Biological Soil Crust Microalgae and Cyanobacteria - Key Players in Polar and Alpine Ecosystems

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Martin Rippin

aus Gera

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Berichterstatter/in:	Prof. Dr. Burkhard Becker
	Prof. Dr. Michael Bonkowski
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List of Abbreviations

AFP	Antifreeze protein
BSC	Biological soil crust
cDNA	Complementary deoxyribonucleic acid
COX1	Cytochrome *c* oxidase I
CTAB	Cetyl trimethylammonium bromide
DEG	Differentially expressed gene
DNA	Deoxyribonucleic acid
ELIP	Early light-induced protein
EPS	Exopolysaccharides
HSP	Heat shock protein
ITS	Internal transcribed spacer
LEA	Late embryogenesis abundant
MAA	Mycosporine-like amino acid
NGS	Next-generation sequencing
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
PVPP	Polyvinylpolypyrrolidone
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
RuBisCO	Ribulose-1,5-bisphosphat carboxylase/oxygenase
UVR	Ultraviolet radiation

Chapter 1

Introduction

Biological soil crusts (BSCs) are unique soil communities which are also referred to as cryptogamic, cryptobiotic, microbiotic, microfloral, microphytic or organogenic crusts as well as simply biocrusts (Belnap et al., 2001a, 2016). They consist of different prokaryotic and eukaryotic organisms which may be either autotrophic, heterotrophic or saprotrophic (Belnap et al., 2001a, 2016). These micro- and macroscopic organisms belong to different groups such as Cyanobacteria (capitalized throughout the thesis as it is a phylum), eukaryotic microalgae, bryophytes, lichens, Fungi (capitalized throughout the thesis as it is a kingdom), heterotrophic Bacteria (capitalized throughout the thesis as it is a domain), non-algal protists (e.g. amoebae, flagellates, ciliates), nematodes, rotifers, tardigrades and microarthropods (Belnap et al., 2001a, 2016; Darby and Neher, 2016; Fiore-Donno et al., 2017). They colonize the top of the soil and form a living crust cover (Belnap et al., 2001a, 2016). The following paragraphs will provide more details on the biodiversity and ecology of BSCs as well as their potential for biological applications and research methods.

1.1 Biodiversity

1.1.1 Cyanobacteria

Cyanobacteria is a phylum of oxygenic photosynthetic prokaryotes which use water as an electron donor during photosynthesis leading to oxygen evolution (Whitton and Potts, 2012). About 2,100 to 2,400 million years ago, these microbes played a key role in the oxygenation of the atmosphere (Hamilton et al., 2016; Stal, 2007; Whitton and Potts, 2012). However, the earliest record of Cyanobacteria extends back to about 3,500 million years ago (Whitton and Potts, 2012). All Cyanobacteria possess chlorophyll *a* and three main accessory pigments: Allophycocyanin, phycocyanin, phycoerythrin (Stal, 2007; Whitton and Potts, 2012). The pigment phycocyanin gives these microbes their typical blueish color (Whitton and Potts, 2012). Certain cyanobacterial genera are capable of fixing molecular nitrogen from the air (diazotrophs) and, thus, make it available to other organisms (Stal, 2007; Whitton and Potts, 2012; Zakhia et al., 2008). Cyanobacteria are morphologically diverse organisms, they can be unicellular or filamentous, may form colonies or sheaths and the cell size ranges from 0.5 to 30 µm (Stal, 2007; Whitton and Potts, 2012). Furthermore, certain Cyanobacteria develop special cell types: heterocysts and diazocytes, which are different types of nitrogen-fixing cells, akinetes, robust resting cells which are able to survive extended periods of unfavorable conditions, and hormogonia, which are small and motile and help the dispersal of the cyanobacterium (Sandh et al., 2012; Stal, 2007; Whitton and Potts, 2012). Their diversity enables Cyanobacteria to occupy a wide range of habitats such as aqueous, both marine and freshwater, and terrestrial environments (Whitton and Potts, 2012; Zakhia et al., 2008). Moreover, these prokaryotes can cope with extreme abiotic stressors and, thus, occur in environments with high salinity (e.g. salt lakes, hyper saline lagoons), extremely acidic and alkaline lakes (down to pH 3.7 and up to pH 13) as well as ecosystems with very high and low temperatures (hot springs, Polar Regions) (Seckbach and Oren, 2007). For example, a huge part of the ice shelf at McMurdo Sound,

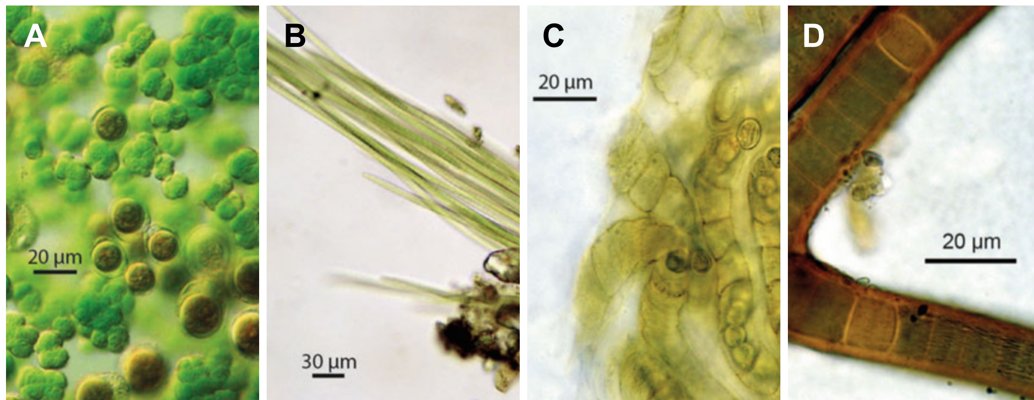

Figure 1: Typical BSC Cyanobacteria. A) *Chroococcidiopsis* sp. B) *Microcoleus vaginatus*. C) *Nostoc commune*. D) *Scytonema* cf. *ocellatum*. Images were taken from Büdel et al. (2016).

Antarctica, is covered by *Oscillatoria* and *Nostoc* (Seckbach and Oren, 2007). The most recent review of cyanobacterial taxonomy was done by Komárek et al. (2014) who used a polyphasic approach and divided the phylum into eight different orders: Chroococcales, Chroococcidiopsidales, Gloeobacterales, Nostocales, Oscillatoriales, Pleurocapsales, Spirulinales and Synechococcales.

In BSC communities, Cyanobacteria fulfill important ecological functions such as the initial colonization of the soil and crust formation, they contribute to the carbon and nitrogen cycle and associate with Fungi to form lichens (please also refer to Section 1.2) (Büdel et al., 2016). By means of morphological and molecular identification, more than 320 species were found in BSC all over the world (Büdel et al., 2016). Typical cyanobacterial genera, which are occurring within these aggregations, are the unicellular *Chroococcidiopsis* as well as the filamentous *Microcoleus*, *Nostoc* and *Scytonema* which are displayed in Figure 1 (Büdel et al., 2016).

1.1.2 Eukaryotic microalgae

Eukaryotic microalgae exhibit a high morphological and physiological diversity which is the key to their ubiquitous distribution (Andersen, 1992). These photosynthetic microorganisms can be found in oceans, lakes, ponds as well

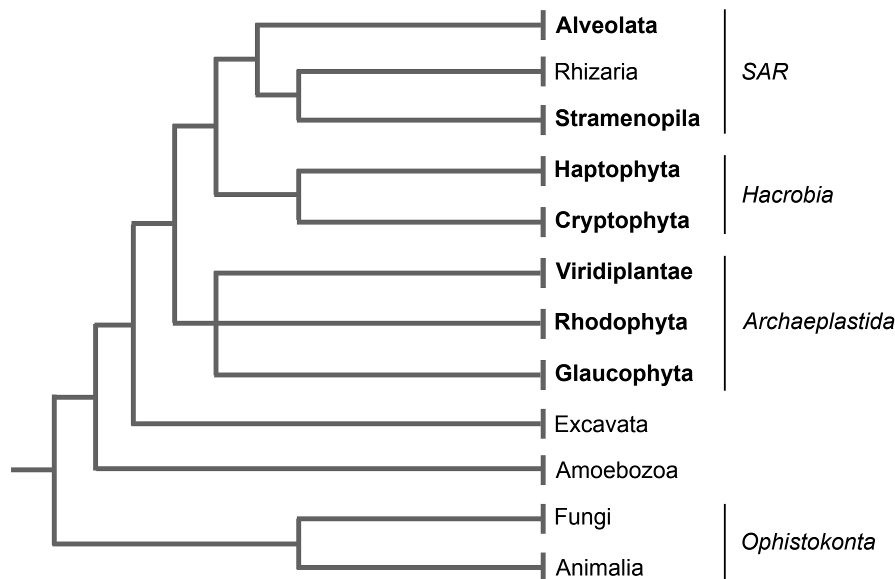

Figure 2: Phylogenetic tree of Eukaryota. The taxa highlighted in bold contain algal genera. The tree was adapted from De Clerck et al. (2012).

as on soil, rocks, snow, ice and other organisms such as plants and animals (Andersen, 1992). Due to special adaptions, certain microalgae are also able to grow in extremely acidic (down to pH 0), alkaline (up to pH 10.6), hypersaline, hot (up to 57°C) and cold environments (even below 0°C) (Seckbach and Oren, 2007). The alga *Chlamydomonas nivalis*, for instance, grows on snow causing a phenomenon called "watermelon snow" which are the red pigmented resting spores of *C. nivalis* (Seckbach and Oren, 2007). Microalgae may be summarized by their ability to perform oxygenic photosynthesis, however, they are polyphyletic and belong to different phyla and subphyla (Andersen, 1992). Figure 2 shows the phylogenetic relationships of eukaryotic taxa containing microalgal genera. Within the Archaeplastida, there are three major taxonomic groups: The Glaucophyta, the Rhodophyta and the Viridiplantae (De Clerck et al., 2012). Rhodophyta or red algae are mostly multicellular and occupy mainly marine habitats (De Clerck et al., 2012). Similar to Cyanobacteria, they use phycobiliproteins (allophycocyanin, phycocyanin, phycoerythrin) as accessory pigments (De Clerck et al., 2012). The same holds true for Glaucophyta, a taxon comprised of unicellular microalgae which can be found in freshwater and terrestrial ecosystems (De Clerck et al., 2012). The clade Viridiplantae

contains a huge diversity of green algae which belong either to the Chlorophyta or the Streptophyta (De Clerck et al., 2012; Leliaert et al., 2012). These organisms colonize both aquatic and terrestrial environments (De Clerck et al., 2012; Leliaert et al., 2012). From an evolutionary point of view, the emergence of streptophytic algae was of major importance as they represent the ancestors of the land plants (De Clerck et al., 2012; Leliaert et al., 2012). Especially, Zygnematophyceae and Coleochaetophyceae are important taxa as they are the closest living relatives of the land plants (Becker and Marin, 2009; De Clerck et al., 2012; Leliaert et al., 2012; Wodniok et al., 2011). In contrast to Glaucophyta and Rhodophyta, Viridiplantae possess chlorophyll *a* and *b* as well as carotenoids and xanthophylls as accessory pigments (De Clerck et al., 2012; Leliaert et al., 2012). The divisions Cryptophyta and Haptophyta encompass unicellular algae possessing two flagella (Gran-Stadniczeñko et al., 2017; Hoef-Emden et al., 2002). Cryptophyta possess chlorophyll *a* and *c* and phycobiliproteins, while Haptophyta contain chlorophyll *a*, *c*1 and *c*2 as well as carotenoids (Pal and Choudhury, 2014). These flagellates occupy both marine and freshwater environments (Gran-Stadniczeñko et al., 2017; Hoef-Emden et al., 2002; Pal and Choudhury, 2014). Similar preferences in terms of habitat are observed for Dinophyta, an algal division within the Alveolata (Pal and Choudhury, 2014; Taylor et al., 2007). Dinoflagellates are mostly symbiotic but about 5% lead a parasitic lifestyle (Taylor et al., 2007). The main pigments of Dinophyta are chlorophyll *a* and *c*2 and the carotenoids peridin and neoperidin (Pal and Choudhury, 2014). Stramenopila, which is also part of the SAR super group, contains several algal taxa within the division Heterokontophyta (De Clerck et al., 2012; Pal and Choudhury, 2014). These organisms are quite diverse and may be found in marine, freshwater and terrestrial ecosystems (Büdel et al., 2016; Heinrich et al., 2016; Pal and Choudhury, 2014). Chlorophyll *a* and *c* as well as fucoxanthin are the major pigments found in Heterokontophyta (Pal and Choudhury, 2014).

Some of the above mentioned microalgal taxa are part of BSCs (Büdel et al., 2016). Streptophytic algae, such as *Klebsormidium* (Klebsormidio-

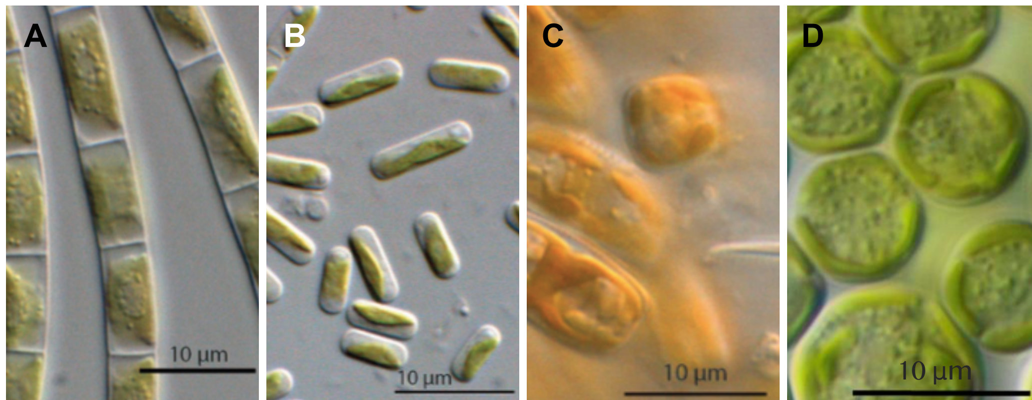

Figure 3: Typical BSC microalgae. A) *Klebsormidium flaccidum.* B) *Stichococcus bacillaris.* C) *Luticola mutica.* D) *Pleurochloris pseudopolychloris.* Images A-C were taken from Büdel et al. (2016) and image D from Borchhardt et al. (2017b).

phyceae, Figure 3A) and *Zygogonium* (Zygnematophyceae), assist the crust formation providing niches for a huge diversity of chlorophytic genera (Chlorophyceae, Trebouxiophyceae, Ulvophyceae) and Heterokontophyta such as Bacillariophyceae (e.g. *Luticola*, see Figure 3C), Eustigmatophyceae and Xanthophyceae (e.g. *Pleurochloris*, see Figure 3D) (Borchhardt et al., 2017b; Büdel et al., 2016). Certain chlorophytes, for example *Stichococcus* (Figure 3B), become the photobionts of lichens (please also refer to Section 1.1.4 and 1.2) (Borchhardt et al., 2017b; Büdel et al., 2016).

1.1.3 Bryophytes

Bryophytes, which traditionally include mosses (Bryophyta), liverworts (Marchantiophyta) and hornworts (Anthocerophyta), are small, non-flowering plants (Prestø et al., 2014; Seppelt et al., 2016; Shaw et al., 2011). These three phyla combined comprise about 15,000-20,000 species and share a haploid-dominant life cycle (Shaw et al., 2011). As most land plants, bryophytes are capable of oxygenic photosynthesis and, thus, have an important ecological role as primary producers (Prestø et al., 2014).

Mosses, liverworts and hornworts are integral parts of BSC communities as they stabilize the soil surface, promote water infiltration as well as chemi-

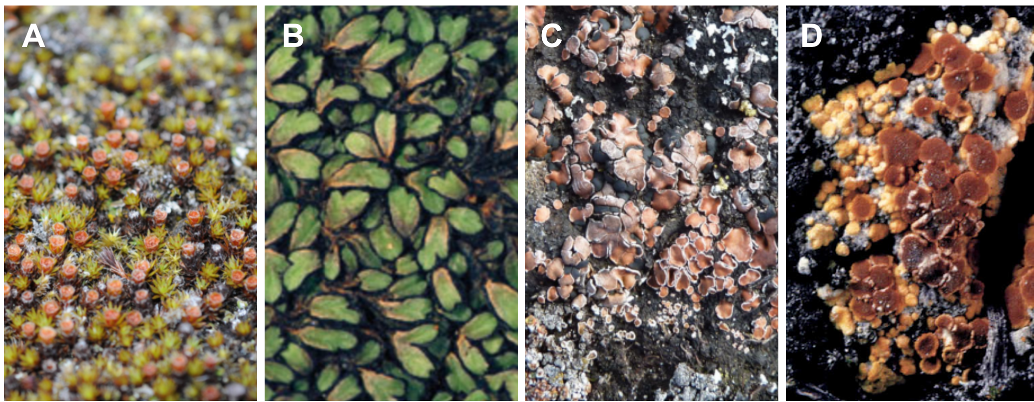

Figure 4: Typical BSC bryophytes and lichens. A) *Polytrichium piliferum*. B) *Riccia* sp. C) *Psora decipiens*. D) *Fulgensia bracteata*. Image A was taken from Prestø et al. (2014), image B from Seppelt et al. (2016), image C from Ruprecht et al. (2014) and image D from Brodo et al. (2001).

cal and physical weathering of soil material (please also refer to Section 1.2) (Seppelt et al., 2016). Typical BSC bryophytes are *Polytrichum* (Bryophyta, see Figure 4A) and *Riccia* (Marchantiophyta, see Figure 4B) (Seppelt et al., 2016). Within BSC, these bryophytes may also serve as habitats for other organisms such as algae or Cyanobacteria (Seppelt et al., 2016).

1.1.4 Fungi and lichens

Fungi are a highly diverse group of eukaryotic microorganisms which play key roles in various ecosystems (Tedersoo et al., 2014). The kingdom is estimated to contain between 800,000 to 5,100,000 species of which about 100,000 have been described (Tedersoo et al., 2014). Certain fungal species may form symbioses with either eukaryotic microalgae (about 86% of the lichen species), prokaryotic Cyanobacteria (about 10%) or both (about 4%) (Maier et al., 2016). The photobiont (alga, cyanobacterium) is surrounded by tight structures, which are formed by the mycobiont (fungus) and extremely robust against changing environmental conditions (Maier et al., 2016; Rosentreter et al., 2016). In return, the photobiont provides nutrients (Rosentreter et al., 2016).

In BSCs, non-lichenized Fungi may recycle nutrients between crust communities and plant vegetation (fungal loop hypothesis) (Maier et al., 2016). A fungus typically associated with BSCs is the hyphomycete *Alternaria* (Maier et al., 2016). Figures 4C and D show the genera *Psora* and *Fulgensia*, repectively, two lichens commonly found in BSC communities across the globe (Rosentreter et al., 2016). The hyphae of *Psora cerebriformis*, for instance, can penetrate the soil, assist particle aggregation and, thereby, crust formation (please also refer to Section 1.2) (Rosentreter et al., 2016).

1.1.5 Soil prokaryotes

Typical prokaryotic phyla found in soil communities are Acidobacteria, Actinobacteria, Bacteroidetes, Planctomycetes, Proteobacteria, Verrucomicrobia (Bacteria), Crenarchaeota, Euryarchaeota and Parvarchaeota (Archaea) (Fierer, 2017). In terms of Bacteria, the same taxa as well as Armatimonadetes and Gemmatimonadetes colonize BSCs (Maier et al., 2016). Archaea seem to be more abundant below the crust (Maier et al., 2016). Soil microbes are involved in nutrient cycling (N, P, S, Fe), biogeochemical processes and affect soil water availability (Fierer, 2017). Certain taxa produce and excrete exopolysaccharides (EPS) which are crucial for soil adhesion and crust formation (please also refer to Section 1.2) (Gundlapally and Garcia-Pichel, 2006; Kielak et al., 2016).

1.1.6 Microfauna

BSCs are inhabited by a diverse microfauna including protists, such as amoeba, flagellates and ciliates, nematodes, tardigrades, rotifers and microarthropods (Darby and Neher, 2016; Fiore-Donno et al., 2017). These organisms are heterotrophs, feeding on Bacteria, Fungi, algae and/or plants or lead a predatory lifestyle (Darby and Neher, 2016). Thus, they regulate microbe populations and mediate nutrient cycling (Darby and Neher, 2016). Moreover, they them-

selves are preyed on by macrofauna representing an important link in the food web (Darby and Neher, 2016).

1.1.7 Biological succession of BSCs

The community composition of BSCs changes over time from the initiation by pioneer species to the terminal succession which is dependent on the environmental conditions (Belnap et al., 2008; Frey et al., 2013; Garcia-Pichel et al., 2016; Lan et al., 2013). Barren soil, which may originate from disturbance but also glacier retreat, is colonized by filamentous Cyanobacteria (see also Section 1.1.1) which aggregate soil particles and stabilize them (Frey et al., 2013; Garcia-Pichel et al., 2016; Lan et al., 2013). Frey et al. (2013) analyzed the initialization of BSC communities along the chronosequence of a retreating glacier. Alpha- and Betaproteobacteria dominated the microbiome of the pristine soil while Cyanobacteria, mostly Oscillatoriales, were only present in low numbers (Frey et al., 2013). These young microbial communities are mainly restricted by carbon and nitrogen availability (Frey et al., 2013). Thus, Cyanobacteria and eukaryotic algae are crucial for the crust development as they fix carbon and make it available for other organisms (Frey et al., 2013). Young cyanobacterial crusts are often dominated by the filamentous cyanobacterium *Microleus vaginatus* which is regarded as a keystone species for the formation of BSCs (Couradeau et al., 2016; Garcia-Pichel et al., 2016). At this early stage, the BSC is still prone to erosion and exhibits a low water and nutrient content (Lan et al., 2013). Over time, soil stability and carbon input are enhanced by an increase of cyanobacterial biomass (Belnap et al., 2008). Subsequently, heterocystous, non-motile Cyanobacteria become more abundant and increase nitrogen availability (Belnap et al., 2008; Garcia-Pichel et al., 2016). The appearance of the crust changes to darker shades due to the accumulation of ultraviolet radiation (UVR) protection pigments such as scytonemin (Belnap et al., 2008; Couradeau et al., 2016; Garcia-Pichel et al., 2016). These alkaloids are produced by certain late-successional Cyanobacteria and cause a decrease of the surface albedo (Couradeau et al., 2016; Garcia-

Pichel et al., 2016). As a result, the local temperature rises which is leading to changes in species composition (Couradeau et al., 2016; Garcia-Pichel et al., 2016). Couradeau et al. (2016) measured an temperature increase of up to 10°C in the crust causing the replacement of *Microcoleus vaginatus* by the more thermotolerant *M. steenstrupii*. During this stage, eukaryotic algae as well as heterotrophic microbes become more abundant (Garcia-Pichel et al., 2016; Lan et al., 2013). As heterotrophs convert organic into inorganic compounds, they improve the conditions for photoautotrophs (positive feedback loop) (Lan et al., 2013). Finally, when the top soil is cemented, lichens and/or bryophytes appear and shape the community: The metabolic efficiency and protection against erosion is higher; the physical structure and porosity of the crust is altered; changes in water and gas fluxes occur (Garcia-Pichel et al., 2016; Lan et al., 2013). Furthermore, bryophytes provide microhabitats for epiphytic Cyanobacteria which increase crust fertility (Garcia-Pichel et al., 2016).

