg^{-1}) (see Supporting Information; Tab. S11.1). No plastic item was found in blank samples. Fibres represented 65.2% and fragments 34.8% of items in all samples pooled. Both microplastic fragments and fibres were of diverse colour (Fig. 11.1). Overall, most plastic items were made from polyester, followed by polyamide, polyethylene and polypropylene (Fig. 11.1). Unfortunately, 39% of plastic items could not be characterized because their size was below the limit of detectability (~100 µm) of the FTIR we used (see Methods and Tab. S11.1).

By using image processing (see Methods), we estimated that the ablation area of the Forni Glacier covered by cryoconite and sparse debris was 0.21-0.23 km^2. Cryoconite and sparse debris weighed 1,640-2,307 tons. According to the amount of plastic items we found in our samples, the ablation area of the glacier should therefore include 131-162 million of microplastics, corresponding to 570-801 million items km^{-2}.

This is the first report published on a scientific journal of microplastic contamination in any terrestrial glacier environment. The amount of microplastics we detected is within the range of variability of contamination observed in marine and coastal sediments in Europe (Hamid et al. 2018). It is also similar to those detected in lakeshore sediments from four lakes located in a region with very low population density and lacking of industrial and agricultural activities within the Siling Co basin in northern Tibet, where a microplastic abundance up to 563 ± 1,219 million items km^{-2} was observed (Zhang et al. 2016). Microplastic contamination on Forni was also intermediate between that measured in soils from mountain floodplains in different parts of Switzerland, where up to 593 items kg^{-1} were found (Scheurer et al. 2018) and that observed in the pelagic water of Lake Hovsgol, a large, remote mountain lake in Mongolia, where an average microplastic density of 20,264 items km^{-2} was found (Free et al. 2014). Clearly, comparisons among levels of microplastic contaminations from very different environmental matrices should be taken very cautiously. However, these results suggest that microplastic contamination on Forni Glacier is within the range of contamination observed in the few remote mountain areas investigated worldwide to date.

Plastic can reach high elevation areas from several paths. Human activities in the mountain environment produce large amount of garbage, which sometimes can hardly be transported down valley. Most alpinist equipment, for instance, is made of plastic polymers, and can be abandoned

on mountains in emergency or deliberately. For instance, in 1990, the “Free K2” expedition recovered about 2 tons of garbage from the second highest mountain of the planet, most of which were plastic (Ardito 1995). Forni Glacier is visited each year by hundreds of tourists whose clothes and equipment may release plastic fibres and items. The presence of polyesters and polyamide fibres in glacier debris seems consistent with this hypothesis. Equipment used during scientific surveys conducted on Forni Glacier can also have determined plastic fibres release (Azzoni, personal information). However, Forni Glacier is also close to densely inhabited areas like the Po Plain in northern Italy, which can contribute to the amount of plastic fragments observed. Indeed, small plastic items can be transported by wind to high altitude, where they can be deposited by both wet and dry deposition (Dris et al. 2016; Allen et al. 2019). Indeed, a recent study by Allen and co-authors (2019) performed in a pristine mountain catchment of French Pyrenees, confirmed that fibres up to ~750 µm long and fragments ≤ 300 µm can be transported by air and deposited in remote areas up to 95 km from potential sources (Allen et al. 2019). This distance is slightly less than that between Forni Glacier and towns in the the densely inhabited Po Plain. Atmospheric transport of plastic items might therefore contribute to the amount of microplastic we found on Forni Glacier. Unfortunately, the relative amount of plastic items that reaches the Forni Glacier from local or remote sources could not be assessed with the data available for the present study and should be further investigated.