1.2 Ecology

1.2.1 Distribution

BSCs are the predominant vegetation cover in hostile environments such as hot and dry steppes and deserts, alpine and subalpine areas as well as the Polar Regions (Bu et al., 2013; Büdel, 2001; Colesie et al., 2014). These arid and semiarid regions are the Earth's biggest terrestrial ecosystem comprising about 41% of the Earth's terrestrial area as well as 27% and 95% of the total soil organic and inorganic carbon reservoir, respectively (Belnap et al., 2016; Chiquoine et al., 2016; Rutherford et al., 2017). Thus, drylands have a significant influence on the global climate (Chiquoine et al., 2016). In general, the vascular plant cover in these ecosystems is sparse or absent, restricted by extreme temperatures and low water availability (Belnap et al., 2016; Colesie et al., 2014). However, the soil surface is not barren but colonized by BSCs which can contribute up to 70% of the total vegetational cover in drylands

(Colesie et al., 2014; Rutherford et al., 2017). Hence, the presence of BSCs is an important characteristic of the Earth's terrestrial land (Belnap et al., 2016). Several studies confirmed the presence of BSCs in North and South America, Europe, the Middle East, Africa, Asia, Australia and Antarctica (Abed et al., 2013; Bu et al., 2013; Büdel et al., 2009; Ponzetti and McCune, 2001; Rosentreter et al., 2014; Williams et al., 2016). However, BSC communities, collected from different places, vary in structure and species composition (Bowker et al., 2016; Büdel, 2001). A number of factors determine the type of crust present in a certain ecosystem (Bowker et al., 2016). Age and degree of land mass isolation has a significant influence on BSC distribution (Bowker et al., 2016). In regards to the distribution of BSC Cyanobacteria, for example, Antarctica has a similarity of more than 10% with all other continents, although it is the most isolated one (Bowker et al., 2016). This might not be surprising as these prokaryotes are an ancient group of crust organisms that colonized the land 3,500 million years ago, thus, before the breakup of Pangaea (Rogers and Santosh, 2004; Whitton and Potts, 2012). In contrast, the similarity for lichens between Antarctica and the other continents lies below 10% (Bowker et al., 2016). Not only biogeography but also climatic conditions, such as the thermal regime as well as the amount and frequency of precipitation prevailing in a specific ecoregion, affect BSC distribution and abundance (Belnap et al., 2001b; Garcia-Pichel et al., 2013; Reed et al., 2012). For instance, the cyanobacterium *Microcoleus vaginatus* prefers cooler soils and winter precipitation, while *Microcoleus steenstrupii* colonizes hot drylands with summer precipitation (Garcia-Pichel et al., 2013). Moreover, the distribution and composition of BSCs is also determined by soil properties such as texture, pH, salinity, sodicity and fertility (Belnap et al., 2001b; Steven et al., 2013a). Examples include the promotion of cyanobacterial richness by sandy and poorly aggregated soils, while highly developed moss and lichen crusts dominate calcareous or gypsiferous soils (Belnap and Lange, 2001; Martínez et al., 2006; Root and McCune, 2012). As the photosynthetic biomass of BSCs is high, these communities are dependent on sunlight (Lange, 2001). Nevertheless, ex-

cessive solar radiation causes the soil to dry out more quickly affecting BSCs negatively (Bowker et al., 2016). Plant canopies can protect crust communities from strong radiation and drying out by providing shade (Belnap et al., 2001b). Furthermore, higher plants may increase soil stability, moisture and fertility, promoting BSC diversity (Bowker et al., 2016). In some cases, however, crust richness is limited by higher vegetation (Eldridge et al., 2006; Langhans et al., 2010). Another important factor influencing BSC cover in drylands is geomorphologies (Bowker et al., 2016). The distribution of BSCs across the Negev Desert, Israel, illustrates the point (Veste et al., 2001). Crust cover is absent on top of the dunes, but lower slopes are colonized by BSCs (Veste et al., 2001).

The Polar Regions

The Polar Regions, the Arctic and Antarctica, may be defined as the areas confined by the Polar Circles at 66° 33' N/S, by the 10°C summer isotherm (mean temperature of the warmest month is below 10°C) or the tree line (Thomas et al., 2008). These regions, also referred to as the *cryosphere*, are dominated by extremely low temperatures, are covered by ice and snow for the most part and exhibit a high seasonality (Thomas et al., 2008). During summer, these ecosystems receive continuous irradiance displaced by long periods of darkness in winter (Thomas et al., 2008). Albeit the huge number of similarities, the Arctic and Antarctica differ significantly from each other (Thomas et al., 2008). The biggest part of the Arctic consists of land-locked sea, which is covered by ice, but also expands into the North of Canada, Alaska and Siberia, and includes Greenland and Svalbard (Thomas et al., 2008). Antarctica, on the other hand, is a continent that is permanently covered by ice and snow (up to 99.2%) surrounded by the Southern Ocean (Green and Broady, 2001; Thomas et al., 2008).

Despite the hostile conditions, which are prevailing in the cryosphere, these ecosystems are not dead, but inhabited by a number of survivalists (Thomas et al., 2008). For example, Antarctica, the coldest, windiest and driest conti-

nent, features two species of seed plants, *Colobanthus quitensis* and *Deschampsia antarctica* (Green and Broady, 2001). However, there is an overall trend to a lower degree of biodiversity (Green and Broady, 2001). BSCs are the dominant vegetation unit in these areas, albeit with a distribution restricted to ice-free terrestrial surfaces (Borchhardt et al., 2017a; Colesie et al., 2014; Green and Broady, 2001). Take, for instance, the case of Arctic Svalbard which exhibits a BSC coverage of up to 90%, with bryophytes and Cyanobacteria being most dominant (Williams et al., 2017). In contrast, Livingston Island, a part of the Antarctic Peninsula, is covered up to 55% by lichen dominated communities (Williams et al., 2017).

Alpine and subalpine areas

Alpine zones span the elevated areas above the treeline, while subalpine refers to the zone between the borders of the montane forest and upper limits of scattered, wind-shaped trees growing on open land (Martin, 2001). Alpine habitats account for approximately 3% of Earth's terrestrial area (Martin, 2001). These rugged ecosystems are characterized by rocky ridges and steep terrain, strong winds, extreme temperatures, high radiation, low water availability and permanent or temporary snow cover which causes the vegetation to be small and patchy (Karsten and Holzinger, 2014; Martin, 2001). Furthermore, biodiversity is lower compared to lower altitudes which might also be caused by hypoxic conditions at higher elevations, a high climatic stochasticity and short growing seasons (Martin, 2001).

Vascular vegetation is poorly developed in alpine and subalpine areas, however, BSCs colonize the open ground between the vegetation patches (Türk and Gärtner, 2001). Records of alpine BSCs exist from the Andes, Olympic Mountains (Washington, USA) and the Austrian Alps, among others (Colesie et al., 2016; Williams et al., 2017). For instance, at Hohe Tauern National Park, a part of the Austrian Alps, 60% of the soil surface is covered by crust communities which exhibit a high abundance of Cyanobacteria (Williams et al., 2017)

1.2.2 Abiotic stress

As described in Section 1.2.1, BSCs have to cope with extreme conditions in these hostile environments: High and low temperatures, high irradiance and low water availability (Belnap et al., 2016). A plethora of physiological and molecular mechanisms enable the crust-inhabiting organisms to survive and adapt to this harsh abiotic stress.

Temperature

Elevated temperatures cause proteins and nucleic acids to denature and destabilize membranes, threatening the viability of exposed organisms (Kobayashi et al., 2014). The general response after heat exposure are changes in the carbohydrate profile, membrane modifications, as well as the expression and/or upregulation of heat shock proteins (HSPs) (Ianutsevich et al., 2016). The green alga *Chlamydomonas reinhardtii* and the red alga *Cyanidioschyzon merolae*, for example, increase the transcript pool of small HSPs upon heat shock treatment (Kobayashi et al., 2014). An example for changes in membrane composition are the two fungal species *Rhizomucor tauricus* and *Myceliophthora thermophila* which increased the amount of phosphatidic acids and sterols in response to elevated temperatures while the phosphatidylcholine and phosphatidylethanolamine contents are reduced (Ianutsevich et al., 2016). Moreover, Ianutsevich et al. (2016) observed a shift in the trehalose concentration, suggesting that this sugar plays an important role in this process. Oukarroum et al. (2012) investigated the heat shock response of the lichen *Parmelina tiliacea* in different physiological states. Interestingly, the lichen is more heat-tolerant in a desiccated than in a wet state (Oukarroum et al., 2012).

The other extreme, low temperatures close to the freezing point, is accompanied by a number of constraints such as decreased transport and enzyme reaction rates and reduced membrane fluidity (Tsuji, 2016). During freezing, dehydration occurs and the formation of ice crystals can even damage the cell (Jung et al., 2014; Park et al., 1997). To counteract low temperatures and freezing, organisms accumulate high- and low-molecular-weight solutes, which

act as cryoprotectants, antioxidants and change their membrane structure and composition (Minami et al., 2005; Quesada and Vincent, 2012; Rütten and Santarius, 1992; Zakhia et al., 2008). Bryophytes, for instance *Polytrichum formosum*, exhibit increased sucrose levels, a known cryoprotectant, during winter (Rütten and Santarius, 1992). Certain polar organisms also synthesize antifreeze proteins, e.g. the Antarctic green alga *Pyramimonas gelidicola* and the diatom *Chaetoceros neogracile*, which bind to ice-crystals and lower the freezing point (Jung et al., 2014; Kim et al., 2017).

Light and UV radiation

Intensive solar radiation may cause deoxyribonucleic acid (DNA) damage, promotes reactive oxygen species (ROS) generation and impairs growth, photosynthesis, respiration and reproduction (Holzinger et al., 2009; Pichrtová et al., 2013; Rajeev et al., 2013). To cope with DNA damage induced by UVR, Cyanobacteria use excision repair and photo-reactivation (Zakhia et al., 2008). Furthermore, these microorganisms synthesize a number of sunscreen compounds, such as scytonemin and mycosporine, to mitigate the effect of UVR (Couradeau et al., 2016; Zakhia et al., 2008). Eukaryotic algae, e.g. *Zygnema* and *Tetracystis*, use mycosporine-like amino acids (MAAs), carotenoids, phenolics or sporopollenin to attenuate harmful UVR (Pichrtová et al., 2013). Singaravelan et al. (2008) found that the soil fungus *Aspergillus niger* gains an increased UVR resistance by melanin formation. Highly pigmented organisms, such as *Zygnema*, *Tetracystis* and *Aspergillus*, can then shade other light- and UVR-sensitive organisms in the BSC community (Belnap and Lange, 2001; Karsten and Holzinger, 2014).

Drought

The loss of water is a lethal threat to all organisms as it causes the desintegration of membranes, organelles and biomolecules, as well as the aggregation of macromolecules such as proteins (Holzinger and Karsten, 2013; Karsten and Holzinger, 2014; Wang et al., 2004). However, certain, dessication-tolerant

organisms can withstand severe dehydration without compromising their viability (Potts, 1994). Cameron (1962), for example, could revive the cyanobacterium *Nostoc commune* after it had been dried out for 87 years. The mechanisms, underlying desiccation tolerance, are complex and manifold (Alpert, 2005; Fernández-Marín et al., 2013; Holzinger and Karsten, 2013; Potts, 1994). For photosynthetic organisms, the availability of water is crucial as it maintains cellular integrity, functionality and acts as an electron donor in the electron transport chain (Fernández-Marín et al., 2013). Some organisms, such as the streptophytic alga *Klebsormidium crenulatum* and chlorophytic alga *Trebouxia gelatinosa*, increase the pool of photosynthetic transcripts to counteract desiccation, while the bryophyte *Syntrichya ruralis* reduces the expression of photosynthetic genes (Carniel et al., 2016; Holzinger et al., 2014). Furthermore, early light-induced proteins (ELIPs) are commonly upregulated in response to dehydration (Fernández-Marín et al., 2013). Holzinger et al. (2014) and Zeng et al. (2002) observed an increase of ELIP transcripts in the alga *K. crenulatum* and the moss *S. ruralis*, repectively, when exposed to drought. ELIPs are low-molecular-weight proteins that belong to the chlorophyll a/b-binding superfamily and protect the photosynthetic apparatus by preventing the formation of free radicals (Fernández-Marín et al., 2013; Norén et al., 2003). Water stress is also associated with the production of the disaccharides sucrose and trehalose which act as osmolytes and membrane stabilizers (Alpert, 2005; Fernández-Marín et al., 2013; Potts, 1994). For instance, trehalose concentration is increased in the cyanobacterium *Nostoc commune* when desiccated (Potts, 1994). Two ecotypes of the nematode *Steinernema feltiae*, one originating from a temperate and the other from a semi-arid region, were compared in terms of desiccation tolerance (Alpert, 2005). The temperate nematode exhibited a higher rate of trehalose removal compared to the semi-arid one, suggesting a lower tolerance of the former (Alpert, 2005). Sadowsky et al. (2016) studied the desiccation-induced response of the photobiont in the Antarctic lichen *Usnea lambii* and found that sucrose levels were increased. Dehydration and rehydration also affect the integrity of membranes negatively

(Perlikowski et al., 2016). Thus, desiccation tolerant organisms are required to protect their biomembranes by modification (Perlikowski et al., 2016). To counteract dehydration, the cyanobacterium *Nostoc* increases the portion of unsaturated fatty acids of its cytoplasmic membrane (Potts, 1994). The streptophyte *K. crenulatum* increases its pool of transcripts involved in the lipid metabolism suggesting membrane modification (Holzinger et al., 2014). Desiccation also induces the formation of ROS, which is extremely harmful to the cell as these radicals can cause nucleic acids, proteins, polysaccharides and lipids to denature (Heinrich et al., 2015; Potts, 1994). Cyanobacteria, for example, express the enzymes ascorbate peroxidase and catalase during dehydration to scavenge harmful ROS (Potts, 1994). The alga *T. gelatinosa* also responds with the induction of the ROS-scavengers to desiccation stress (Carniel et al., 2016). As mentioned before, water stress compromises the structural integrity and functionality of proteins (Wang et al., 2004). Hence, organisms counteract this stress by expressing molecular chaperones, such as HSPs and late embryogenesis abundant (LEA) proteins (Alpert, 2005; Wang et al., 2004). Homologues of LEA proteins, which are typically associated with water stress, were found in a number of Bacteria, nematodes, bryophytes and algae (Alpert, 2005; Gasulla et al., 2013; Goyal et al., 2005; Holzinger et al., 2014). Examples include the moss *Physcomitrella patens*, which exhibits an increased abundance of LEA transcripts upon desiccation stress (Shinde et al., 2012). Furthermore, *P. patens* induced the expression of HSPs and other chaperones (Wang et al., 2009a). Desiccation may also cause the transition to permanent stages, e.g. cysts, akinetes, zygo- or oospores, as an avoidance mechanism (Holzinger and Karsten, 2013).

1.2.3 Ecological functions

BSC communities are essential components in dryland ecosystems as they contribute to primary production, nitrogen fixation and phosphorus cycling (Belnap, 2003; Belnap et al., 2016; Bu et al., 2013). Furthermore, BSCs offer

erosion protection and influence hydrology, albedo and the surface temperature (Belnap, 2003; Belnap et al., 2016; Bu et al., 2013; Chamizo et al., 2015).

Carbon cycle

The photosynthetic biomass of BSCs, including Cyanobacteria, eukaryotic algae, bryophytes and lichens, contribute greatly to primary production in arid and semi-arid regions where vascular plants are rare or absent (Baran et al., 2015; Belnap, 2003; Belnap et al., 2001b; Su et al., 2013). On a global scale, BSCs fix 3.9 Pg carbon per year corresponding to 7% of the net primary production of the terrestrial vegetation (Elbert et al., 2012). However, the carbon fixation by the autotrophs inhabiting BSCs is restricted by available water, which is coming from rain, fog, dew, increased humidity or snow melt (Belnap, 2003; Belnap and Lange, 2001; Steven et al., 2013b). When exposed to moisture, metabolic processes resume almost directly; respiratory activity starts after about 3 min and photosynthesis takes 30 min to become fully activated (Belnap et al., 2001b). Hence, prolonged wet periods lead to carbon accumulation while short wet periods as well as dry periods may result in a negative carbon budget (Belnap et al., 2001b). Photosynthesis is also controlled by temperature (Belnap et al., 2001b). Up to 28°C carbon fixation is increasing, however, it experiences a drop above this temperature (Belnap et al., 2001b). There are some exceptions, such as the lichen *Collema tenax*, which does not arrest photosynthesis up to a temperature of 36°C (Belnap et al., 2001b). Overall, BSCs contribute carbon and nutrients to heterotrophic organisms, such as Fungi and Bacteria, fertilize the soil and contribute to humus formation (Belnap, 2003; Belnap and Lange, 2001; Belnap et al., 2001b).

Nitrogen cycle

Nitrogen fixation in drylands is mainly carried out by lichenized and free-living Cyanobacteria which are part of BSC communities with lichens to be most important (Belnap, 2003; Belnap and Lange, 2001; Belnap et al., 2001b). As primary production is limited by nitrogen availability, continuous fixation

is crucial for soil fertility and to prevent desertification (Belnap et al., 2001b). Similar to carbon fixation, nitrogen fixation depends not only on water but also community composition (Belnap, 2003; Belnap et al., 2001b). Lichens reach the highest turnover rates once the carbon reservoirs are full (Belnap et al., 2001b). Moreover, temperature affects nitrogen fixation rates (Belnap and Lange, 2001; Belnap et al., 2001b). For most lichen species, an increase can be observed up to $25 - 30°C$ (Belnap and Lange, 2001; Belnap et al., 2001b). Consequently, fixation rates are higher during cooler seasons in hot climates, while temperate and Polar Regions have the highest rates in warmer seasons (Belnap and Lange, 2001). A large part of the fixed nitrogen leaks directly into the surroundings and is taken up by vascular plants, as well as heterotrophs such as Fungi and Bacteria (Belnap, 2003; Belnap and Lange, 2001; Belnap et al., 2001b). For instance, the cyanobacterium *Nostoc* releases 88% of the fixed nitrogen into the environment (Belnap et al., 2001b). In total, BSCs contribute about 50% of the nitrogen fixed on land, which corresponds to 49 Tg per year (Elbert et al., 2012).

Phosporus cycle

BSCs also increase phosphorus bioavailability in the adjacent soil and are involved in the conversion of inorganic to organic phosphorus (Baumann et al., 2017; Zhang et al., 2016). Hence, they play an essential role in the phosphorus cycle, however, the underlying mechanisms are not well studied yet (Baumann et al., 2017). It is anticipated that associated Fungi supply phosphorus to lichens and bryophytes or secrete enzymes, such as phosphatase, into the soil to generate bioavailable phosphorus (Belnap et al., 2016).

Wind and water erosion

The process of soil development in arid regions is slow and the formed soil is extremely erodible (Belnap, 2003). Biologically crusted soils are less prone to erosion as the EPS produced by Cyanobacteria and green algae, as well as the rhizines of lichens and the rhizoids of bryophytes aggregate soil particles

which makes it more difficult for wind and water to move them (Belnap, 2003; Belnap et al., 2001b). The more developed a crust is, e.g. lichen and moss crusts, the better it can provide protection against erosive forces (Belnap, 2003; Belnap et al., 2001b). Hence, BSCs increase soil stability and minimize soil loss (Belnap and Lange, 2001; Belnap et al., 2001b).

Hydrology

The presence and type of BSC community have an influence on water infiltration, water retention and, thus, soil moisture (Belnap, 2003, 2006; Belnap and Lange, 2001; Belnap et al., 2001b; Rodríguez-Caballero et al., 2013). Depending on the morphology of the crust, water infiltration can be increased or decreased (Belnap, 2003; Belnap and Lange, 2001). BSCs with a smooth or rugose appearance exhibit smaller and fewer pores which decreases water residence time and, in turn, infiltration (Belnap, 2003; Belnap et al., 2001b). Furthermore, mucilaginous Cyanobacteria can amplify this effect as they excrete hydrophobic substances and due to their ability to swell and clog pores upon wetting (Belnap et al., 2001b; Rodríguez-Caballero et al., 2013). Pinnacled and rolling morphologies, on the other hand, which are more common in cooler ecosystems, feature a higher porosity and rougher surface resulting in an enhanced water infiltration (Belnap, 2003; Rodríguez-Caballero et al., 2013). However, factors such as site and soil texture can overrule the effect of biocrusts (Belnap, 2003; Belnap et al., 2001b). Clay-rich soils, for example, exhibit an impaired infiltration of water whether or not BSCs are present (Belnap et al., 2001b).

Albedo and surface temperature

Soil albedo is the reflective power of the soil surface which is affected by the crust cover (Belnap et al., 2001b; Couradeau et al., 2016). Well-developed, darker BSCs have a lower albedo than uncrusted soil or younger crusts (Belnap, 2003; Belnap et al., 2001b). Lichen or moss dominated crusts, for instance, reflect only 50% of the incoming light compared to bare soil (Belnap, 2003).

As a consequence, surface temperatures are increasing by $10 - 14°C$ (Belnap, 2003). Many ecosystem functions and processes, such as carbon and nitrogen fixation, microbial activity, seed germination and plant growth, are directly controlled by temperature (Belnap, 2003; Belnap et al., 2001b). Surface temperature also affects the microfauna (Belnap, 2003; Belnap et al., 2001b).

1.2.4 Disturbance

Disturbance influences ecosystem structure and composition and is the main reason for spatial heterogeneity (Bu et al., 2013). It also affects competition and the availability of resources (Bu et al., 2013). Disturbance can be caused by grazing, fire, human activity or invasive species (Belnap and Lange, 2001; Bu et al., 2013; Kuske et al., 2012). The disturbance of biocrusts leads to changes in surface albedo and temperature, alters nutrient cycles and metabolic processes, decreases soil fertility, increases the erosion risk as well as the composition of the crust cover (Belnap and Lange, 2001; Belnap et al., 2001b; Bu et al., 2013; Eldridge et al., 2015; Kuske et al., 2012). Severely disturbed BSCs may take decades or even centuries to recover, which influences the performance of the whole ecosystem (Belnap and Lange, 2001; Kuske et al., 2012). Factors, such as climate, soil type and the degree of disturbance, determine the recovery rate (Belnap and Lange, 2001).

1.3 Global change scenarios

Climate change is a major threat to biodiversity and ecosystem functionality worldwide (Frenot et al., 2005; Lee et al., 2017; Walther et al., 2002). Over the past 50 years, the mean temperature on Earth has increased by 0.17°C per decade (NOAA National Centers for Environmental Information, 2017). In the Arctic, for instance, the increase was even two to three times higher (Post et al., 2009). These changes in temperature and also precipitation regimes are likely to affect arid and semi-arid regions across the globe (Rutherford et al., 2017). Global change is expected to promote the development of deserts

which is also linked to a regression of vegetation and an accumulation of mobile sand (Fischer and Subbotina, 2014). Furthermore, biodiversity and ecosystem interactions are highly affected, e.g. by the invasion of alien species and the disruption of the trophic hierarchy (Frenot et al., 2005; Walther et al., 2002). BSCs are no exception, being extremely sensitive to alterations in temperature as well as precipitation amount and frequency (Fischer and Subbotina, 2014; Rutherford et al., 2017). Consequently, their community composition and ecological functionality are altered (Fischer and Subbotina, 2014; Rutherford et al., 2017). Increased temperatures and changes in precipitation, for example, can lead to a dramatic increase in moss mortality and a decline in lichen coverage (Bowker et al., 2014; Rutherford et al., 2017). In turn, the surface albedo can increase which may be a positive feedback loop for further changes (Rutherford et al., 2017).

Looking at a global scale, both Polar Regions have experienced rather strong warming events in the past (Lee et al., 2017; Post et al., 2009). Besides freezing later and breaking up earlier, the Arctic sea ice has retreated for the past two to three decades (Post et al., 2009). These changes in temperature, snow and ice cover as well as nutrient availability cause shifts in vegetation and even extinctions (Post et al., 2009). The terrestrial biodiversity of Antarctica is mostly bound to ice-free zones (Lee et al., 2017). However, increased temperatures may cause these zones to expand and coalesce which could abolish ancient species boundaries (Lee et al., 2017).

1.4 Applications

BSCs offer several possibilities for application such as artificial cultivation to counteract desertification or to rehabilitate agricultural fields after overexploitation (Bu et al., 2013; Chen et al., 2006; Rossi et al., 2016; Xiao et al., 2015). Chen et al. (2006) used cultures of the cyanobacterium *Microcoleus vaginatus* to inoculate sand dunes in Inner Mongolia, China, to increase the resistance against soil erosion. After 22 days, crusts were established and less

prone to wind and water erosion (Chen et al., 2006). Xiao et al. (2015) cultured moss crusts artificially on the Loess Plateau, China, and monitored the effects on soil stabilization as well as water infiltration and retention over eight years. The results indicate a positive impact, however, the effect on the surface water conditions was only negligible (Xiao et al., 2015). BSCs are also sources of biotechnological products such as the microbial sunscreen scytonemin (Siezen, 2011). As mentioned in Section 1.2.1, BSCs occupy extremely cold environments such as the Arctic and Antarctica (Williams et al., 2017). These cold-adapted and cold-acclimated organisms possess unique features in terms of transcriptome, proteome and lipidome (Jung et al., 2014). In order to cope with low temperatures and freezing, they produce antifreeze proteins (AFPs) which can be biotechnologically exploited (Jung et al., 2014). AFPs can be added to red blood cells to prevent hemolysis during freeze-thaw cycles or to food, such as meat and ice cream, to reduce ice crystal size (Jung et al., 2014).

1.5 Methodology

Studying BSC communities involves a plethora of methods as they may be analyzed in an ecological context as well as in terms of biodiversity and functionality (Borchhardt et al., 2017b; Liu et al., 2014; Williams et al., 2017). There are non-invasive methods, which can be carried out in the field, but most techniques involve sampling and further analysis of the material in a laboratory (Belnap et al., 2001b; Borchhardt et al., 2017a; Rajeev et al., 2013).

There are three different methods commonly used to assess the BSC cover along a transect in a certain area: Quadrats, line-point intercept and line intercept (Belnap et al., 2001b; Bowker et al., 2013; Williams et al., 2017). Each methodology has advantages and drawbacks and the choice should be dependent on the degree of accuracy and detail required (Belnap et al., 2001b). Within these predefined sampling areas, so-called *vegetation plots*, either individual taxa or morphological groups are determined (Belnap et al., 2001b;

Castillo-Monroy et al., 2016). The classification of morphological groups is quick, simple and gives information about the ecological role (Belnap et al., 2001b; Williams et al., 2017). The identification of taxa, on the other hand, is more precise but in the field it is limited to bryophytes and lichens (Belnap et al., 2001b; Bowker et al., 2013; Castillo-Monroy et al., 2016; Chiquoine et al., 2016). Microorganisms, such as Cyanobacteria and algae, have to be sampled and analyzed in the laboratory (Borchhardt et al., 2017a; Chiquoine et al., 2016). Portable devices enable researches to measure several parameters directly in the field (Colesie et al., 2016). Colesie et al. (2016), for example, monitored the activity of BSCs using an IMAGING-PAM and a gas exchange system, both portable.

Sampled BSC material can be analyzed in different ways. Commonly, soil properties, such as carbon, nitrogen, phosphorus, sulfur, sand, silt and clay content as well as pH, are determined (Baumann et al., 2017; Borchhardt et al., 2017a; Bowker et al., 2013; Castillo-Monroy et al., 2016). Moreover, the chlorophyll *a*, EPS and scytonemin concentration are measured giving information about biomass, crust stability and stage of development, respectively (Belnap et al., 2008; Chiquoine et al., 2016; Couradeau et al., 2016; Kuske et al., 2012). In order to study nutrient cycles, activity assays of key enzymes, such as phosphatase, urease, invertase and catalase, are carried out (Bowker et al., 2013; Liu et al., 2014). However, collecting BSC samples also enables the identification of microorganisms inhabiting the crust (Borchhardt et al., 2017a; Chiquoine et al., 2016; Williams et al., 2016). Using light microscopy, a great variety of taxa can be identified either directly in the sample or after the preparation of enrichment cultures and isolates (Baumann et al., 2017; Borchhardt et al., 2017a; Cameron and Devaney, 1970; Williams et al., 2016). This type of identification is based on the recognition of morphological traits such as color, cell size and shape, or motility (Ab Majid et al., 2015; Cox, 1996; John et al., 2002; Lányi, 1988). For instance, Borchhardt et al. (2017a) isolated eukaryotic microalgae from BSCs by cultivating them on agar plates and used the morphological key by Ettl and Gärtner (2014) to identify the taxa. Despite

this method being relatively fast and cheap, morphological features are often ambiguous and, thus, difficult to identify (Albrecht et al., 2017; Manoylov, 2014; Misawa, 1999). Molecular techniques, such as barcoding, offer a reliable alternative (Vieira et al., 2016; Wang et al., 2016). A *barcode* is a molecular marker gene, such as the 16S/18S ribosomal ribonucleic acid (rRNA) gene and the internal transcribed spacer (ITS) region, the ribulose-1,5-bisphosphat carboxylase/oxygenase (RuBisCO) large subunit or the cytochrome *c* oxidase I (COX1), which is amplified and sequenced (An et al., 1999; Doyle et al., 1997; Evans et al., 2007; Wilmotte, 1994). Commonly, these amplified products are sequenced using the chain-termination method originally developed by Sanger et al. (1977). The resulting sequence can be identified by performing a sequence homology search against a suitable database (Altschul et al., 1990; Raja et al., 2017). However, these databases can contain incorrect and wrongly annotated sequences or even lack the reference sequence required for identification (Taberlet et al., 2012). To circumvent this problem, the query sequence may be combined with an appropriate taxon sampling and analyzed using e.g. maximum parsimony or maximum likelihood based phylogenetic inference methods (Komárek et al., 2014; Mount, 2008; Wang et al., 2016).

Culture isolates also provide the possibility to perform physiological experiments with BSC organisms. For example, typical abiotic stressors, which are prevailing in terrestrial habitats (e.g. high radiation, drought), can be mimicked and the effects recorded (Herburger and Holzinger, 2015; Holzinger et al., 2009; Karsten and Holzinger, 2014). A recent study by Herburger and Holzinger (2015) compared the physiological response to desiccation stress of different *Klebsormidium* and *Zygnema* strains. Additionally, underlying molecular mechanisms can be studied by using approaches such as transcriptomics or proteomics (Carniel et al., 2016; Holzinger et al., 2014; Wang et al., 2009a). Proteomic studies focus on all proteins of an organism at a certain time, which constantly change in response to environmental conditions, while transcriptomics are based on gene expression levels (Han et al., 2008; Liang and Zeng, 2016). For transcriptomic studies, total ribonucleic acid (RNA) is isolated

and transcribed into complementary deoxyribonucleic acid (cDNA) which is subsequently sequenced using next-generation sequencing (NGS) tools such as Illumina or Roche 454 (Bentley et al., 2008; Holzinger et al., 2014; Liang and Zeng, 2016; Margulies et al., 2005). The raw data is filtered and expression levels are determined using different software, e.g. the *R* package Bioconductor (Liang and Zeng, 2016). If annotations for the differentially expressed genes (DEGs) are available, conclusions on functional relations can be drawn (Carniel et al., 2016; Holzinger et al., 2014; Liang and Zeng, 2016). Ideally, physiological data is also recorded and used for comparison and validation (Iñiguez et al., 2017). Holzinger et al. (2014), for example, studied the desiccation stress response of the streptophyte *Klebsormidium crenulatum* using the effective quantum yield of photosystem II as a fitness parameter and transcriptomics to shed light on the molecular mechanisms.

Unfortunately, most organisms inhabiting natural habitats, such as BSCs, are unculturable and, thus, cannot be studied individually as described above (Massana et al., 2014; Schloss and Handelsman, 2005; Shi et al., 2009; Ward et al., 1990). Furthermore, the interactions between all organisms within a community should also be considered for a holistic understanding (Urich et al., 2014). A barcode amplicon (e.g. the 16S rRNA gene) can be produced from the whole community and used in a metabarcoding approach, either cloned into vectors and sequenced using the chain-termination technique, or directly applied to a NGS platform (Couradeau et al., 2016; Eldridge et al., 2015; Fierer, 2017; Fiore-Donno et al., 2017; Frey et al., 2013; Kuske et al., 2012). The latter is also referred to as amplicon sequencing and can give insights into biodiversity and relative abundance of individual organisms in a sample (Lange et al., 2015; Taberlet et al., 2012). The generated sequences are counted, normalized and annotated to obtain the percentage per taxa, also known as operational taxonomic unit (OTU) (Caporaso et al., 2010; Couradeau et al., 2016; Fiore-Donno et al., 2017; Taberlet et al., 2012). To minimize spurious OTUs and overestimation, a so-called *mock community*, which is an artificial sample with known diversity and abundance, should be included in the sequencing run

(Fiore-Donno et al., 2017). The bioinformatic tool kit for amplicon sequencing is vast, including software such as QIIME, mothur and the *R* package vegan (Caporaso et al., 2010; Fiore-Donno et al., 2017; Oksanen et al., 2017; Steven et al., 2013b). These tools help to reduce errors introduced during the amplification and produce sound results (Caporaso et al., 2010; Fiore-Donno et al., 2017; Oksanen et al., 2017; Steven et al., 2013b).

Metabarcoding gives insights into BSC diversity and abundance (Taberlet et al., 2012). However, the usage of barcoding genes limits the results to organisms which possess the corresponding gene (Taberlet et al., 2012). To overcome this problem, total DNA or RNA, extracted from environmental samples, can be used for metagenomics or metatranscriptomics, respectively (Rajeev et al., 2013; Urich et al., 2008, 2014). The DNA or cDNA is processed employing NGS techniques and the outcome is subjected to a bioinformatic pipeline which gives results on community composition as well as functionality (Rajeev et al., 2013; Urich et al., 2008, 2014). Moreover, the proteome or even the metabolome of BSCs may be examined using metaproteomics or metabolomics, respectively (Bastida et al., 2014; Jones et al., 2014; Zampieri et al., 2016). However, all these *omic* techniques highly depend on high-quality biomolecules, which are often difficult to isolate from soil (Chen et al., 2009; Wang et al., 2009b). Soil almost always contains humic substances (humin, humic acid, fulvic acid) which are partly coextracted with nucleic acids (Wang et al., 2012, 2009b). These compounds inhibit enzymatic reactions and, therefore, have to be removed during, or directly after the extraction (Wang et al., 2012, 2009b). Hence, the establishment of reliable protocols is indispensable when working with soil communities such as BSCs.

1.6 Objectives

This thesis aims to shed light on the biodiversity and functionality of BSCs which are the dominant vegetation type in alpine regions and polar deserts. These communities are of major importance as they contribute to carbon and

nitrogen fixation, protect the soil against erosion, increase soil fertility and provide the nutritional base for higher trophic levels. The main focus lies on terrestrial microalgae and Cyanobacteria which play a key role as primary producers in these ecosystems.

First and foremost, a method for nucleic acid extraction, which produces high-quality DNA and RNA, had to be established and refined. BSC samples were collected from the Arctic Svalbard (August 2014) and Livingston Island which is part of the Antarctic Peninsula (February 2015). These communities were studied in terms of biodiversity using metatranscriptomics (RNA-Seq) and metabarcoding (amplicon sequencing). Furthermore, an alpine isolate of the hydro-terrestrial alga *Zygnema circumcarinatum* was exposed to desiccation stress and analyzed by applying transcriptomics.

Chapter 2

Paper I

RNA isolation from biological soil crusts: Methodological aspects

Rippin M, Komsic-Buchmann K, Becker B

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2.1 Abstract

Biological soil crust (BSC) communities can be found in almost all environments except for the tropics. These microbial mats are especially predominant in ecosystems, which exhibit harsh conditions, in which they play a key role as primary producers. Studying their metatranscriptomic data enables scientists to shed light on taxa composition, the interactions between those organisms and their ability to cope with abiotic stressors in the extreme environments, e.g. the polar regions. The basis of a successful metatranscriptomic analysis is the isolation of pure RNA of high quality and integrity. Nucleic acid extraction from soil samples is challenging due the diverse chemical and physical properties of soil. Humic substances are often co-extracted and, subsequently, contaminate the sample. In this study, different RNA extraction techniques were tested and evaluated for the isolation of high quality RNA from four BSC samples, three isolated in Germany and one polar BSC: a CTAB based protocol, the Spectrum™ Plant Total RNA Kit and the Precellys Plant RNA Kit. The CTAB based method provides high quality RNA at a low yield and with an average degree of contamination for all tested samples. RNA obtained by using the Spectrum™ Plant Total RNA Kit is of high quality with only little contamination but only half of the samples could be processed successfully. A high throughput approach is the Precellys Plant RNA Kit resulting in a fair RNA quality but with the highest level of contamination. Furthermore, only three out of four samples yielded any results. Further purification is often necessary as DNA and humic substances often cannot be easily removed. Using enzymatic digestion DNA can be specifically cleaved while humic substances can be separated from the RNA fraction by using either gel filtration or RNA affinity binding columns.

2.2 Keywords

Biological soil crust, *metatranscriptomics*, *RNA extraction*, *RNA purification*, *CTAB*

2.3 Introduction

About 40% of terrestrial ecosystems exhibit harsh conditions, such as rather low or high temperatures, high irradiance or low water as well as nutrient availability that prevents any vascular plant cover. However, the soil surface of these habitats is mostly not bare but overgrown by green, red, brown or black patches which are termed biological soil crusts (BSC) (Belnap, 2006; Belnap and Eldridge, 2001; Büdel, 2001; Rindi et al., 2010). BSC are microbial communities consisting of phototrophic organisms, such as cyanobacteria, green eukaryotic algae, lichens and bryophytes (mosses and liverworts), and heterotrophic microbes, including nonlichenised microfungi and heterotrophic bacteria, in varying proportions (Belnap, 2006; Belnap et al., 2001b; Belnap and Weber, 2013; Evans and Johansen, 1999). By producing a matrix of filaments, which weave through the top layer of soil binding particles together, a living crust cover is formed either on the surface or within the top centimeter of soil (Belnap, 2006; Belnap et al., 2001a,b; Evans and Johansen, 1999). These soil crusts are rather dominant in arid and semiarid regions, where they cover up to 70% of the surface, but occur as well in various other habitats from temperate to boreal and even polar latitudes as they are able to cope with extreme conditions such as heat, cold, high UV irradiation, changing salinity and desiccation (Belnap and Eldridge, 2001; Belnap and Lange, 2001; Belnap et al., 2001b; Belnap and Weber, 2013; Hagemann et al., 2015; Raanan et al., 2015). Only the tropical rain forest appears to be free of BSC (Belnap, 2006; Büdel, 2001). Due to their robustness and adaptability to various climates BSC play a key role as pioneering organisms in harsh environments (Breen and Lévesque, 2008; Mikhailyuk et al., 2015; Rajeev et al., 2013). Apart from primary production, these microbial communities recycle nutrients, mineralise the soil and retain water which increases soil fertility. In turn, seed germination and survival as well as growth of vascular plants in general is enhanced which promotes biodiversity in sparse regions (Belnap et al., 2001b; Büdel et al., 2009; Eldridge, 2000; Evans and Johansen, 1999; Karsten

and Holzinger, 2014; Mikhailyuk et al., 2015). Furthermore, the extracellular polysaccharides production of the crust organisms leads to the formation of soil aggregates which are consequently less prone to wind and water erosion (Belnap and Lange, 2001; Belnap et al., 2008, 2001b; Evans and Johansen, 1999; Hagemann et al., 2015). Another important ecological aspect of BSC is their ability to fix atmospheric carbon and nitrogen into organic compounds (Belnap et al., 2001b; Elbert et al., 2009; Hagemann et al., 2015; Karsten and Holzinger, 2014; Lenhardt et al., 2015). Elbert et al. (2012) argues that crust communities even play a major role in global carbon and nitrogen cycles as they contribute approximately 7% of the estimated net primary production of terrestrial vegetation and about 50% of the global terrestrial nitrogen fixation.

As a consequence of extreme conditions, such as drought, high intensity solar radiation and short vegetation periods as well as temperatures around or below the freezing point causing freezing-thawing-cycles, the terrestrial vegetation in the Arctic and Antarctica is rather limited. In Antarctica, for example, only two species of phanerogams occur which might not be surprising considering the fact that Antarctica is the coldest, windiest as well as driest continent (Breen and Lévesque, 2008; Green and Broady, 2001; Pichrtová et al., 2013; Remias et al., 2011). Nevertheless, both polar regions exhibit a multitude of BSC on the ice-free soil surfaces (Belnap and Lange, 2001; Green and Broady, 2001; Kaplan et al., 2013). It was recently found that in the Arctic (Spitsbergen) BSC cover up to 90% of the soil surface whereas the Antarctic Peninsula (Livingston Island) exhibits a lower coverage of up to 55% (Williams et al., 2017). Typical green microalgae found in polar BSC are, for example, *Prasiola crispa*, *Zygnema* and *Klebsormidium* (Elster et al., 2008; Green and Broady, 2001; Mikhailyuk et al., 2015; Pichrtová et al., 2013; Rindi et al., 2010). Cyanobacterial genera, which are usually encountered in the Arctic and Antarctica, are *Anabaena, Aphanothece*, *Chroococcidiopsis*, *Gloeocapsa*, *Leptolyngbya*, *Lyngbya*, *Microcoleus*, *Nostoc*, *Oscillatoria*, *Phormidium* and *Synechococcus* (Quesada and Vincent, 2012).

2.3.1 Metatranscriptomic analysis of BSC

Biological soil crust communities are complex in terms of microorganism diversity but also functionality as described above. They possess many unique features and are extremely well adapted to the barren environments they are usually located in. To study both the different taxa and their mechanisms of adaption a cultivation approach will only give limited insights. Up to 99% of the microbes found in certain environmental samples cannot be cultivated under laboratory conditions and the method does not provide an adequate degree of sensitivity (Bailly et al., 2007; Geisen et al., 2015; Rajendhran and Gunasekaran, 2008; Sessitsch et al., 2002; Wang et al., 2012). However, the extraction of nucleic acids directly from environmental samples may give a broader picture of the microbes comprising the BSC as well as their interactions and enables scientists to learn about their strategies to cope with stressors such as heat or drought. While the isolation of DNA for metagenomic purposes allows genome analysis and its organisation for all taxa present in the crust, RNA-based metatranscriptomic approaches provide a method to investigate the viable and active part of the BSC and their expressed set of genes (Damon et al., 2012; Duarte et al., 1998; Rajendhran and Gunasekaran, 2008; Sessitsch et al., 2002; Urich et al., 2008; Wang et al., 2012). The analysis of rRNA, for example, reveals microbe diversity and abundance, whereas studying eukaryotic poly-A mRNA provides a focused view on active eukaryotic cellular functions (Damon et al., 2012; Sessitsch et al., 2002). Moreover, the influence of soil characteristics, climate and type of vegetation, which are associated with different habitats, on soil communities and their development can be evaluated (Damon et al., 2012). Apart from providing new insights for a more profound understanding of these organisms, the knowledge gained by metagenomics and -transcriptomics can be also used for biotechnological applications. Sequences coding for novel enzymes or whole pathways may be revealed building the foundation for new bioprocesses (Bailly et al., 2007; Damon et al., 2012; Rajendhran and Gunasekaran, 2008).