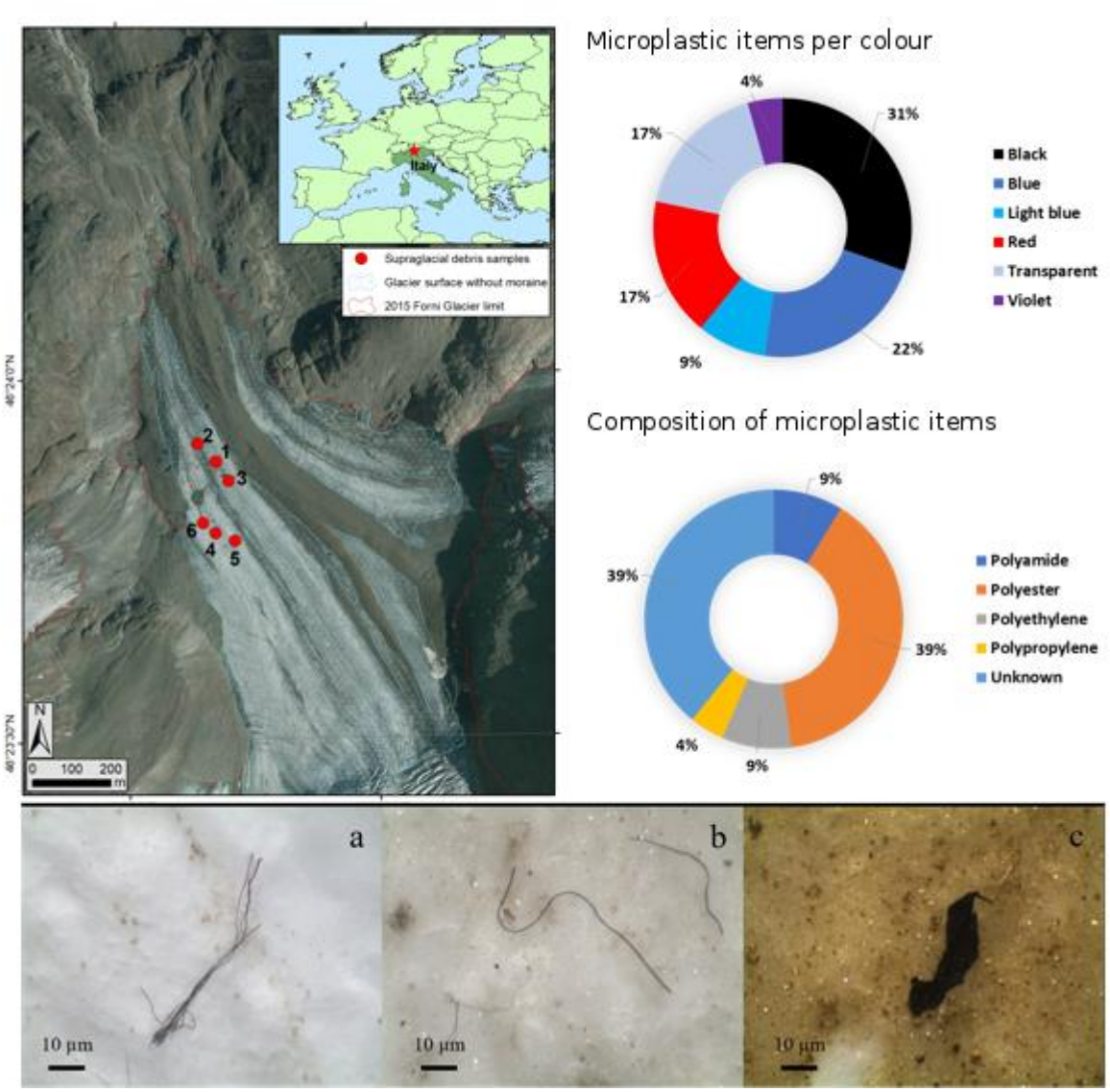

Figure 11.1 Study area and microplastic composition. Top-left panel: the ablation area of the Forni glacier with sample collection sites (red dots) and its geographical location within Europe. The blue shaded area indicates the part of the glacier tongue covered by scattered and sparse debris. Top-right panel: proportion of plastic items per colour (upper diagram) and per polymer composition (lower diagram). Bottom line: polyester fibres (a and b) and an unknown fragment (c).