Despite the clear advantages of metatranscriptomic analysis there are some general drawbacks which should be considered. Firstly, RNA is, compared to DNA, easily degraded which makes it more laborious to isolate appropriate amounts of high quality RNA (Sessitsch et al., 2002). Furthermore, metatranscriptomics of environmental samples feature a bias if the regions of interest might be unaccessible to primers and enzymes due to secondary structure formation (Geisen et al., 2015; Rajendhran and Gunasekaran, 2008). However, the biggest challenge might be metatranscriptomic analysis of soil communities such as BSC. The first difficulties may be encountered when sampling microbial mats from the environment. Depending on the crust type and its adhesion to the surface as well as the soil type, texture and moisture, a larger or smaller amount of soil particles will contaminate the sample. These particles can present a major threat to successful metranscriptomic analysis as they may contain humic substances, rather persistent contaminants in RNA preparations, or even allophane (found in volcanic ash soils) which can absorb RNA prior to extraction (Wang et al., 2012).

Humic substances are a group of structurally diverse, yellow to dark brownish, polyelectrolytic organic compounds with a molecular mass of 0.1 to about 300 kDa. They possess various reactive functional groups allowing them to absorb water, ions and organic molecules. Hence, almost any kind of organic molecule, i.e. nucleic acids, might become attached to humic substances. To classify them, their solubility properties are considered: Humin is insoluble at any pH, humic acids are only soluble at a lower pH and the fraction of fulvic acids is soluble at any pH. Due to their physico-chemical characteristics, which are similar to nucleic acids, humic and fulvic acids are often coextracted with RNA. Contaminated RNA cannot be used for various molecular biological methods as humic acids inhibit enzymatic reactions, such as digestion using restriction enzymes, DNases or RNases, hybridisation, PCR and RT-PCR, transformation as well as nucleic acid detection and measurement (Peršoh et al., 2008; Rajendhran and Gunasekaran, 2008; Wang et al., 2012, 2009).

Naturally, different soil samples have different physicochemical properties causing different problems when extracting nucleic acids (Wang et al., 2012). Ideally, a universal protocol is sought which could be applied for all kinds of samples. At the moment, however, miscellaneous methods have been reported which might work, or not, for particular BSC. Hereinafter, certain RNA isolation methods are evaluated and discussed which may help to overcome the previously described problems and enable the extraction of high purity RNA from BSC samples.

2.4 Material and methods

2.4.1 BSC sampling

Four different soil crust communities were isolated. The samples were retrieved from the horticulture of the Botanical Institute of the University of Cologne, North Rhine-Westphalia, Germany (BSC C1 & C2), from limestone gravel in close vicinity to Frammersbach, Bavaria, Germany (BSC FR), and from the surroundings of the Ny-Ålesund Research Station on Spitsbergen, Svalbard, Arctic (BSC NÅ). The corresponding sampling sites are displayed in Figure 1. The sample C1, C2 and NÅ were crusts dominated by eukaryotic green microalgae while sample FR was a cyanobacterial crust community. The samples were preserved with LifeGuard™ Soil Preservation Solution (MO BIO Laboratories, Inc., Carlsbad, CA, USA). The reagent was used according to manufacturer's instructions and the samples stored at −80°C.

2.4.2 RNA extraction

The samples BSC C1, C2, FR and NÅ were processed using three RNA extraction methods: a CTAB based protocol (Chang et al., 1993), the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, Inc., St. Louis, MO, USA) and the Precellys Plant RNA Kit (peqlab/VWR International, GmbH, Erlangen, Germany).

Figure 1: Sampling Sites of the tested BSC samples. 1 - BSC C1 isolated from the horticulture of the Botanical Institute of the University of Cologne, North Rhine-Westphalia, Germany, high water and peat content; 2 - BSC C2 isolated from the horticulture of the University of Cologne, North Rhine-Westphalia, Germany, low water and peat content; 3 - BSC FR isolated from the top of limestone gravel close to Frammersbach, Bavaria, Germany, high water content; 4 - BSC NÅ isolated in close vicinity of the Ny-Ålesund Research Station on Spitsbergen, Svalbard, Arctic; high water and peat content.

CTAB protocol: The extraction buffer (2% CTAB, 2% PVPP, 100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 2.0 M NaCl, 0.5 g/L spermidine, adjusted to pH 7.0, amended freshly with 2% β-mercaptoethanol before use) was pre-heated to 60°C prior to cell disruption. Initially, the BSC material was thawed at 4°C and the LifeGuard^{TM} Soil Preservation Solution removed after a centrifugation step (2,500 *g*, 4°C, 10 min). For homogenisation 1 g of the sample was moved to a mortar, chilled with liquid nitrogen, and ground with a cooled pestle as soon as all the nitrogen evaporated. Subsequently, the ground and frozen material was gradually transferred to the preheated buffer solution (10 mL) and immediately dispersed by vortexing. To extract nucleic acids from the

cell lysate, an equal volume of chloroform:isoamyl alcohol 24:1 (V:V) was added and mixed with the solution by inverting the tube. Incubating the sample for 10-15 min at 65°C was followed by a 10 min centrifugation step at 4,000 *g* resulting in phase separation. The aqueous phase was collected and once more extracted using chloroform:isoamyl alcohol. By means of a 8 M LiCl stock solution the sample's concentration was set to approximately 2 M and kept at 4°C over night. Precipitated DNA and RNA were collected by centrifugation for 30 min at 17,000 *g* and 4°C in an Eppendorf centrifuge. After supernatant removal and air drying, the pellet was resuspended in 200-500 µL SSTE buffer (1.0 M NaCl, 0.5% SDS, 10 mM Tris-HCl at pH 8.0, 1 mM EDTA, preheated if white precipitates visible). Several tubes were combined (up to 600 µL) to facilitate handling. An additional extraction with chloroform:isoamyl alcohol was carried out with subsequent collection of the aqueous phase by centrifugation at 12,000 *g* for 10 min. A second RNA precipitation was performed by adding two volumes of ice-cold absolute ethanol to the solution and incubating the samples at −20°C for at least 2 h. Nucleic acids precipitate and were spun down for 30 min at 17,000 *g* and 4°C. The Supernatant was discarded. The nucleic acid pellet was washed with 70% ethanol, dried and finally dissolved in nuclease-free water.

Spectrum™ Plant Total RNA Kit: The kit was used according to manufacturer's instructions following protocol A. The BSC sample was prepared as described in the CTAB based method.

Precellys Plant RNA Kit: The protocol of the kit was obeyed applying buffer T-P for BSC sample treatment. The bead tube was loaded with 250 mg of the crust sample and 600 µL buffer T-P for cell lysis. After homogenisation and incubation at room temperature according to the manufacturer's instructions, an additional centrifugation step was performed to remove all of the insoluble material. Spinning the tubes at 12,000 *g* for 10 min was sufficient. The supernatant was carefully transferred to the DNA removal column without disturbing the pellet. Subsequently, all steps of the Precellys Plant RNA Kit protocol were carried out.

RNA quantity and quality were determined using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and performing an agarose gel electrophoresis loading at least 300 ng of the sample onto a 1% agarose gel stained with ethidium bromide.

2.4.3 Further purification steps

The nucleic acid solution was subjected to enzymatic digestion with DNase I (Thermo Fisher Scientific, Waltham, MA, USA) following manufacturer's instructions.

To separate humic substances from the isolated RNA, MicroSpin S-400 HR columns (GE Healthcare, Little Chalfont, UK) were used according to manufacturer's protocol. Samples were solved in water or 10 mM, 25 mM, 50 mM and 100 mM Tris buffer adjusted to pH 8.0 and a sample volume of 50 µL was loaded onto the column.

Finally, samples were concentrated and purified using the RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany).

2.5 Results and discussion

2.5.1 Sampling

Metatranscriptomic and metagenomics analysis is only possible if the nucleic acid integrity of the sample is preserved until extraction is due. This is of particular importance when working with RNA considering its liability to degradation. Quenching the cells down to low temperatures is often not enough, thus, utilising a preserving agent is indispensable. Early studies employed RNAlater® Storage Solution (Ambion, Austin, TX, USA) to preserve RNA (Cai et al., 2013; Florell et al., 2001; Linke et al., 2010; Sikulu et al., 2011; Wang et al., 2012), however, a range of other solutions became available recently (McCarthy et al., 2015). During this study the LifeGuard™ Soil Preservation Solution (MO BIO Laboratories, Inc., Carlsbad, CA, USA) was the first

and only tested preserving agent. This choice was based on the fact that it was specifically designed to stabilise nucleic acids in soil samples (Goffredi et al., 2015; Mondav et al., 2014; Peay et al., 2013; Simmons et al., 2014). The effectiveness of the LifeGuard^{TM} Soil Preservation Solution was evaluated using a liquid culture of *Zygnema* sp. and the BSC sample C1. In order to investigate the influence of soil particles on the integrity of RNA two aliquots of the algae culture was blended with soil. The samples were then treated with the agent and stored at −20°C for several days. Subsequently, RNA was extracted applying the CTAB protocol. Figure 2-1, lanes b_1 and b_2, indicate that the presence of soil particles in the *Zygnema* sample prevents the isolation of RNA. However, if the sample was pretreated with LifeGuard^{TM} Soil Preservation Solution prior to extraction intact RNA was obtained (Figure 2-1, lanes c_1 and c_2). Similarly, nucleic acids from BSC sample C1 could only be obtained if protected by the preserving agent (Figure 2-2, lanes b_1 and b_2). These findings suggest that the LifeGuard^{TM} Soil Preservation Solution is a suitable RNA preserving agent applicable to BSC samples. Whether other RNA preservation solutions achieve similar or better results was not evaluated due to the positive outcome of these experiments. Based on this result the LifeGuard^{TM} Soil Preservation Solution was used for all RNA preparations in this study.

2.5.2 Extraction protocols

CTAB protocol

The original CTAB (Cetyl trimethylammonium bromide) protocol, which was published by Chang et al. (1993), is widely used for RNA isolation. Depending on sample type and desired quality, modifications were introduced to optimise the method. Different studies already confirmed the effectiveness of processing soil samples with the CTAB based method (Griffiths et al., 2000; Robe et al., 2003; Sessitsch et al., 2002; Urich et al., 2008). Due to the CTAB and the polyvinylpolypyrrolidone (PVPP) in the extraction buffer co-precipitation of

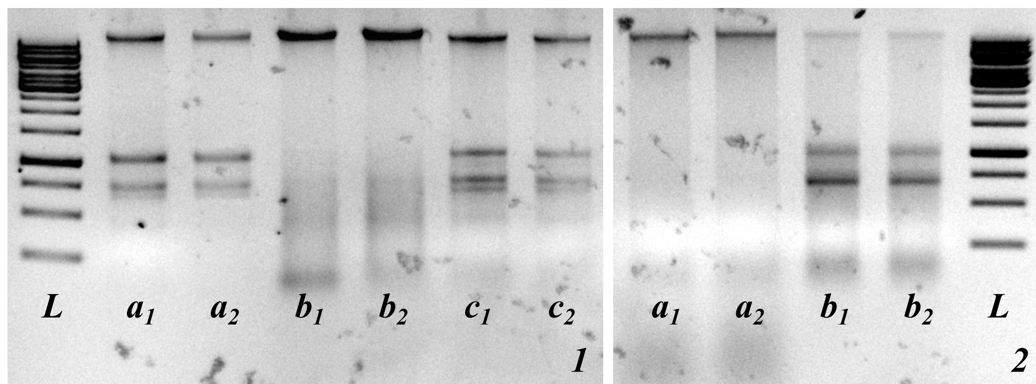

Figure 2: RNA extraction in the presence of soil. A 1% agarose gel image of RNA extracted from biological duplicates (1/2) of a liquid culture of *Zygnema* sp. (1) and BSC sample C1 (2) is shown. RNA was extracted using the CTAB based method. 1 - L = Ladder, $a_{1/2}$ = pure culture, $b_{1/2}$ = culture + soil, $c_{1/2}$ = culture + soil + LifeGuard^{TM} Soil Preservation Solution; 2 - L = Ladder, $a_{1/2}$ = BSC C1, $b_{1/2}$ = BSC C1 + LifeGuard^{TM} Soil Preservation Solution.

humic substances is inhibited and the nucleic acid purity is enhanced (Rajendhran and Gunasekaran, 2008; Wang et al., 2012; Zhou et al., 1996). The composition of the extraction buffer differs among various publications. We used the original extraction buffer recipe (Chang et al., 1993) adjusted to pH 7.0 (Wang et al., 2009) in this study.

Bead beating is a very convenient way of lysing cells intended for nucleic acid extraction. Many novel studies suggest bead based cell disruption (Urich et al., 2008; Wang et al., 2008) as it is fast and allows a high throughput of various samples (Leite et al., 2012). However, the agitating beads may cause shear forces which break the nucleic acids into smaller fragments decreasing their integrity (Robe et al., 2003; Yuan et al., 2012). During this study, the isolation of high quality RNA from BSC using any bead beating protocol was not achieved (see also Precellys Plant RNA kit). Thus, homogenization with mortar and pestle is recommended. Unfortunately, only one sample at a time can be processed which makes this treatment more tedious. Subsequently, the ground and frozen material was gradually transferred to the preheated buffer solution and immediately dispersed by vortexing. This step was shown to be

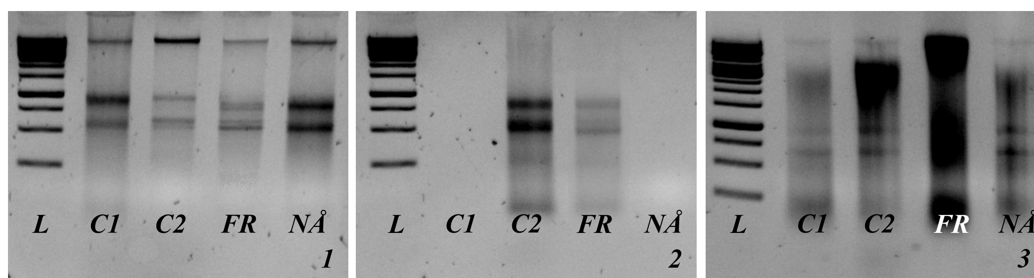

Figure 3: Comparison of RNA extracted from BSC samples C1, C2, FR and NÅ using different methods (1% agarose gel image). 1 - CTAB based protocol, L = Ladder; 2 - Spectrum^{TM} Plant Total RNA Kit, L = Ladder; 3 - Precellys Plant RNA Kit, L = Ladder.

extremely crucial for successful RNA extraction (MacRae, 2007). After extraction with the organic solvents a first RNA precipitation step was performed using LiCl which facilitates the separation of humic substances from RNA.

All four BSC samples yielded RNA when processed with the CTAB protocol as shown in Figure 3-1. Clearly, the protocol is rather time consuming but the integrity of the RNA obtained is higher than for any other method tested (Figure 3). Thus, the CTAB extraction method enables the isolation of high quality RNA. Nevertheless, the samples often needed a further clean up to remove remaining DNA and humic substances.

Spectrum^{TM} Plant Total RNA Kit

A variety of commercial kits is available for RNA extraction from different tissues. However, many of these kits cannot cope with the secondary metabolites and contaminants contained in many samples. Sigma-Aldrich (St. Louis, MO, USA) states that their Spectrum^{TM} Plant Total RNA Kit may overcome this problem while avoiding the usage of hazardous chemicals, such as chloroform or phenol, producing high quality RNA. The system is simple and requires only a short time depending on the number of samples.

Different BSC samples were tested with varying results. Depending on the water and peat content, RNA isolation was successful or not. Generally, a lower sample volume per tube is recommended if handling problematic tissue.

The manufacturer suggests protocol A for root or stem tissue, material which contains plenty of water and low levels of RNA or extremely difficult samples in general. Thus, protocol A was followed yielding rather pure, intact RNA at high quantities if successful. The resulting gel image is shown in Figure 3-2 suggesting that DNA was completely removed as no DNA band is observable. Nevertheless, only from two out of four BSC samples, processed with the Spectrum^{TM} Plant Total RNA Kit, RNA could be isolated.

If an appropriate sample volume is available the kit should be considered since satisfying amounts of RNA of good quality may be obtained. Unfortunately, the Spectrum^{TM} Plant Total RNA Kit is not a universal method for processing BSC samples.

Precellys Plant RNA Kit

Another commercially available kit for rapid RNA extraction from plant tissue tested was the Precellys Plant RNA Kit marketed by peqlab/VWR International, GmbH (Erlangen, Germany). This system uses bead beating (not successful for the BSC samples tested, see above) for cell disruption allowing the processing of various samples at a time. Moreover, handling of hazardous chemicals is not required. Certain modifications in the protocol enabled RNA extraction from soil crust samples with decent yield and quality.

Both supplied buffers, buffer T-P and P-P, were compared indicating that the utilisation of buffer T-P resulted on average in a 3.5 times higher RNA amount per sample (data not shown). Hence, buffer T-P was applied for BSC sample treatment, although, a higher quantity of humic substances was co-extracted compared to buffer P-P. It is recommended to load the tube with 250 mg of the crust sample and 600 µL buffer for cell lysis. This ratio was found to be ideal for extracting the highest amount of nucleic acids per sample volume. The final eluate might still contain DNA and humic substances which need to be separated from the RNA. Furthermore, the method did not yield satisfying RNA quantities for certain BSC material as displayed in Figure 3-

Table 1: Comparison of CTAB based method, Spectrum^{TM} Plant Total RNA Kit and the Precellys Plant RNA Kit in terms of RNA yield, quality/integrity, robustness, working time and contamination. (n = number of replicates per BSC)

	CTAB based method	**Spectrum^{TM} Plant Total RNA Kit Sigma-Aldrich**	**Precellys Plant RNA Kit peqlab**
RNA Yield in µg/mg sample (n)	9 ± 3.4 (4)	52.9 ± 34 (4)	52.7 ± 22 (4)
RNA Quality/Integrity	High	High	Low
Number of BSC samples yielding RNA	4/4	2/4	3/4
Average time to process 1 g of sample in h	20	1	1
DNA contamination	yes	no	yes
Average A260/280 (n)	1.55 ± 0.08 (4)	1.86 ± 0.15 (4)	1.43 ± 0.03 (4)
Average A260/230 (n)	1.03 ± 0.06 (4)	1.44 ± 0.37 (4)	0.76 ± 0.05 (4)

3. Optimising the lysis conditions may increase the outcome, however, if the sample volume is limited optimisation might not be an option.

The Precellys Plant RNA Kit allows RNA extraction from several samples in parallel while handling is fairly easy. Albeit the produced RNA lacks purity and may be partly sheared.

There are several other protocols and commercial kits available for RNA extraction from soil samples available (Bailly et al., 2007; Wang et al., 2009, 2008). The commonly used RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) as well as the RNA PowerSoil® Total RNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA), which was especially designed for soil samples, did not yield any usable RNA with our BSC samples. In Table 1, all three successfully applied methods are compared in terms of RNA yield and quality, successful RNA isolation, processing time and contamination. Thus,

depending on desired quality and available time the appropriate protocol may be chosen. Although, a final clean up to remove all humic substances may be necessary before using the RNA preparation for further enzymatic reactions, e.g. cDNA synthesis (Wang et al., 2012).

2.5.3 Further purification

Due to the co-extraction of DNA as well as humic and fulvic acids, the obtained RNA might need additional purification steps after successful isolation (Wang et al., 2012, 2009). A variety of methods is available to remove any contaminants using molecular differences between the different substances.

Both the CTAB based protocol and the Precellys Plant RNA Kit do not separate DNA completely from the RNA fraction. Hence, the remaining traces of DNA have to be removed. For this purpose, the RNA sample was subjected to an enzymatic digestion with DNase removing DNA completely while RNA was not degraded as the enzyme is specifically cleaving DNA molecules. However, many down-stream applications require the subsequent elimination of the enzyme as well as a high RNA concentration. During this study, the RNeasy MinElute Cleanup Kit was commonly used which allows both a concentration of RNA and separation of contaminants to increase the sample purity which is assessed by photometric measurement. Pure RNA corresponds to an A 260/280 value of 2.00. The rather low average values for A 260/280 given in Table 1 were often improved by applying this kit (up to 0.21 units was observed).

Gel filtration provides a suitable tool to separate molecules depending on their size. As humic and fulvic acids in soil possess a molecular weight average of less than about 20 kDa and, thus, less than rRNA and mRNA, an appropriate size exclusion column may be able to separate these contaminants from the nucleic acid fraction. Studies by Wang et al. (2012, 2009) found the MicroSpin S-400 HR column (GE Healthcare, Little Chalfont, UK) to be appropriate for this purpose as the balance between product recovery and contaminant removal was optimal compared to similar columns. Figure 4 shows a very effi-

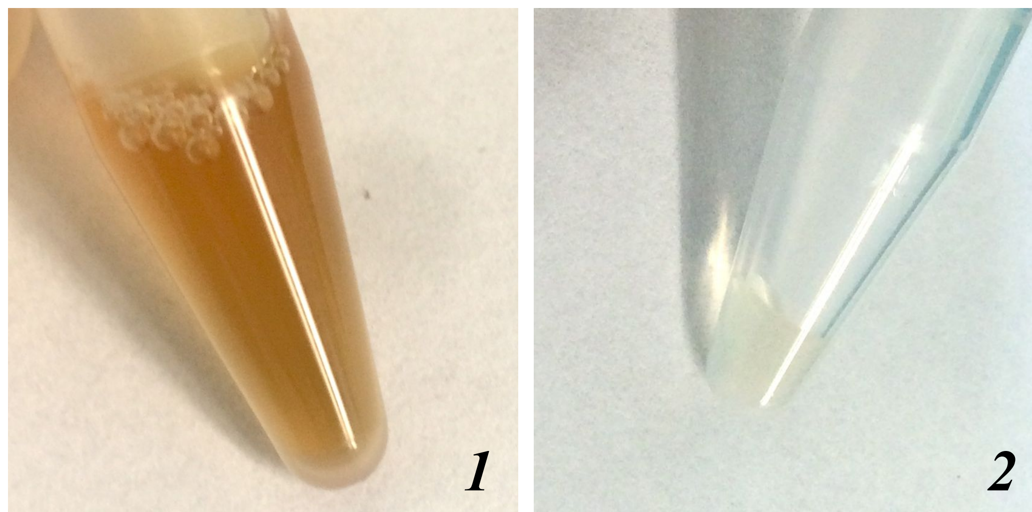

Figure 4: Removal of humic substances using MicroSpin S-400 HR columns. A RNA solution of BSC C1. 1 - before application to spin column; 2 - and after application of the spin column.

cient separation of humic contaminants which is also supported by an increased A 260/280 value (up to 0.39 units) in the filtrated sample. Unfortunately, the RNA recovery is rather low considering a loss of up to 80%. It was not possible to improve the RNA yield by modifying buffer composition and pH. RNA preparations from microbial crust communities mostly contain higher levels of contaminants compared to nucleic acids extracted from most cultivated microbes. Therefore, subsequent purification might become necessary depending on the intended employment. To yield the desired RNA quality and quantity, the techniques described above should be combined in an appropriate matter.

2.6 Conclusion

RNA extraction from biological soil crust communities is not as trivial as nucleic acid isolation from most pure cultures. Still RNA from environmental samples may provide a more distinct picture of the interactions of the community and its adaptions to location and climate. A major problem during RNA extraction from soil-containing samples is the co-extraction of humic substances, which are challenging to remove. The three presented protocols in

combination with an appropriate downstream purification form a good foundation to deal with these obstacles and isolate high quality RNA in sufficient amounts. The decision for a certain method should be based on required purity and quality of the final product, but also on the amount of sample available. The CTAB extraction protocol allows only a rather slow sample throughput and does not remove a sufficient amount of contaminants but yields RNA of good quality from most samples (all samples tested were successfully processed). In contrast, the Spectrum^{TM} Plant Total RNA Kit provides a platform to handle several samples in parallel and yields RNA of high purity and quality. However, not all of the tested BSC isolates yielded RNA. The lowest quality of RNA may be obtained using the Precellys Plant RNA Kit, but the handling is fairly easy and allows parallel runs of different samples. In combination with DNA digestion as well as humic contaminants removal by gel filtration and/or affinity binding columns the resulting RNA should possess a sufficient quality for sequencing purposes. RNA extracted from the BSC sample NÅ by applying the CTAB method has been used successfully for metatranscriptomic analysis (unpublished own results). Thus, the RNA quality was sufficient for down-stream applications (e.g. NGS).

In conclusion, many protocols and commercial kits are available for RNA extraction, however, many do not yield any, or only yield unsatisfactory, results. The selected techniques discussed above were proven to work for at least half of the selected BSC samples making them feasible for RNA isolation. The best working technique was the CTAB based protocol. In combination with DNA digestion and the RNeasy Cleanup Kit the RNA samples will possess a fair to good quality and high integrity.

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Chapter 3

Paper II
Genus richness of microalgae and Cyanobacteria in biological soil crusts from Svalbard and Livingston Island: Morphological vs. molecular approaches

Rippin M, Borchhardt N, Williams L, Colesie C, Jung, P, Büdel B, Karsten U, Becker B

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3.1 Abstract

Biological soil crusts (BSCs) are key components of polar ecosystems. These complex communities are important for terrestrial polar habitats as they include major primary producers that fix nitrogen, prevent soil erosion and can be regarded as indicators for climate change. To study the genus richness of microalgae and Cyanobacteria in BSCs, two different methodologies were employed and the outcome compared: Morphological identification using light microscopy and the annotation of ribosomal sequences taken from metatranscriptomes. The analyzed samples were collected from Ny-Ålesund, Svalbard, Norway, and the Juan Carlos I Antarctic Base, Livingston Island, Antarctica. This study focused on the following taxonomic groups: Klebsormidiophyceae, Chlorophyceae, Trebouxiophyceae, Xanthophyceae and Cyanobacteria. In total, combining both approaches, 143 and 103 genera were identified in the Arctic and Antarctic samples, respectively. Furthermore, both techniques concordantly determined 15 taxa in the Arctic and 7 taxa in the Antarctic BSC. In general, the molecular analysis indicated a higher microalgal and cyanobacterial genus richness (about 11 times higher) than the morphological approach. In terms of eukaryotic algae, the two sampling sites displayed comparable genus counts while the cyanobacterial genus richness was much higher in the BSC from Ny-Ålesund. For the first time, the presence of the genera *Chloroidium*, *Ankistrodesmus* and *Dunaliell*a in Polar Regions was determined by the metatranscriptomic analysis. Overall, these findings illustrate that only the combination of morphological and molecular techniques, in contrast to one single approach, reveals higher genus richness for complex communities such as polar BSCs.

3.2 Keywords

Biological soil crust, *eukaryotic algae*, *Cyanobacteria*, *morphological identification*, *metatranscriptomics*

3.3 Introduction

The Arctic region and Antarctica are extreme environments defined by low temperatures year-round, low water availability, and large seasonal fluctuations with long periods of darkness during winter vs. continuous irradiance in summer (Thomas et al., 2008a). Thus, plant cover is sparse and biodiversity is generally low; e.g. only two autochthonous flowering plants occur in Antarctica and these are mainly found in a few suitable areas along the coast (Pointing et al., 2015; Thomas et al., 2008b). These ecosystems are instead dominated by biological soil crusts (BSCs), which are the main terrestrial primary producers and also important ecosystem engineers (Evans and Johansen, 1999; Pointing and Belnap, 2012; Williams et al., 2017; Yoshitake et al., 2010). BSCs are communities of eukaryotic algae, Cyanobacteria, lichens, bryophytes, as well as heterotrophic fungi and bacteria which colonize the surface and top millimeters of the soil (Belnap, 2006; Belnap et al., 2001a; Büdel et al., 2016). Typically occurring phyla of eukaryotic microalgae are Streptophyta (Klebsormidiophyceae, Zygnematophyceae), Chlorophyta (Chlorophyceae, Trebouxiophyceae, Ulvophyceae) and Ochrophyta (Bacillariophyceae, Eustigmatophyceae, Xanthophyceae) (Büdel et al., 2016). Certain crust organisms, such as Cyanobacteria and eukaryotic algae, form a matrix which aggregates the living cells and soil particles (Belnap, 2006; Breen and Lévesque, 2008). Hence, BSCs provide protection against soil erosion and cryoturbation (Evans and Johansen, 1999). Moreover, BSCs enhance water and nutrient retention and the crust-associated Cyanobacteria fix nitrogen which in turn creates improved conditions (fertilization) for seed germination and plant growth (Belnap et al., 2001b; Breen and Lévesque, 2008). This accumulation of biomass forms the nutritional basis for higher trophic levels such as the Svalbard reindeer (Rangifer tarandus platyrhynchus) (Cooper and Wookey, 2001; Elster et al., 1999). In general, BSCs are essential components of sparse ecosystems such as the polar deserts. These communities are valuable biological indicators for abiotic factors, ecological health and climate change (Belnap et al., 2001b; Pushkareva

et al., 2016). Due to global warming which particularly affects the Arctic and the Antarctic Peninsula, an invasion by alien species is likely, which in turn might alter the BSC composition (Chown et al., 2012; Frenot et al., 2005; Lee et al., 2017; Pushkareva et al., 2016). Hence, studying individual BSC organisms, as well as the whole community, is important to fully address biodiversity, which is a prerequisite for monitoring changes, as well as for predictions of future climate change (Pushkareva et al., 2016).

The identification of autotrophic microorganisms in BSCs, such as eukaryotic algae and Cyanobacteria, is traditionally performed using light microscopy to examine BSCs *in situ*, or through established cultures of selected crust organisms (Bischoff and Bold, 1963; Büdel et al., 2009; Skinner, 1932; Waterbury and Stanier, 1978). The taxa of interest are typically identified using morphological traits such as color, cell size and shape, or motility (Cox, 1996; John et al., 2002; Lind and Brook, 1980; Prescott, 1964). The utilization of light microscopy for identification is rather quick and inexpensive compared to molecular techniques (Misawa, 1999), but requires expert knowledge for many taxa as morphological features are often difficult to recognize and distinguish (Manoylov, 2014). In addition, morphological features are not always stable as they can change in response to environmental factors (Albrecht et al., 2017). As an alternative, a number of molecular markers, so called barcodes, can be used if unialgal cultures are available (Vieira et al., 2016). Typical barcode sequences are the 16S/18S rRNA gene and the internal transcribed spacer (ITS) rDNA, the RuBisCO large subunit (rbcL), the plastid elongation factor tufA, the cytochrome oxidase I (COX I), as well as internal simple sequence repeats (ISSR) of microsatellite regions (An et al., 1999; Buchheim and Chapman, 1991; Doyle et al., 1997; Evans et al., 2007; Hall et al., 2010; Shen, 2008; Sherwood et al., 2008; Wilmotte, 1994). A huge pitfall is that the majority of microorganisms present in environmental samples, such as BSCs, are unculturable (Massana et al., 2014; Schloss and Handelsman, 2005; Shi et al., 2009; Ward et al., 1990). To overcome the limitation of cultivation, biodiversity assessments can be performed by metabarcoding (Elferink et al., 2017; Taberlet

et al., 2012; Yoon et al., 2016). For this purpose, total DNA is extracted from an environmental sample (eDNA) and used as a template to generate an amplicon mixture from a barcoding gene (Taberlet et al., 2012). Subsequently, the generated PCR products are sequenced with a high-throughput sequencing (HTS) technique, such as Roche 454 or Illumina (Bentley et al., 2008; Margulies et al., 2005; Taberlet et al., 2012). The identification of taxa is carried out by annotating the obtained sequence reads against an adequate database and sequence counts provide information about taxonomic abundance in the sample (Pawlowski et al., 2011; Taberlet et al., 2012). However, metabarcoding also exhibits various pitfalls such as the introduction of sequence errors during PCR, the design of suitable metabarcoding primers, covering all taxa of interest, and, again, the need for appropriate reference databases (Taberlet et al., 2012). Fortunately, PCR-dependent bias and the dependency on single barcodes can be avoided by exploiting shotgun metagenomics and metatranscriptomics (Urich et al., 2008, 2014). Similar to metabarcoding, total nucleic acids are isolated from an environmental sample but the amplification step is omitted (Urich et al., 2008, 2014). Instead, total DNA or cDNA is directly applied to HTS and the resulting sequences are assembled for any desired gene or transcript of interest, e.g. the small ribosomal subunit RNA (SSU) (Geisen et al., 2015). This powerful approach enables a more reliable taxonomic identification compared to metabarcoding but relies on the availability of correctly determined sequence data (Klimke et al., 2011; Urich et al., 2008). Additionally, the isolation of high quality nucleic acids from soil samples, suitable for HTS, can be difficult due to the presence of humic substances and exocellular RNase activity (Greaves and Wilson, 1970; Rippin et al., 2016).

The present study focused on a methodological comparison of morphological and molecular approaches to analyze the microalgal and cyanobacterial genus richness of Arctic and Antarctic BSCs. Studying polar BSCs improves the knowledge on these microecosystems, their biodiversity and ecological structure. In this study, the identification of various groups of eukaryotic algae (Klebsormidiophyceae, Chlorophyceae, Trebouxiophyceae, Xantho-

phyceae) and Cyanobacteria in one Arctic and one Antarctic BSC sample was carried out. Eukaryotic and prokaryotic algae were identified using light microscopy in combination with suitable taxonomic keys. Furthermore, active species in the crust were targeted by applying a metatranscriptomic approach and algal as well as cyanobacterial taxa were identified using fully assembled SSUs. The final results were combined and compared to assess the comprehensiveness of both techniques regarding the genus richness.

3.4 Material and methods

3.4.1 Sampling

BSC samples were collected during expeditions to the Arctic and Antarctica in August 2014 and February 2015, respectively. The Arctic BSC (NÅ; cf. Borchhardt et al. (2017a); Rippin et al. (2016); Williams et al. (2017); Jung et al. (in preparation)) was sampled in close vicinity to Ny-Ålesund, Svalbard, Norway (78° 55' 26.33" N 11° 55' 23.84" E; Figure 1a). The research station Ny-Ålesund is located at Kongsfjorden and its climate is classified as polar tundra (ET) according to the Köppen-Geiger system (Peel et al., 2007; Vogel et al., 2012). The temperature is low year-round with an annual average of -4.5°C, the highest monthly temperature is in July with 5.8°C and the lowest in March with -12°C (all temperatures are averaged from August 1993 to July 2011) (Maturilli et al., 2013). The mean annual precipitation is 433 mm (Førland et al., 2011). Williams et al. (2017) found 90% of the area to be covered by BSCs. The Antarctic BSC (Gr1; cf. Williams et al. (2017)) was collected close by the Spanish Juan Carlos I Antarctic Base at Livingston Island, South Shetland Islands (62° 39' 55.44" S 60° 23' 42.76" W; Figure 1b). Similar to Svalbard, the South Shetland Islands rank as polar tundra (ET) using the Köppen-Geiger system (Michel et al., 2014; Pereira et al., 2006). The temperature at the Juan Carlos I Antarctic Base averages -1°C annually, with the highest mean temperature (2.1°C) in austral summer and the lowest mean temperature (-4.6°C) in winter (all temperatures are averaged from De-

cember 2001 to April 2003 and January 2007 to February 2011) (Bañón et al., 2013). The site receives on average 444.5 mm of precipitation annually (Bañón et al., 2013). The BSC coverage is approximately 43% with skeletal soils being predominant (Williams et al., 2017). BSC samples for metatranscriptomic analysis (1 g) were preserved using the LifeGuard^{TM} Soil Preservation Solution (MO BIO Laboratories, Carlsbad, CA, USA).

Figure 1: Overview map of sampling sites in the Arctic and Antarctica. a - Svalbard in the Arctic. Samples were taken at Ny-Ålesund (NÅ). b - Livingston Island which is part of the South Shetland Islands. Isolates were collected in close vicinity of the Juan Carlos I Antarctic Base (Gr1). Maps were created using snazzymaps.com.

3.4.2 Cultivation and microscopy

Enrichment cultures of eukaryotic algae were established by transferring the sampled BSCs (NÅ, Gr1) onto solid 1.5% Difco™ Agar (Becton Dickonson, Heidelberg, Germany) containing Bold's Basal Medium modified according to Starr and Jeffrey (1993). The cultures were kept at 15°C, 30 µmol photons m^{-2} s^{-1} under a 16:8 hour light-dark regime. A detailed outline on the procedure is given in Borchhardt et al. (2017b). The identification of genera or species was carried out by light microscopy (BX51, Olympus, Tokyo, Japan) with oil-immersion and a 1000-fold magnification. Relevant identification keys are listed in Borchhardt et al. (2017b). A complete list of the identified Arctic algae (NÅ) was published by Borchhardt et al. (2017a) and is used for comparison in the present study.

Cyanobacterial genera, present in the Arctic (NÅ) and Antarctic (Gr1) BSC were directly determined using light microscopy with oil immersion and a 630-fold magnification by using identification keys (Geitler, 1932; Komárek and Anagnostidis, 1998, 2005). Cyanobacteria were additionally pre-cultivated on BG-11 medium at 15 – 17°C, 20-50 µm photons m^{-2} s^{-1} under a 14:10 hour light-dark cycle and identified using suitable taxonomic keys as described by Williams et al. (2016). The data of Jung et al. (in preparation), which examined the cyanobacterial diversity of both sites, was compared to the molecular dataset.

3.4.3 RNA Isolation and sequencing

The Arctic sample (NÅ) was processed according to Rippin et al. (2016) using the CTAB protocol, DNase I (Thermo Fisher Scientific, Waltham, MA, USA) and the RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany). Due to low RNA yields, a total of six biological replicates were extracted and combined to obtain three pooled replicates. RNA from three biological replicates, collected at Livingston Island (Gr1), was isolated using the Spectrum™ Plant Total RNA Kit (Sigma Aldrich, St. Louis, MO, USA), treated with DNase I (Thermo

Fisher Scientific, Waltham, MA, USA) and purified using the RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany) as described by Rippin et al. (2016). Single samples yielded sufficient amounts of RNA.

All RNA samples were further processed by Eurofins Genomics (Ebersberg, Germany). The processing included quality control utilizing the BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA) and library preparation for both triplicates. Eukaryotic mRNA was enriched by using oligo-(dT) beads, fragmented and, subsequently, cDNA was synthesized using random hexamers. Finally, Illumina compatible adapters were ligated. The libraries of the individual samples NÅ and Gr1 were applied to an Illumina HiSeq 2500, all triplicates multiplexed on one lane, using 125 bp paired-end and single-end mode, respectively. For sample NÅ, the HiSeq Control Software 2.2.58, RTA 1.18.64 and bcl2fastq-1.8.4 were used while the detected signals from sample Gr1 were processed by operating the HiSeq Control Software 2.2.38, RTA 1.18.61 and bcl2fastq-1.8.4 (Illumina, San Diego, CA, USA).

3.4.4 Bioinformatics

The obtained raw reads (SRA bioproject PRJNA421095) were quality trimmed using Trimmomatics 0.35 (Bolger et al., 2014) and rRNA gene reads were filtered out using SortMeRNA 2.1 (Kopylova et al., 2012) and the SILVA SSU NR Ref 119. All rRNA SSU reads were assembled using EMIRGE 0.61.0 (Miller et al., 2011) combined with the SILVA SSU NR Ref 128. The script emirge_amplicon.py for single-end reads with the flag *phred33* was run. Only successfully assembled SSUs were considered. The assigned genera for Klebsormidiophyceae, Chlorophyceae, Trebouxiophyceae, Xanthophyceae and Cyanobacteria were extracted and compiled with an in-house *R*-script (Version 3.3.2).

3.5 Results

The genus richness of phototrophic organisms in BSCs, isolated from Svalbard (NÅ) and Livingston Island (Gr1), was assessed using morphological features and annotated environmental rRNA SSU sequences. We obtained 18,041,893, 28,446,946 and 116,112,134 forward raw reads for sample NÅ and 62,235,763, 70,163,318 and 48,605,923 for Gr1. After quality trimming and rRNA SSU filtering we ended up with 8,936, 7,818 and 38,320 sequences for NÅ and 706,549, 1,081,090 and 8,646 for Gr1. This study focused on genera belonging to the Klebsormidiophyceae, Chlorophyceae, Trebouxiophyceae, Xanthophyceae or Cyanobacteria. All the taxa that were identified are included in the supplementary information (Online Resource 1). Additional taxonomic groups, for example Fungi, that were identified in the molecular analysis were not further investigated in this study.

3.5.1 The Arctic BSC

The Arctic sample contained 26 genera of eukaryotic algae and Cyanobacteria identified by light microscopy while 132 were assigned by metatranscriptomic analysis. The average number of genera in the metatranscriptomic triplicates was 71.7 ± 36.1 (SD). In total, 15 genera were determined by both methodologies, compared to 117 and 11 genera which were solely found by molecular and morphological identification, respectively (Figure 2a). The utilized 18S reference database contained sequences for 5 of the 11 genera identified by morphology. Figures 3a and 4a display all genera identified with 1, 44, 25, 6 and 67 taxa belonging to Klebsormidiophyceae, Chlorophyceae, Trebouxiophyceae, Xanthophyceae and Cyanobacteria, respectively. The genera *Coccomyxa*, *Elliptochloris*, *Stichococcus* (Trebouxiophyceae), *Leptolyngbya*, *Microcoleus*, *Nostoc* and *Phormidium* (Cyanobacteria) were found in the culture isolates and in all three metatranscriptomes while *Klebsormidium* (Klebsormidiophyceae), *Chlorococcum*, *Coelastrella*, *Desmodesmus*, *Lobochlamys* (Chlorophyceae), *Botrydiopsis*, *Heterococcus* (Xanthophyceae) and *Stigonema* (Cyano-

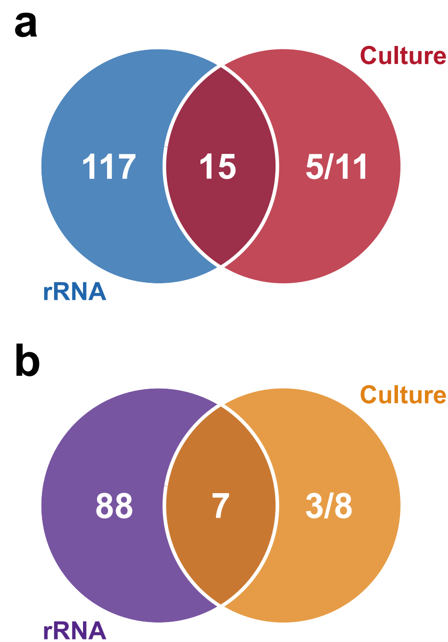

Figure 2: Comparing the molecular (blue, violet) and morphological (red, orange) approach in terms of identified algal genera, both eukaryotic and prokaryotic. The numbers divided by a slash represent the taxa present in the reference database (left) and all genera identified (right). a - NÅ; b - Gr1.

bacteria) were identified in at least one of the three replicates and by light microscopy. All three metatranscriptomes per site included rRNA SSU sequences of the following genera, which could not be cultivated: *Chloromonas*, *Dictyococcus*, *Oedogonium*, *Scenedesmus* (Chlorophyceae), *Chlorella*, *Chloroidium*, *Dictyochloropsis* (Trebouxiophyceae), *Anabaena*, *Aphanizonemon*, *Arthronema*, *Calothrix*, *Chroococcidiopsis*, *Crinalium*, *Cyanothece*, *Cylindrospermum*, *Gloeobacter*, *Nodularia*, *Tolypothrix* and *Trichormus* (Cyanobacteria). The morphology based method identified the eukaryotic genera *Gloeocystis**, *Macrochloris**, *Neocystis**, *Tetracystis* (Chlorophyceae), *Desmococcus**, *Koliella*, *Lobosphaera* (Trebouxiophyceae), *Pleurochloris**, *Pleurogaster**, *Tribonema* and *Xanthonema* (Xanthophyceae), of which 6 were not represented by 18S rRNA gene sequences in the reference database (asterisk).