11.4. Conclusion

Our findings of microplastic contamination on glaciers, albeit not unexpected, demonstrated that also this contaminant can reach remote, high mountain areas. When trapped in the sediment, microplastics can persist on glaciers for an unknown amount of time and there is therefore the potential for long-term persistence of microplastic on glaciers, which may have already accumulated an unknown amount of plastic since the 1950s, when plastics have started to be released in the environment. These particles might be potentially released by glaciers, entering melting waters and contributing to freshwater contamination and, ultimately, also to marine contamination. The current amount of contamination and the fate of microplastics in glaciers should therefore be carefully evaluated by further studies beside this very preliminary investigation.

11.5. Supplementary material

Table S11.1 Plastic items observed in both supraglacial debris and cryoconite samples from the Forni Glacier. Sample ID, debris typo, number, colour, shape, size and polymer typology of all plastic items are reported. The term unknown refers to polymers that could not be chemically characterized.

Samples			**Plastic item**			
ID sample	**Debris**	**Dry weight (g)**	**Colour**	**Shape**	**Size (µm)**	**Polymer**
S1	Supraglacial	50.87	Red	Fragment	73.36	Unknown
			Black	Fragment	106.70	Unknown
			Red	Fragment	68.45	Unknown
			Transparent (partly blue)	Fibre	1235.46	Polyester
			Transparent	Fibre	1695.24	Polyamide
			Transparent	Fragment	764.39	Polypropylen
			Transparent	Fibre	1280.41	Polyamide
S2	Supraglacial	51.34	Blue	Fibre	1401.81	Polyester
			Black	Fragment	547.64	Polyethylene
			Blue	Fragment	105.38	Unknown
S3	Supraglacial	51.30	Blue	Fibre	15.62	Unknown
			Red	Fibre	1547.80	Polyester
S4	Cryoconite	52.66	Light blue	Fragment	65.05	Unknown
			Red	Fibre	348.96	Polyester
S5	Cryoconite	51.37	Black	Fibre	1117.03	Polyester
			Blue	Fibre	808.22	Polyester
			Black	Fibre	889.33	Polyester
			Black	Fibre	85.47	Unknown
			Violet	Fibre	42.18	Unknown
			Black	Fragment	1385.78	Polyethylene
			Black	Fibre	1142.32	Polyester
S6	Supraglacial	53.52	Light blue	Fibre	177.44	Unknown
			Red (partly blue)	Fibre	900.47	Polyester

Figure S11.1 Cryoconite sample collection on the surface of Forni Glacier. To avoid contamination, samples were collected in accurately cleaned glass jars with a metal laboratory spoon (cryoconite) or a gardening metal shovel (supraglacial debris) by an operator wearing a 100% cotton surgical gown, woollen socks, cotton trousers and wooden clogs.

12. CONCLUSIONS

The aim of this PhD project was to investigate bacterial communities processes in supraglacial ecosystems and provide more data about the anthropic impact on glaciers. Overall, our results bring to light important new aspects.

First, we confirmed that cryoconite holes host dynamic bacterial communities. Indeed, they show differences not only along the ablation season and among years, but also during the same day (**Chapters 3** and **4**). Therefore, these bacterial communities proved to be extremely versatile and to rapidly react to environmental changes. Indeed, they proved to modify their composition according to temperature changes along the ablation season, but also according to differences in light exposure during the day, showing a behaviour similar to other environments, i.e. photoinhibition (already observed in marine waters (Hihara et al. 2001; Ogawa et al. 2018)). Cryoconite holes represent a unique microhabitat and an ideal structure for biogeographical studies, and an important result is that their bacterial communities are glacier-specific, indeed looking at both large scale (**Chapter 7**) and small scale (**Chapter 6**) areas, they were significantly different among glaciers. On the other side they were also significantly different from the bacterial communities inhabiting the same type of sediment (fine grained matter), but not immersed into melting water, on the same glacier. This is an important aspect, it brings to light an important question: to which extent cryoconite holes are unique microbial habitat and, at the same time, how much do they differ according to local condition? They are not only glacier specific, but they look also cryoconite hole specific.

Indeed, the differences between the communities of the two type of habitat (pit full of melting water and dry sediment) are likely due to the protection that cryoconite holes provide (*e.g.*, from high UV radiation, desiccation, harsh wind) that is absent on the dry ice, and even if the sparse sediment can come from the sediment previously contained in cryoconite holes and vice versa, the differences shown can be a consequence of the response of the bacterial community to the habitat switch that can be very rapid, as they were demonstrated to be able to vary also within the same day for their metabolic expression and along the ablation season for their composition (**Chapter 3** and **4**). Therefore, the fast response of bacterial communities that was demonstrated looking at their temporal trends is further supported by its changes from wet to dry condition.