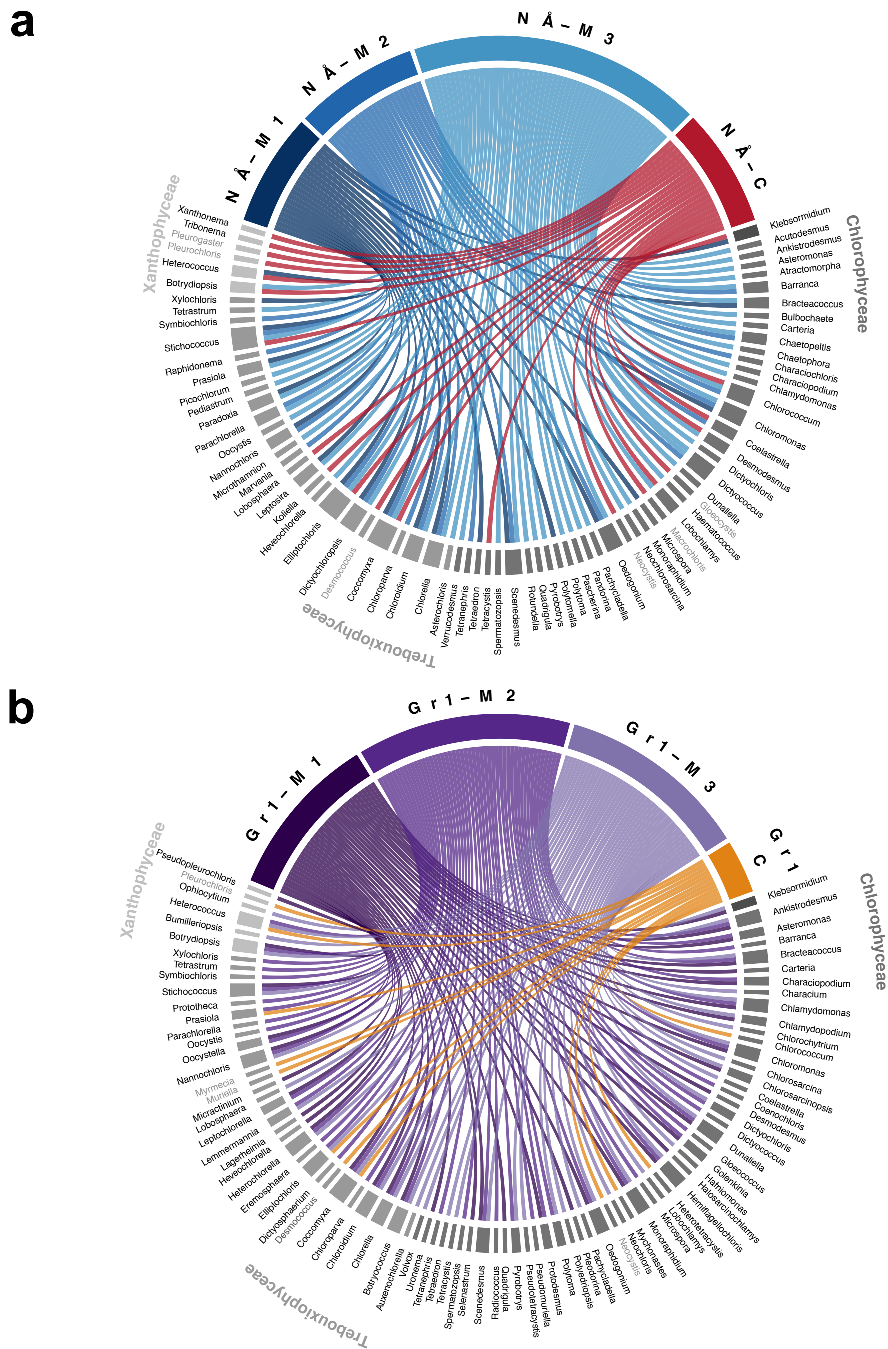

Figure 3: All genera identified belonging to either Klebsormidiophyceae (*Klebsormidium*), Chlorophyceae, Trebouxiophyceae or Xanthophyceae. The metatranscriptome (blue or violet shades) is separated in three replicates while the culture isolates are represented by a single category (red, orange). Chords connect the methodologies with the identified genera. Genera displayed in grey were absent from the database used for the metatranscriptomic analyses. a - NÅ; b - Gr1.

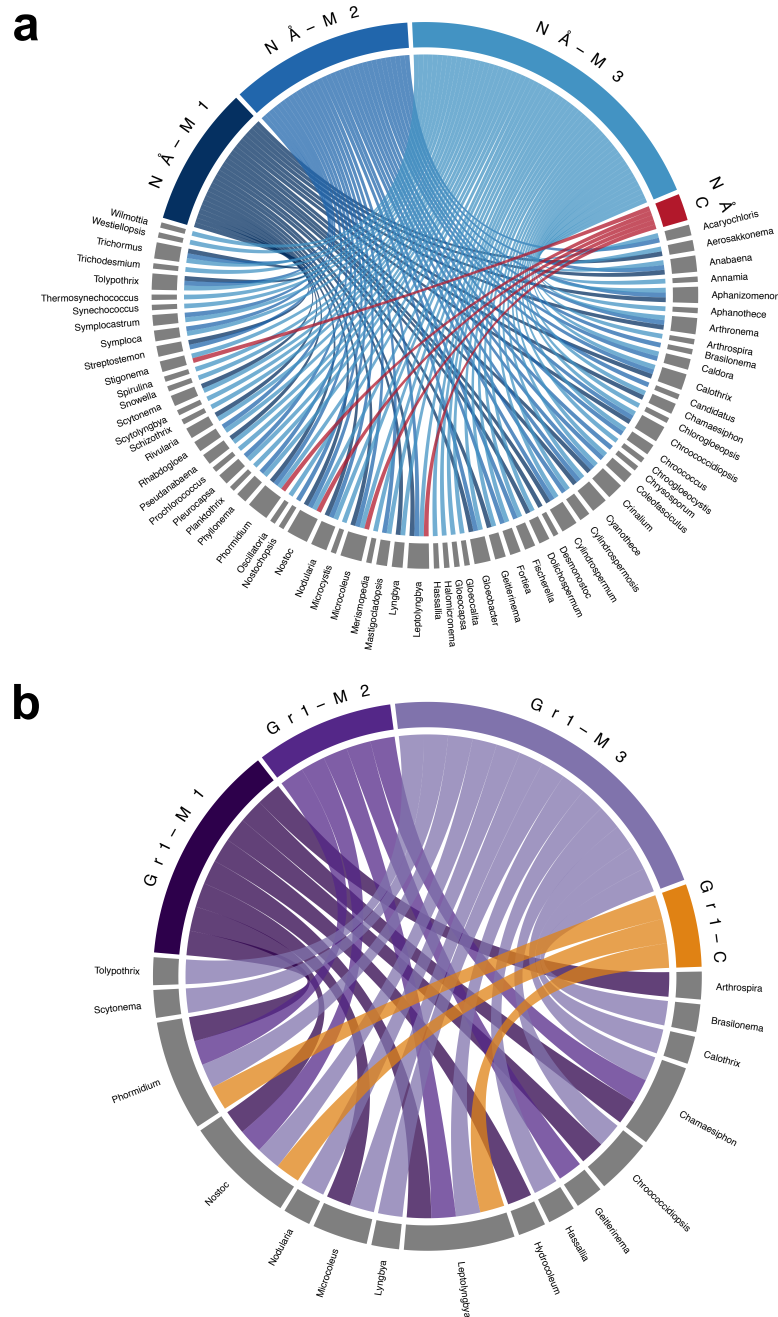

Figure 4: All genera identified belonging to Cyanobacteria. The metatranscriptome (blue or violet shades) is separated in three replicates while the culture isolates are represented by a single category (red, orange). Chords connect the methodologies with the genera identified. a - NÅ; b - Gr1.

3.5.2 The Antarctic BSC

A total of 15 genera, present in the Antarctic BSC, were determined based on morphology while molecular identification yielded 95 taxa. The genera mean value of the three molecular replicates was 51.3 ± 5.7 (SD). Both techniques found 7 genera while 8 and 88 genera were solely determined by morphology or sequence homology search, respectively (Figure 2b). For 3 genera, identified from culture isolates, rRNA reference sequences were present in the database. Regarding the different taxonomic groups, several genera were determined: 1 genus of Klebsormidiophyceae, 53 genera of Chlorophyceae, 28 genera of Trebouxiophyceae, 6 genera of Xanthophyceae and 16 genera of Cyanobacteria (Figure 3b and 4b). The genera *Coccomyxa* (Trebouxiophyceae), *Leptolyngbya*, *Nostoc* and *Phormidium* (Cyanobacteria) could be determined morphologically and were identified in all replicates used for the molecular approach. In addition, the genera *Elliptochloris*, *Prasiola* (Trebouxiophyceae), *Heterococcus* (Xanthophyceae) were determined by morphology and present in at least one out of three metatranscriptomic replicates. All three replicates prepared for molecular identification included the genera *Ankistrodesmus*, *Bracteacoccus*, *Chlamydomonas*, *Chloromonas*, *Dunaliella*, *Hemiflagellochloris*, *Monoraphidium*, *Oedogonium*, *Scenedesmus* (Chlorophyceae), *Botryococcus*, *Chlorella*, *Chloroidium*, *Heterochlorella*, *Nannochloris*, *Stichococcus* (Trebouxiophyceae), *Botrydiopsis* (Xanthophyceae) and *Chamaesiphon* (Cyanobacteria), however, cultivation failed for these organisms. In contrast, cultures were established for the genera *Chlorococcum*, *Heterotetracystis*, *Mychonastes*, *Neocystis** (Chlorophyceae), *Desmococcus**, *Muriella**, *Myrmecia** (Trebouxiophyceae) and *Pleurochloris** (Xanthophyceae) which are missing in the rRNA dataset. Five of these genera were not represented by 18S sequences in the used database (asterisk).

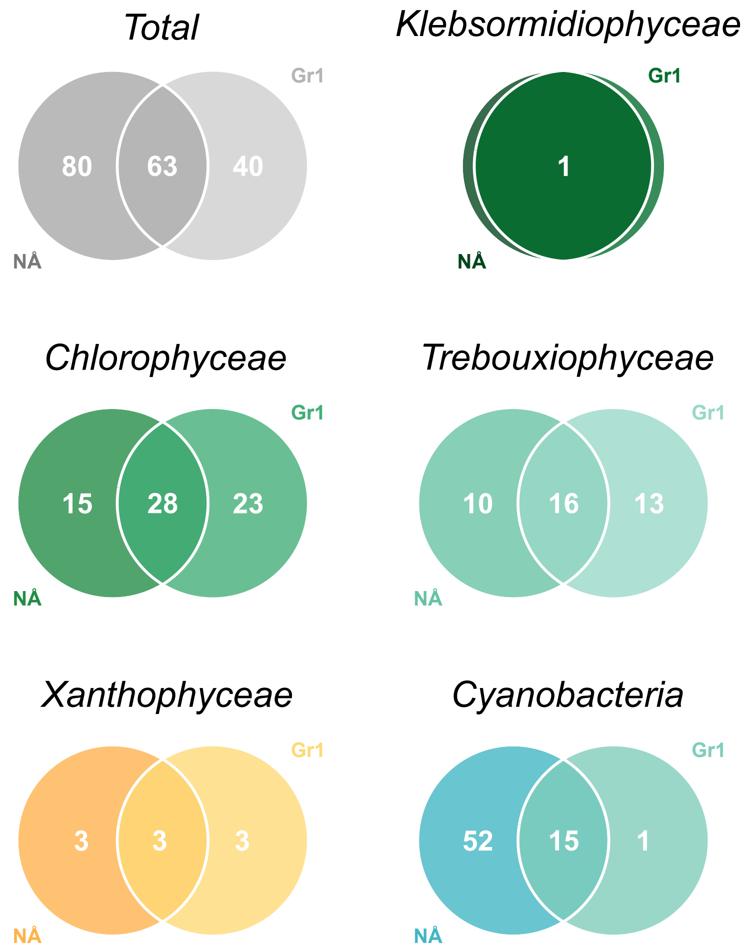

Figure 5: Comparing the Arctic sample (NÅ) and the Antarctic BSC (Gr1) in terms of identified total genera (grey) and genera belonging to either Klebsormidiophyceae (dark green), Chlorophyceae (green), Trebouxiophyceae (light green), Xanthophyceae (yellow) or Cyanobacteria (turquoise).

3.5.3 Site comparison

By comparing the Arctic and Antarctic crust samples we found 63 genera that were shared between both locations (Figure 5). However, the BSC collected from Svalbard, included 80 microalgae and Cyanobacteria which were not shared with the Antarctic sample, although the sample from Livingston Island contained 40 additional taxa. The genus *Klebsormidium* occurred at both sites. The biggest portion of chlorophycean (28) and trebouxiophycean

(16) genera was found in both samples. Regarding Xanthophyceae, both BSCs contained 6 genera of which 3 were shared between sites. The analyses of the Arctic and Antarctic samples revealed 67 and 16 cyanobacterial genera to be present, respectively. An overlap of 15 cyanobacterial genera was detected.

3.6 Discussion

Overall, the molecular survey of the BSC samples revealed a higher degree of genera richness for Chlorophyceae, Trebouxiophyceae and Cyanobacteria compared to the culture and morphology based methodology. However, the rRNA SSU identification did not recover all genera determined by light microscopy. Regarding Cyanobacteria, the Arctic crust sample exhibited a much higher variety compared to the sample collected at Livingston Island.

3.6.1 Assessment of microalgal and cyanobacterial genera richness

The genus *Klebsormidium* (Klebsormidiophyceae) is typically found in BSC communities and has been previously found to colonize soils of both Livingston Island and Svalbard (Borchhardt et al., 2017b; Büdel, 2001; Kastovská et al., 2005; Zidarova, 2008). Our metatranscriptomic analyses confirmed the presence of this streptophytic taxon in the Polar Regions, although *Klebsormidium* was not identified by light microscopy in the sample collected from Livingston Island.

Several chlorophycean genera were identified in both the Arctic and the Antarctic samples by metatranscriptomic analysis: *Ankistrodesmus*, *Chlamydomonas*, *Chloromonas*, *Coelastrella*, *Desmodesmus*, *Dictyococcus*, *Dunaliella*, *Lobochlamys*, *Monoraphidium*, *Oedogonium* and *Scenedesmus*. *Coelastrella*, *Desmodesmus* and *Lobochlamys* were also found in Arctic culture isolates. All the aforementioned genera are known to occupy terrestrial habitats from temperate to cold climates (Borchhardt et al., 2017b; Borie and Ibraheem, 2003; Büdel, 2001; Büdel et al., 2016; De Wever et al., 2009; Lürling, 2003; Matuła

et al., 2007; Pushkareva et al., 2016; Schulz et al., 2016; Thorn and Lynch, 2007; Tschaikner et al., 2007; Uzunov et al., 2008; Wu et al., 2016; Zidarova, 2008). For *Chlamydomonas*, *Chloromonas*, *Coelastrella* and *Monoraphidium*, earlier records exist confirming their occurrence in both Polar Regions, however, *Dictyococcus*, *Oedogonium* and *Scenedesmus* have only been identified from Arctic BSCs (Borchhardt et al., 2017b; Matuła et al., 2007; Pushkareva et al., 2016; Zidarova, 2008). The genera *Chlamydomonas*, *Coelastrella* and *Scenedesmus* were previously identified from Svalbard and *Monoraphidium* from Livingston Island (Matuła et al., 2007; Pushkareva et al., 2016; Zidarova, 2008). Previous research, describing the genera *Ankistrodesmus* and *Dunaliella* in polar habitats was not found, therefore, this is the first study which confirms their presence in Svalbard and Livingston Island BSCs. Species of *Chlorococcum* were found in culture isolates prepared from both the Arctic and Antarctic BSCs as well as in the metatranscriptomic dataset generated from the NÅ sample. Kastovská et al. (2005) also found *Chlorococcum* in close vicinity to Ny-Ålesund and Borchhardt et al. (2017b) identified this genus from samples collected from Ardley and King George Island, Antarctica. The genus *Tetracystis* was identified from both BSC communities. Based on morphology, *Tetracystis* was found in the Arctic sample and metatranscriptomics confirmed its presence in the BSC from Livingston Island. Evidence exists that suggests *Tetracystis* colonizes the soil of Arctic and Antarctica ecosystems (Broady, 1986; Giełwanowska and Olech, 2012; Patova et al., 2015; Pushkareva et al., 2016). The following eukaryotic algae were only identified in culture isolates: *Gloeocystis* and *Macrochloris* were present in isolates from the Arctic while *Heterotetracystis* and *Mychonastes* grew in the Antarctic enrichment cultures. The genus *Neocystis* was found in both samples. For almost five genera their presence in polar and alpine ecosystems has been reported previously (Andreyeva and Kurbatova, 2014; Borchhardt et al., 2017b; Lukešová et al., 2010; Matuła et al., 2007; Patova et al., 2015; Pushkareva et al., 2016; Zidarova, 2008).

Both the Arctic and Antarctic metatranscriptomic datasets and culture isolates contained the trebouxiophycean genus *Coccomyxa*. Previous studies also found that this genus is an inhabitant of Svalbard and Livingston Island ecosystems (Matuła et al., 2007; Pushkareva et al., 2016; Zidarova, 2008). *Coccomyxa* has a ubiquitous distribution and exhibits an extraordinary versatility, it occurs in terrestrial mats, in associations with soil, Fungi and bryophytes, and is also planktonic in freshwater systems (Büdel, 2001; Darienko et al., 2015; Peveling and Galun, 1976; Schmidle, 1901; Vogel, 1955). An ecophysiological study on three *Coccomyxa* strains isolated from Antarctic BSC samples points to pronounced cold and drought tolerance (Pfaff et al., 2016). Rivasseau et al. (2016) even found a new species of *Coccomyxa*, *C. actinabiotis*, in the cooling pool of a nuclear power plant. Thus, it is not surprising that members of this genus can colonize the extreme habitats which prevail in the Polar Regions. The green alga *Elliptochloris* generally occupies terrestrial habitats and associates with Fungi to form lichens (Ettl and Gärtner, 2014). Furthermore, this genus was found in the Canadian Arctic and King George Island, Antarctica (Borchhardt et al., 2017b; Pushkareva et al., 2016). Both methodologies that were used in this study confirm that *Elliptochloris* is present in Svalbard and Livingston Island BSCs. In contrast, the genus *Stichococcus* was found in both the Arctic and the Antarctic metatranscriptomic data and in isolates from Livingston Island but not from Svalbard. This alga has often been identified as a member of BSC communities (Kastovská et al., 2005). Different species of *Stichococcus* have been previously found to colonize the soils of both Livingston Island and Svalbard (Kastovská et al., 2005; Zidarova, 2008). The green alga *Prasiola*, was identified through both the metatranscriptomic and microscopic identification for Livingston Island terrestrial habitats. However, only the Arctic metatranscriptomes showed evidence for *Prasiola* species. These findings are supported by a previous survey on species richness which reported *Prasiola* from both Livingston Island and Svalbard (Matuła et al., 2007; Zidarova, 2008). Based solely on the metatranscriptomic analysis, the genera *Chlorella*, *Chloroidium* and *Nannochloris* were identified in both the

Arctic and Antarctic BSC samples. Different species of *Chlorella* have been previously isolated from Svalbard and Livingston Island (Matuła et al., 2007; Pushkareva et al., 2016; Zidarova, 2008). Vishnivetskaya (2009) identified *Nannochloris* in Siberian permafrost using molecular techniques, which suggests that this alga can survive in extreme conditions. The genus *Chloroidium* has been previously identified in BSCs, however, it has not been reported from polar habitats so far (Schulz et al., 2016). Therefore, this study provides the first molecular record of *Chloroidium* in Arctic and Antarctic ecosystems. The presence of *Lobosphaera* in the Arctic and Antarctic BSC samples was supported by the morphological and metatranscriptomic identification. Additionally, Giełwanowska and Olech (2012) identified *Lobosphaera* as a photobiont of Antarctic lichens. Based on morphological identification, the genus *Desmococcus* was found in BSC samples from both sites, *Koliella* was identified in isolates from the Arctic, while *Muriella* and *Myrmecia* grew in the Antarctic enrichment cultures. These findings are in accordance with previous studies reporting the presence of these genera in polar and alpine habitats (Andreyeva and Kurbatova, 2014; Borchhardt et al., 2017b; Czerwik-Marcinkowska et al., 2015; Kawecka, 1986; Lukešová et al., 2010; Patova et al., 2015; Pushkareva et al., 2016; Takeuchi, 2001; Zidarova, 2008).

The xanthophyte *Heterococcus* was identified by both techniques from the Svalbard and Livingston Island samples. Pushkareva et al. (2016) and Borchhardt et al. (2017b) also identified this genus in BSCs collected in the Canadian Arctic and King George Island, Antarctica. The genus *Botrydiopsis* is also typically found in BSCs and was previously isolated from other Arctic and Antarctic locations (Broady, 1976; Büdel, 2001; Pushkareva et al., 2016). Our metatranscriptomic analyses support the presence of this xanthophycean alga in the Polar Regions. However, it was identified by light microscopy only in the sample collected from Livingston Island. On the other hand, the genera *Pleurogaster*, *Tribonema* and *Xanthonema* were only present in isolates from the Arctic, while *Pleurochloris* was identified in both samples by means of morphological methods. *Pleurochloris*, *Tribonema* and *Xanthonema* have been

previously found in polar and alpine ecosystems (Borchhardt et al., 2017b; Matuła et al., 2007; Patova et al., 2015; Pushkareva et al., 2016; Zidarova, 2008). However, the genus *Pleurogaster* had previously only been isolated from terrestrial habitats outside of polar regions (Stoyneva, 2000).

All metatranscriptomes and morphological analyses identified the cyanobacterial genera *Leptolyngbya*, *Nostoc* and *Phormidium* from Svalbard and Livingston Island samples, which was also reported by Pushkareva et al. (2016) and Zidarova (2008), respectively. All genera occur in various climates and in aquatic and terrestrial habitats (Büdel, 2001; Casamatta et al., 2005; Dojani et al., 2014; Rippka et al., 1979). The cyanobacterium *Microcoleus* also has a ubiquitous distribution, is often associated with BSCs and has been found in both Arctic and Antarctic environments (Büdel, 2001; Pushkareva et al., 2015). All metatranscriptomic datasets contained evidence of *Microcoleus*, however, it was only identified from Svalbard through the morphological investigation. The genus *Stigonema* was identified from Svalbard through both metatranscriptomic and morphological methods, which is in agreement with other reports (Matuła et al., 2007; Pushkareva et al., 2015). However, in contrast to our results *Stigonema* can also be found in Antarctic ecosystems (Büdel, 2001). The cyanobacterial genera *Calothrix*, *Chamaesiphon*, *Chroococcidiopsis*, *Nodularia* and *Tolypothrix*, typically found in soil biocenoses (Büdel, 2001; Wang et al., 2015), were identified in the Arctic and the Antarctic sample using metatranscriptomics. Earlier studies, assessing the species richness of aeroterrestrial algae from Svalbard, recorded all of these genera (Matuła et al., 2007; Pushkareva et al., 2016, 2015).

Interestingly, the Arctic crust community exhibited higher cyanobacterial genus richness than the BSC from Livingston Island, while the genus richness of eukaryotic algae was similar. Our morphological and molecular analyses found only 16 different genera of Cyanobacteria in the Antarctic sample compared to 67 in the Arctic. BSCs develop over time and undergo changes in species composition (Büdel et al., 2009). Early successional stages of BSCs are usually dominated by Cyanobacteria, however, bryophytes, lichens and/or liverworts

may become the dominant groups in many ecosystems (Büdel et al., 2009). Thus, differences in cyanobacterial genus richness may be explained by the different developmental stages of the communities. Furthermore, Williams et al. (2017) reported a lower abundance of cyanobacterial crusts on Livingston Island than at Ny-Ålesund, Svalbard. Our observation might also be linked to the reduced occurrence of Cyanobacteria in these soil communities which was previously reported by Colesie et al. (2014a,b).

3.6.2 Methodological differences and difficulties

The identification of microalgae through metatrancriptomics yielded a higher number of genera compared to the morphological determination of cultivated and directly observed organisms. This is an indication for the lack of culturability of most microorganisms associated with BSCs (Massana et al., 2014; Schloss and Handelsman, 2005; Shi et al., 2009; Ward et al., 1990). The successful cultivation of microalgae is highly dependent on the applied growth conditions including temperature, irradiance and culture medium (Bold, 1942; Hoham, 1975; Singh and Singh, 2015; Wu et al., 2016). For example, Hoham (1975) tested various cryophilic algae, isolated from alpine habitats in Washington, USA, and discovered that *Chloromonas pichinchae* reached the highest growth rate at 1°C while *Cylindrocystis brébissonii* grew fastest at 10°C. Therefore, the genus *Chloromonas* might have been inhibited during our culturing procedure due to unsuitable temperatures. Regarding the growth medium, one single solution, that is suitable for all algae and Cyanobacteria, does not exist (Bold, 1942; Lee et al., 2014). Even though the medium, used to establish the enrichment cultures, was optimized for a broad range of algae, certain strains may not exhibit growth due to individual nutrient requirements. Some species of the genus *Dunaliella*, for example, prefer higher salt concentrations compared to other algae (Oren, 2005). The salt concentrations of Bold's Basal Medium, used to establish the enrichment cultures, could have been too low to enable the growth of *Dunaliella*. Similar reports exist for the cyanobacterium *Chroococcidiopsis*. Different strains of *Chroococcidiopsis*, isolated from

different habitats, exhibited growth only if the medium was supplied with the appropriate amount of sea salt (Cumbers and Rothschild, 2014). However, growth parameters and culture medium determine the culturability of microorganisms only to a certain extent. For many organisms, especially algae, the co-cultivation of certain helper organisms (other algae, bacteria etc.) is essential for growth and development (Jones et al., 1973; Sanders et al., 2001; Vartoukian et al., 2010). Some Cyanobacteria, which form symbioses with eukaryotic algae, fail to grow in the absence of their partner (Thompson et al., 2012). It is likely that quorum sensing plays an important role for these biotic interactions as certain signal molecules from one organism influence growth and development of another (Poonguzhali et al., 2007; Williams, 2007). Chi et al. (2017) studied the marine bacterium *Ponticoccus* sp. PD-2 and how it controls the growth of the dinoflagellates *Alexandrium tamarense*, *Prorocentrum donghaiense* and the haptophyte *Phaeocystis globosa*. Although this clear indication of microalgae interacting with other microbes exists, hardly anything is known about the underlying mechanisms. BSCs are complex biocoenoses with many dependencies and co-dependencies which are not yet fully understood.

Another potential problem when comparing morphological and molecular methodologies is the false identification of taxa due to ambiguous morphological traits (Hodač et al., 2016; Wu et al., 2001). Some genera, such as *Chlorella*, are difficult to identify as their morphological characters are limited and changeable depending on environmental conditions (Bock et al., 2011; Hodač et al., 2016). This might be one possible explanation why the metatranscriptomic data confirmed the presence of *Chlorella* in the Arctic and the Antarctic sample but the morphology based identification did not. However, it is more likely that the spatial heterogeneity of the sample replicates collected for molecular and light microscopic analysis caused some of the differences (Concostrina-Zubiri et al., 2013; Kim et al., 2015). A clear indication of heterogeneity is the differing results observed for the replicates of the same site in the molecular analysis. For example, the genera *Coelastrella*, *Chlorococcum*

and *Chloromonas* were found in one, two and three replicates of the Arctic metatranscriptome, respectively. Furthermore, the high standard deviation of the averaged genera number for the molecular dataset of the Arctic BSC suggests sample heterogeneity. Differences in taxonomic determination may also arise due to a lack of consensus in the algal taxonomy. Many species concepts are still being revised based on molecular analyses rendering an unambiguous morphological identification difficult (Champenois et al., 2015; Kawasaki et al., 2015; Škaloud et al., 2016; Stoyanov et al., 2014). The genus *Leptolyngbya*, for example, was revealed to be heterogeneous and only recently split up into 15 new genera (Komárek, 2016).

Finally, we came across a number of genera which could be identified by light microscopy but not by the analysis of the rRNA SSU sequences. Regarding the genera *Gloeocystis*, *Macrochloris*, *Neocystis*, *Desmococcus*, *Muriella*, *Myrmecia*, *Pleurochloris* and *Pleurogaster*, the corresponding 18S sequences were missing from the database utilized in the course of identification. Incomplete and wrongly annotated references are a major problem when performing molecular analyses of any kind and impair the correct determination of genera in environmental samples (Klimke et al., 2011; Taberlet et al., 2012). The microbial dark matter, meaning the total of unculturable microorganisms, complicates this matter even more (Solden et al., 2016). However, by studying metagenomics, metatranscriptomics and single cell genomics researchers can overcome the limit of culturability and supply the databases with novel sequences (Solden et al., 2016). A number of algae, such as *Chlorococcum*, *Heterotetracystis*, *Mychonastes*, *Tetracystis*, *Koliella*, *Lobosphaera*, *Tribonema* and *Xanthonema*, were isolated from the BSC samples and were included in the reference database, used for this study. However, they were not detected in the metatranscriptomes. One possible explanation is the spatial heterogeneity of the sample replicates (Concostrina-Zubiri et al., 2013; Kim et al., 2015). Furthermore, these organisms may have been inactive during sampling and were therefore undetectable during total RNA analysis (Geisen et al., 2015). Many algae are known to develop resting cells to survive unfavorable conditions

such as drought (Holzinger and Karsten, 2013; Li et al., 2015). Li et al. (2015) identified 35 different algae in resting stages; among them *Chlorococcum* which surrounds resting cells with a thin layer of mucus. A species of *Mychonastes*, *M. desiccatus*, may produce desiccation-resistant cysts if dried out (Margulis et al., 1988). Massalski et al. (1994) isolated *Lobosphaera* from King George Island, Antarctica, and studied its ultrastructure. The alga, colonizing volcanic rocks, was able to produce resting cysts with thickened cell walls to outlast detrimental conditions. Similar observations were made for *Tribonema bombycinum* which produced akinetes in response to starvation (Nagao et al., 1999). Our crust samples were collected in rather extreme habitats exhibiting low temperatures, high irradiance or complete darkness and low water availability throughout the year resulting in a hostile environment (Thomas et al., 2008a). Thus, the absence of certain genera in the molecular datasets might be attributed to the occurrence of resting stages, which likely occur under the conditions prevailing at Svalbard and Livingston Island.

3.7 Conclusion

BSCs represent an important part of polar ecosystems and serve as indicators for ecological health and climate change. Thus, these complex communities need to be closely examined in order to gain insights into not only taxonomy but also physiological plasticity and functionality. This study focused on comparing methodologies to analyze the genus richness of these interesting biocoenoses. Cultivating microorganisms, present in the BSC, combined with light microscopy offers individual analysis of certain organisms while metatranscriptomics enables investigation of the whole genera richness within the crust. The selection of one or the other technique should be based on the individual research focus as both come with certain benefits, as well as costs. The isolation of microorganisms and establishment of cultures enables detailed investigations and follow-up experiments on the isolated algae. However, unculturable genera will be neglected. In contrast, molecular surveys are always

dependent on appropriate and complete reference datasets as it is difficult to correctly annotate taxa which are absent from, or misidentified in the database. Moreover, PCR biases and sequencing errors may falsify the results. In our opinion, the combination of both approaches will generate reliable results of higher quality. Thus, we recommend conjoined studies to validate findings and maximize outcome.

3.8 Acknowledgements

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3.9 Conflict of interest

The authors declare that they have no conflict of interest.

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Chapter 4

Paper III
Biodiversity of biological soil crusts from the Polar Regions revealed by metabarcoding

Rippin M, Lange S, Sausen N, Becker B

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4.1 Abstract

Biological soil crusts (BSCs) are amalgamations of autotrophic, heterotrophic and saprotrophic organisms. In the Polar Regions, these unique communities occupy essential ecological functions such as primary production, nitrogen fixation and ecosystem engineering. Here we present the first molecular survey of BSCs from the Arctic and Antarctica focused on both eukaryotes and prokaryotes as well as passive and active biodiversity. Considering sequence abundance, *Bryophyta* is among the most abundant taxa in all analyzed BSCs suggesting that they were in a late successional stage. In terms of algal and cyanobacterial biodiversity, the genera *Chloromonas*, *Coccomyxa*, *Elliptochloris* and *Nostoc* were identified in all samples regardless of origin confirming their ubiquitous distribution. For the first time, we found the chrysophyte *Spumella* to be common in polar BSCs as it was present in all analyzed samples. Co-occurrence analysis revealed the presence of sulfur metabolizing microbes indicating that BSCs also play an important role for the sulfur cycle. In general, phototrophs were most abundant within the BSCs but there was also a diverse community of heterotrophs and saprotrophs. Our results show that BSCs are unique microecosystems in polar environments with an unexpectedly high biodiversity.

4.2 Keywords

Biological soil crust, *algae*, *terrestrial* Cyanobacteria, Bryophyta, *Svalbard*, *Livingston Island*

4.3 Introduction

Biological soil crusts (BSCs) are the dominant primary producers in polar deserts of the Arctic and Antarctica (Borchhardt et al., 2017b; Colesie et al., 2014; Williams et al., 2017; Yoshitake et al., 2010). The extreme conditions prevailing in these ecosystems limit the occurrence of vascular plants (Pointing

et al., 2015; Thomas et al., 2008a,b). Instead, BSCs cover the ground, e.g. up to 90% at Arctic Svalbard, adopting various important ecological roles such as primary production (Williams et al., 2017). The diazotrophic microbes inhabiting BSCs fix dinitrogen from the atmosphere and make it available to other organisms (Belnap, 2003; Maier et al., 2016; Yoshitake et al., 2010). Certain BSC species grow in filaments or sheaths, while others excrete extracellular polymeric substances forming a soil binding matrix and, thus, stabilize the soil and prevent erosion and cryoturbation (Belnap et al., 2001a; Evans and Johansen, 1999; Gold and Bliss, 1995). BSCs are ecosystem engineers that develop the soil by increasing its moisture and nutrient content (Belnap, 2006; Bowker et al., 2006; Evans and Johansen, 1999; Pointing and Belnap, 2012). These processes have a positive effect on seed germination and plant growth which in turn promotes biodiversity (Belnap et al., 2001a; Breen and Lévesque, 2008; Evans and Johansen, 1999). Furthermore, BSCs are food resources for higher trophic levels (Cooper and Wookey, 2001; Devetter et al., 2017; Guan et al., 2018).

BSC communities are complex microecosystems comprised of autotrophs, heterotrophs and saprotrophs, all of which can be prokaryotic or eukaryotic (Belnap et al., 2001a, 2016). *Cyanobacteria*, eukaryotic algae and lichens as well as *Bryophyta* and *Marchantiophyta* build the basis of BSCs as they are able to fix carbon into organic compounds (Belnap et al., 2001a; Belnap and Lange, 2001; Yoshitake et al., 2010). Cyanobacterial genera that typically occur in BSCs are *Chroococcus*, *Gloeocapsa*, *Leptolyngbya*, *Microcoleus*, *Nostoc*, *Phormidium* and *Scytonema* (Büdel et al., 2016; Hoffmann, 1989; Pushkareva et al., 2016; Zidarova, 2008). Algal taxa commonly found in these communities are members of the *Klebsormidiophyceae*, *Zygnematophyceae* (*Streptophyta*), *Chlorophyceae*, *Trebouxiophyceae*, *Ulvophyceae* (*Chlorophyta*), *Diatomea*, *Eustigmatophyceae* and *Xanthophyceae* (*Ochrophyta*) (Büdel et al., 2016; Hoffmann, 1989; Pushkareva et al., 2016; Zidarova, 2008). Typical prokaryotic taxa, representing heterotrophic BSC members, are *Archaea* and the bacterial phyla *Acidobacteria*, *Actinobacteria* and *Proteobacteria* (Maier et al., 2016).

On the other hand, eukaryotic heterotrophs present in BSCs are *Ascomycota* and *Hyphomycetes* as well as *Bilateria* and the taxonomically diverse Protozoa (Darby and Neher, 2016; Fiore-Donno et al., 2017; Maier et al., 2016; Soule et al., 2009). Certain *Bacteria*, *Fungi* and *Bilateria* are decomposers closing the nutrient cycle (Darby and Neher, 2016; Hendriksen, 1990).

Establishing and developing BSC communities depends on several biotic and abiotic factors such as pedological properties and climate (Belnap, 2006; Büdel et al., 2009; Elster et al., 1999; Pushkareva et al., 2016). Initially, *Cyanobacteria* colonize the barren soil forming the basis of BSCs that are subsequently populated by algae, lichens, mosses and liverworts (Belnap, 2006; Belnap et al., 2008; Büdel et al., 2009). Polar BSCs are generally dominated by lichens, mosses and liverworts in later successional stages (Belnap, 2006).

Despite the resilience of these unique biocoenoses to extreme abiotic stress, climate change is a major threat to BSCs as it can dramatically affect their composition and abundance (Evans and Lange, 2001; Johnson et al., 2012; Zelikova et al., 2012). Increased temperatures and irradiance as well as altered precipitation patterns in the Arctic and Antarctica are expected to change the species diversity and community structure of BSCs by an invasion of foreign species (Chown et al., 2012; Evans and Lange, 2001; Lee et al., 2017; Pushkareva et al., 2016). Hence, the investigation and monitoring of BSCs, one of the key players in polar desert ecosystems, is essential to evaluate and even predict the effects of the ongoing climate change.

According to Elster et al. (1999) there is only a small number of studies focusing on terrestrial compared to the aquatic microalgal flora in the Polar Regions. To contribute new insights into the microalgal and cyanobacterial diversity, we performed a comprehensive metabarcoding survey on BSCs from Svalbard, Norway, and Livingston Island, Antarctic Peninsula. As general prokaryotic and eukaryotic markers (16S and 18S rRNA gene) were used to study these biocrust isolates, other organism groups, such as bryophytes, were covered as well. The prepared amplicon pools were sequenced together with a mock community which served as an intrinsic control. The generated data was

also used to identify influential abiotic parameters and co-occurrence patterns. Furthermore, these results complement the morphological, molecular and ecological studies of Borchhardt et al. (2017a), Rippin et al. (2018) and Williams et al. (2017), respectively.

4.4 Material and methods

4.4.1 BSC sampling

BSC samples were collected from the Arctic Svalbard, Norway, and Livingston Island, Antarctic Peninsula, in August 2014 and January 2015, respectively (refer to Borchhardt et al. (2017a) and Williams et al. (2017) for details). Both islands are located in the polar tundra zone according to the Köppen-Geiger classification system (Pereira et al., 2006; Vogel et al., 2012). Details on the individual samples are given in Table 1, while images of the analyzed BSCs and the corresponding sampling sites are depicted in Figure 1. The nucleic acid content of the BSC samples was preserved using the LifeGuard™ Soil Preservation Solution (MO BIO Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions and stored at −80°C.

4.4.2 Algal cultivation

To establish a mock community (MC), the following algal strains were retrieved from the Culture Collection of Algae at the University of Cologne: *Chlorella sorokiniana* (CCAC 5791), *Chlorococcum* sp. (CCAC 3846 B), *Chloromonas vacuolata* (CCAC 2316), *Gloeocystis* sp. (CCAC 4293 B), *Microthamnion kuetzingianum* cf. (CCAC 5521 B), *Oocystis* sp. (CCAC 1782 B), *Stichococcus bacillaris* (CCAC 1896 B), *Zygnema* sp. (CCAC 1384 B). *Klebsormidium crenulatum* (SAG 2415) was obtained from the Culture Collection of Algae at Goettingen University. *Chlamydomonas reinhardtii* (CC3395) was taken from the stock commonly used in our lab. All algae, except *C. reinhardtii*, were cultivated in liquid Waris-H medium (McFadden and Melkonian, 1986)

Table 1: Sampling Locations. BSCs were collected from the Arctic Svalbard, Norway (BG, ED, NÅ, TD), and Livingston Island, Antarctic Peninsula (JC). Sampling location and the corresponding coordinates as well as previous studies, which were focused on samples from the same sites, are included.

Sample	Sampling location	Coordinates	Publications
BG	Breinosa Gruve 7, close to Longyearbyen, Svalbard, Norway	78.1485000 N 16.0481500 E	Borchhardt et al. (2017a); Williams et al. (2017)
ED	Endalen, close to Longyearbyen, Svalbard, Norway	78.1863400 N 15.7610330 E	Borchhardt et al. (2017a); Williams et al. (2017)
JC	Spanish Juan Carlos I Antarctic Base, Antarctic Peninsula	62.6653889 S 60.3952200 W	Rippin et al. (2018); Williams et al. (2017)
NÅ	Ny-Ålesund, Svalbard, Norway	78.9233167 N 11.9245833 E	Borchhardt et al. (2017a); Rippin et al. (2018); Williams et al. (2017)
TD	Todalen, close to Longyearbyen, Svalbard, Norway	78.9233167 N 11.9245833 E	Borchhardt et al. (2017a)

at 20°C and 60 µmol photons m^{-2} s^{-1} with an light:dark cycle of 14:10 h. *C. reinhardtii* was grown in TAP medium (Gorman and Levine, 1965) using the same temperature and light settings.

4.4.3 Nucleic acid extraction and amplification

Total nucleic acids were extracted from the BSC samples (all in triplicates, except NÅ which was only one sample) using the cetyltrimethylammonium bromide protocol according to Rippin et al. (2016) with several modifications: The LifeGuard™ Soil Preservation Solution was not removed prior to the ex-

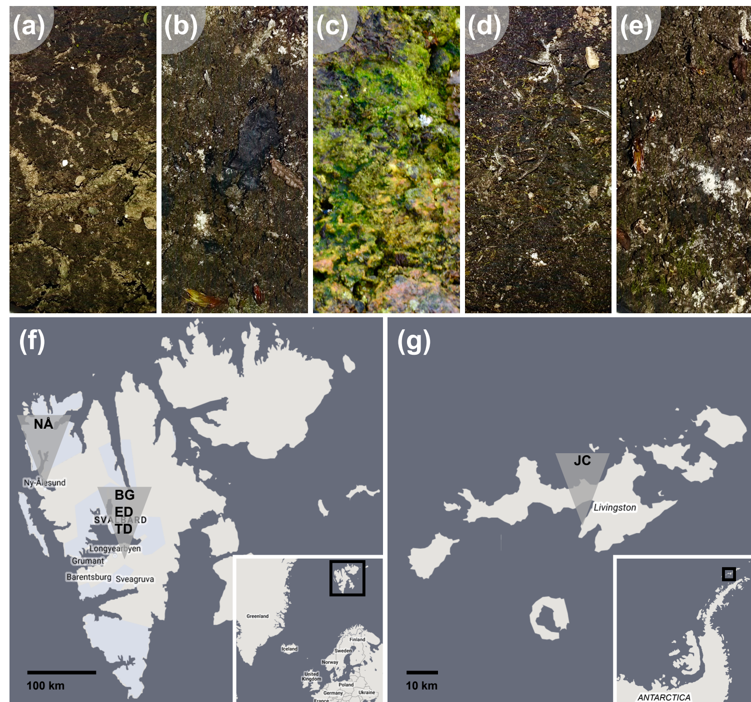

Figure 1: BSC samples and locations. Images of the sampled BSCs BG (a), ED (b), JC (c) NÅ(d) and TD (e). BG, ED and TD were collected in close vicinity to Longyearbyen, while NÅ was isolated from Ny-Ålesund as indicated on the map (f). JC was sampled at Livingston Island close by the Spanish Juan Carlos I Antarctic Base (g). (photos courtesy of N. Borchhardt & C. Colesie, maps were created with *snazzymaps.com*)

traction and the aqueous phase, retained after the second chloroform washing step, was further processed using the peqGOLD Plant RNA Kit (peqlab/VWR International, Erlangen, Germany) according to the manufacturer's instructions.