So, we understood so far how different bacterial communities of cryoconite holes can be, but how similar are they? The answer is given by the fact that the most representative orders of these microhabitats are always the same all over the world (**Chapter 7** and **8**) and also by the results of

Chapter 3 and **6**. Indeed, in **Chapter 3** we can see that in the late ablation season bacterial communities look more similar than at the beginning, and in **Chapter 6** results show that communities of cryoconite holes of different glaciers are more similar between them than to the sparse debris of the same glacier. So, in an ideal situation, in absence of perturbations, we can guess that bacterial communities may tend to a climax situation, with a final composition very similar for all of them, maybe also including samples from all over the world.

Supraglacial bacterial communities are important also because they contribute in part to the colonization of recently deglaciated areas. Indeed, our results reported in **Chapter 4** show that bacteria typical of cold environment inhabit the most recently deglaciated areas and put the basis for the consequent colonization. It seems in fact that their metabolisms foster nutrients availability and organic matter production, promoting the development of a more and more complex community.

Another aspect that was taken into consideration concerns the problem of glaciers decrease, due to supraglacial algal communities. Our results proved that the main responsible of algal bloom and the consequent decrease of the albedo is *A. nordenskioeldii*, and that it is possible to quantify with remote sensing techniques algae concentration in algal bloom with a high resolution excluding inorganic impurities (**Chapter 9**).

Unfortunately, biological impurities are not the only ones to negatively affect the glacial ecosystems, indeed different pollutants (both legacy pollutants and currently used ones) have been reported in glacial ice and supraglacial sediment (**Chapter 10**). Furthermore, also microplastics have been found in cryoconite (**Chapter 11**), with an amount of particles per gram comparable to that reported in marine sediments (Hamid et al. 2018). This is an important aspect than needs to be further investigated in the future, since microplastics can be biomagnified in the trophic chain and at the same time have the potential to adsorb other pollutants that normally are not biomagnified (Auta et al. 2017; Eriksen et al. 2014).

Therefore , it is of fundamental importance to keep studying glaciers ecosystems in all their aspects, and to keep the focus on the fact that global warming is irreversibly affecting them, and timing is important. For this reason it is important to study now the biodiversity correlated with these environments, because we may not have other chances, and these kind of studies may help to understand what will be the future of these ecosystems.

13. References

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14. Publications

1. Pittino F, Maglio M, Gandolfi I, et al (2018) Bacterial communities of cryoconite holes of a temperate alpine glacier show both seasonal trends and year-to-year variability. 59:1–9. https://doi.org/10.1017/aog.2018.16
2. Pittino F, Ambrosini R, Azzoni R, et al (2018) Post-Depositional Biodegradation Processes of Pollutants on Glacier Surfaces. Condens Matter 3:24. https://doi.org/10.3390/condmat3030024
3. Ambrosini R, Azzoni R, Pittino F, et al (2019) First evidence of microplastic contamination in the supraglacial debris of an alpine glacier *. Environ Pollut 253:297–301. https://doi.org/10.1016/j.envpol.2019.07.005
4. Franzetti A, Pittino F, Gandolfi I, et al (2020) Early ecological succession patterns of bacterial, fungal and plant communities along a chronosequence in a recently deglaciated area of the Italian Alps. FEMS Microbiol Ecol 96:1–12. https://doi.org/10.1093/femsec/fiaa165
5. Di Mauro B, Garzonio R, Baccolo G, et al (2020) Glacier algae foster ice-albedo feedback in the European Alps. Sci Rep 1–9. https://doi.org/10.1038/s41598-020-61762-0
6. Zawierucha K, Porazinska DL, Ficetola GF, et al (2020) A hole in the nematosphere tardigrades and rotifersdominate the cryoconite hole environment, whereas nematodes are missing. J Zool. https://doi.org/10.1111/jzo.12832