To obtain DNA, the extract was treated with RNase A (Thermo Fisher Scientific, Waltham, MA, USA) as suggested in the manual and purified using the illustra MicroSpin S-400 HR Columns (GE Healthcare, Little Chalfont, UK).

RNA based metabarcoding was only performed for the samples NÅ and JC as the sampling sites are most comparable in terms of climate (seaside,

similar precipitation patterns etc.). RNA was purified by DNA removal using DNase I (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. cDNA synthesis was performed using the Revert Aid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA).

The extraction of DNA from all cultivated algae except *K. crenulatum* was carried out using the DNeasy Plant Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The harvested filaments of *K. crenulatum* were processed using a modified version of the cetyltrimethylammonium bromide protocol described by Rippin et al. (2016). After the second chloroform washing step, the upper phase was collected. The DNA was precipitated with isopropanol at −20°C for at least 1 h. After a washing step with 75% ethanol, the DNA was eluted in RNase-free water.

Amplicons were generated for all triplicates (for NÅ three technical replicates were produced) using the Kapa HiFi HotStart DNA Polymerase (Roche, Basel, Switzerland). To analyze the eukaryotic diversity, we targeted the ribosomal small subunit (SSU) V4 region with the universal eukaryotic primers TAReuk454FWD1 (5'-CCAGCASCYGCGGTAATTCC-3') and TAReukREV3 (5'-ACTTTCGTTCTTGATYRA-3') designed by Stoeck et al. (2010) (Amplicon size $\approx$ 380 bp). Similarly, the prokaryotic 16S rRNA gene V2-V3 region was amplified using the universal primers 104F (5'-GGCGVACGGGTGMGTAA-3') and 515R (5'-TTACCGCGGCKGCTGGCAC-3') taken from Lange et al. (2015) (Amplicon size $\approx$ 400 bp). The eukaryotic amplicons of the BSC samples were amplified employing the following protocol: an initial denaturation step at 95°C for 3 min and 25 3-step cycles at 98°C for 20 s, at 49°C for 15 s and 72°C for 35 s were followed by a final elongation at 72°C for 1 min. The prokaryotic target was amplified in a similar way, however, the 25 cycles included 20 s at 98°C, 10 s at 65°C and 15 s at 72°C. Each template was amplified three times (NÅ was amplified nine times) with each primer pair and these triplicates were pooled in an equimolar manner to minimize PCR bias. For the mock community, the templates, obtained from the cultivated algae,

the bryophyte *Physcomitrella patens* and the liverwort *Marchantia polymorpha* (both supplied by O. Artz, AG Höcker, Botanical Institute, University of Cologne), were only targeted with eukaryotic primers and the PCR protocol consisted of 30 cycles of 20 s at 98°C, 15 s at 51.8°C and 35 s at 72°C. All PCR products were purified using the Agencourt® AMPure® XP system (Beckman Coulter, Brea, CA, USA) according to the manufacturer's protocol.

4.4.4 Library preparation and sequencing

The eukaryotic amplicons of the cultivated algae, *P. patens* and *M. polymorpha* were quantified, using the Qubit (Thermo Fisher Scientific, Waltham, MA, USA), and combined in equimolar portions to yield a mock community (MC). This synthetic community was only prepared for the eukaryotic barcode as it was used to minimize sequencing bias and to adjust the bioinformatic pipeline. All BSC amplicons and MC were subjected to A-tailing using the DreamTaq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, libraries were prepared employing the Qiagen QIAseq 1-Step Amplicon Library Kit (Qiagen, Hilden, Germany). The libraries were multiplexed and sequenced on the Illumina MiSeq platform (300 bp, paired-end) at the MPI for Plant Breeding Research, Cologne.

4.4.5 Bioinformatic pipeline

The demultiplexed raw reads were quality checked with VSEARCH 2.4 (Rognes et al., 2016) and read pairs with a minimal overlap of 100 bp and a minimum Phred score of 33 were merged with PEAR 0.9.10 (Zhang et al., 2014). Subsequently, primer sequences and sequences containing ambiguous bases were removed and all sequences were dereplicated using VSEARCH. After pooling all samples, clustering of operational taxonomic units (OTUs) was performed using Swarm 2.1.13 (Mahé et al., 2014, 2015) with flag d = 1. Chimera detection was carried out with UCHIME (Edgar et al., 2011), integrated in VSEARCH, and taxonomic annotations were assigned employing blastn 2.3

Table 2: Environmental parameters at the Arctic sampling sites. Precipitation of the Arctic sampling sites as well as chemical parameters (C = carbon, N = nitrogen, S = sulfur, P = phosphorus) of the soil underneath the sampled Arctic BSCs taken from Borchhardt et al. (2017a) (BG = Berg, ED = EiE, NÅ = NA, TD = TD).

Sample	Precipitation [mm/a]	C [g/kg]	N [g/kg]	S [g/kg]	Total P [mg/kg]	Water-extractable P [mg/kg]
BG	205	26	1.9	0.8	461.9	4.5
ED	205	331.6	9.6	4.9	395	4.4
NÅ	471	17	1.4	0.5	169.1	5.5
TD	205	296.2	12.1	6.4	515.9	108.1

(Altschul et al., 1990) with an E-value of E-10 and the Silva SSURef database 128 (Quast et al., 2013). Finally, an OTU table was constructed by running the script OTU_contingency_table.py which is part of the SWARM package. All chimeric OTUs and OTUs shorter than 300 bp or with less than 72 reads were filtered out. Additionally, a read count threshold of 6 was applied. Rarefaction (Hurlbert, 1971) and MDS plots (Bray and Curtis, 1957; Jaccard, 1912) were produced with the vegan package 2.4.4 in *R* (Oksanen et al., 2017). The MDS plots were based on the Bray-Curtis dissimilarity index and generated individually for the prokaryotic and the eukaryotic dataset. Furthermore, the package was used to calculate the Shannon, Simpson and Inverse Simpson index. Moreover, environmental vectors (the values for each parameter in Table 2 were retrieved from Borchhardt et al. (2017a)) were fitted onto ordination (MDS based on the Jaccard index) using the squared correlation coefficient r^2 and require 999 permutations. The *R* package metagenomeSeq 1.16 (Paulson et al., 2013) was used for several different tasks: Read count normalization employing the CSS method; OTU heat map plotting of all replicates including only the 1,000 OTUs with the largest overall variance; differential abundance testing considering only groups with a fold change of more than 4 and a false discovery rate of less than 0.001. Based on the Spearman correlation coeffi-

cient (Spearman, 1904), co-occurrence patterns were analyzed by running the modified scripts co_occurrence_pairwise_routine.R and edgelist_creation.R written by Williams et al. (2014). The analysis was based on prokaryotic orders and eukaryotic classes, as far as applicable, and the Spearman coefficient cutoff was set to 0.75. All scripts and auxillary files can be accessed via github (https://github.com/Klebsi/BSC_Amplicon). All raw sequences were submitted to the NCBI Sequence Read Archive (SRA) under the Bioproject PRJNA415906.

4.5 Results

4.5.1 Sequencing outcome, OTU table and mock community

The sequencing produced a total of 21,530,106 paired-end reads. After quality filtering and removing 245,825 potential chimeras, 9,978,488 merged sequences remained and were clustered into 9,062 OTUs. On average, each sample contained 230,814 sequences and 1,084 OTUs. Read and OTU counts for the individual sample replicates are summarized in Table S1. By means of rarefaction sampling, we found that all replicates, except one eukaryotic RNA derived JC replicate, reached saturation (Figure S2). This JC replicate also exhibited a low read count (530 reads after clustering and filtering, Table S1) and was subsequently removed from the analysis. For the remaining BSC samples, we were able to recover the full prokaryotic and eukaryotic diversity within the limits of our methodology.

A mock community (MC), consisting of ten different eukaryotic microalgae, a bryophyte and a liverwort, was included in the sequencing run to adjust the bioinformatic pipeline. We ended up with 12 OTUs with 8.3 ± 0.7% relative abundance each after aggregation according to taxonomic affiliation and normalization (Figure S3). Furthermore, the taxonomic annotation of each OTU corresponded to one of the used organisms.

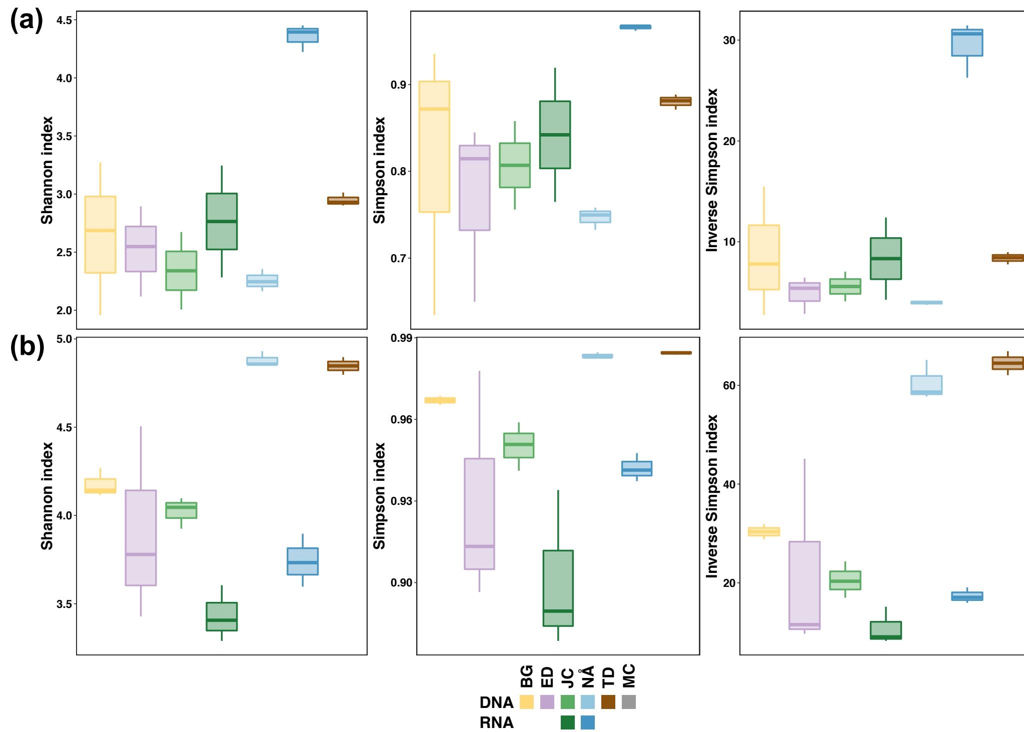

Figure 2: Biodiversity analysis. α-diversity indices (Shannon, Simpson, Inverse Simpson) of the eukaryotic (a) and prokaryotic (b) amplicon dataset given as box plots.

The heatmap in Figure S4a shows that the samples cluster in two main groups, the eukaryotic replicates on the left and the prokaryotic on the right. Furthermore, MDS plots were produced revealing that one replicate of the eukaryotic DNA derived JC sample (Figure S4b) and one of the prokaryotic TD sample (Figure S4c) were clear outliers in both analyses. Hence, they were subsequently removed.

4.5.2 BSC Biodiversity

Biodiversity of the different BSC samples was measured by calculating Shannon, Simpson (1-D) and Inverse Simpson (1/D) indices. The individual indices ranged from approximately 2 to 5, 0.7 to 0.99 and 3 to 65 for Shannon, Simpson and Inverse Simpson, respectively (Figure 2). Overall, the eukaryotic biodiversity (Figure 2a), as measured by these indices, had a lower range compared to the prokaryotic diversity (Figure 2b). The RNA derived NÅ amplicon exhib-

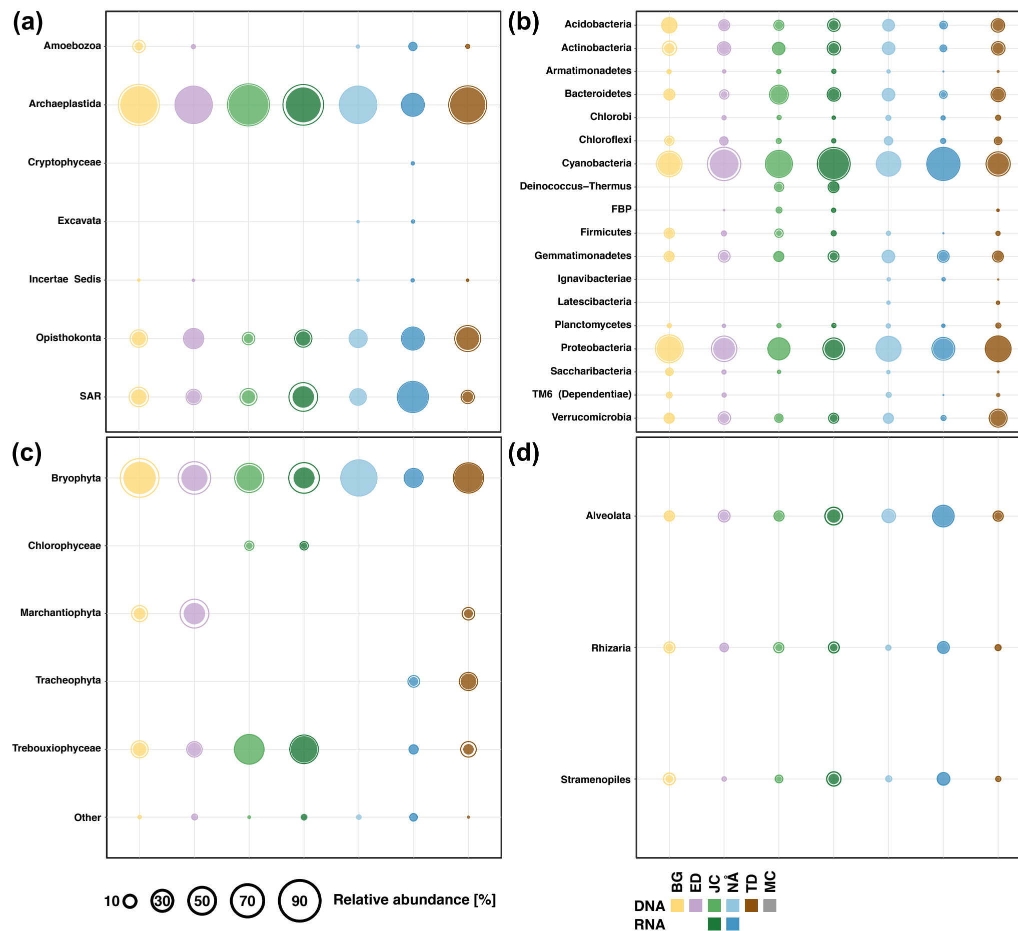

Figure 3: Relative taxonomic abundance. Relative abundance of eukaryotic (a) and prokaryotic phyla (b) and taxonomic groups within *Archaeplastida* (c) and *SAR* (d). For *Chloroplastida*, groups with a relative abundance of less than 1% were summarized as "Others". Relative abundance is given as bubble size and SD as rings.

ited the highest mean values across all eukaryotic indices. DNA derived from TD and NÅ showed the highest average indices among the prokaryotic indices.

The bubble plots in Figure 3 depict the relative abundances, meaning the percentage of sequences per OTU, of eukaryotic phyla, other lower taxonomic categories within *Archaeplastida* and *SAR* as well as prokaryotic phyla. All samples exhibited similar patterns for the relative abundances of the targeted taxonomical groups with minor differences. The most abundant eukaryotic phyla were *Archaeplastida*, *Opisthokonta* and the supergroup *SAR* (Figure 3a). Within the the *Archaeplastida*, *Bryophyta* were most dominant, while *Tre-*

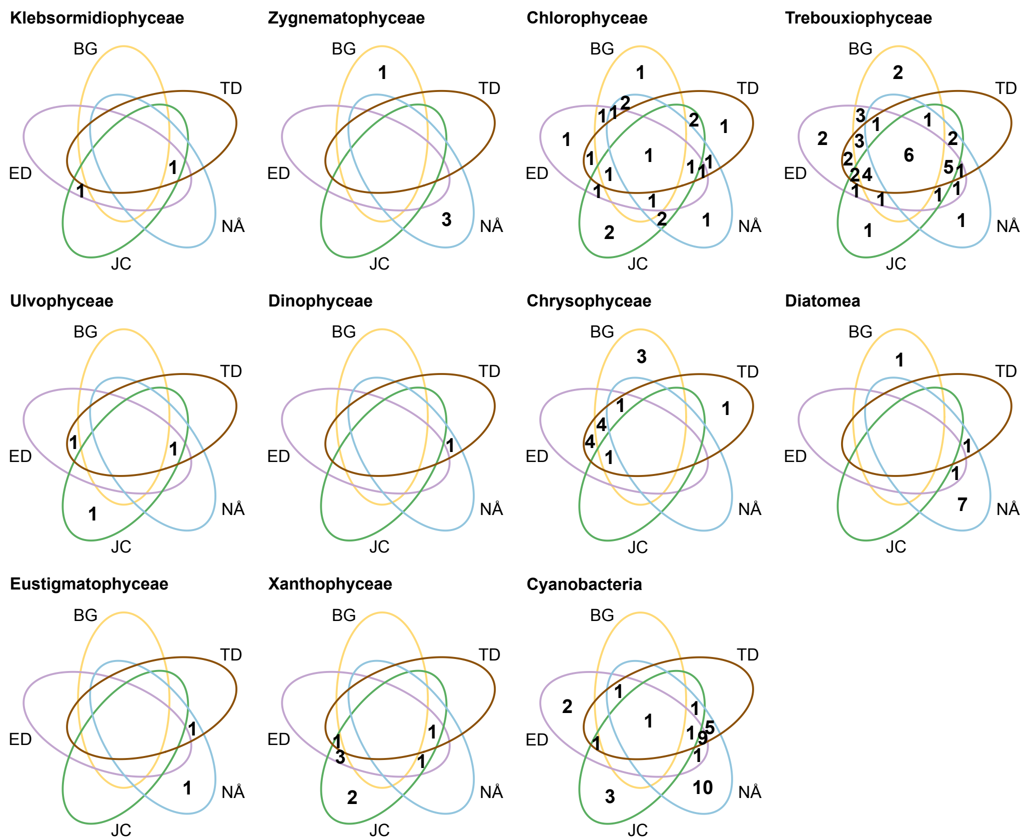

Figure 4: Algal and cyanobacterial diversity. Venn diagrams depicting how many OTUs, belonging to the *Klebsormidiophyceae*, *Zygnematophyceae*, *Chlorophyceae*, *Trebouxiophyceae*, *Chrysophyceae*, *Diatomea*, *Eustigmatophyceae*, *Xanthophyceae* (*Eukaryota*) and *Cyanobacteria* (*Bacteria*), were shared between the DNA derived amplicons. Zeros are not displayed for better comprehension.

bouxiophyceae and *Marchantiophyta* were the second and third most abundant taxa (Figure 3c). The relative abundances of the taxa *Stramenopiles*, *Alveolata* and *Rhizaria* within the *SAR* supergroup were in similar ranges (Figure 3d). *Cyanobacteria* and *Proteobacteria* were the most abundant prokaryotic phyla in all samples (Figure 3b). Furthermore, *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Gemmatimonadetes* and *Verrucomicrobia* were also dominant in the analyzed BSCs.

In Figure 4, the distribution of selected OTUs among the analyzed DNA derived samples is presented. The taxa *Chlorophyceae*, *Trebouxiophyceae*, *Chrysophyceae* and the phylum *Cyanobacteria* were present in all BSC sam-

ples, while *Klebsormidiophyceae*, *Ulvophyceae* and *Xanthophyceae* were absent from BG, *Zygnematophyceae* were missing in ED, JC and TD, JC was lacking *Diatomea* and *Dinophyceae* and *Eustigmatophyceae* were only found in NÅ and TD. The highest diversity was found within the *Chlorophyceae*, *Trebouxiophyceae* and *Cyanobacteria*.

BSC samples collected from the same spots were analyzed previously. Morphological identification of *Klebsormidiophyceae*, *Zygnematophyceae*, *Chlorophyceae*, *Trebouxiophyceae*, *Eustigmatophyceae* and *Xanthophyceae* was performed for BG, ED, NÅ and TD by Borchhardt et al. (2017a). Rippin et al. (2018) published the morphological identification of the same algal taxa and Cyanobacteria for JC and NÅ. Additionally, the authors used the full SSU rRNA gene assembled from metatranscriptomes for the identification of *Klebsormidiophyceae*, *Zygnematophyceae*, *Chlorophyceae*, *Trebouxiophyceae*, *Ulvophyceae*, *Dinophyceae*, *Chrysophyceae*, *Diatomea*, *Eustigmatophyceae*, *Xanthophyceae* and *Cyanobacteria*. Both methodologies and the results presented in this study confirm the presence of *Klebsormidium*, *Stichococcus* and *Microcoleus* in NÅ, *Prasiola* and *Heterococcus* in JC as well as *Coccomyxa*, *Elliptochloris*, *Leptolyngbya*, *Nostoc* and *Phormidium* in both samples (Table S5). In addition, amplicon sequencing found the genera *Chloromonas*, *Coccomyxa*, *Elliptochloris*, *Spumella* and *Nostoc* to be present in all analyzed BSCs. In total, morphological identification and the amplicon data shared 32 genera across all samples (excluding *Ulvophyceae*, *Dinophyceae*, *Chrysophyceae* and *Diatomea*), while the two molecular techniques (amplicon, metatranscriptome) concordantly confirmed 40 genera within NÅ and JC (including *Ulvophyceae*, *Dinophyceae*, *Chrysophyceae* and *Diatomea*).

4.5.3 Passive vs. active community

DNA and RNA derived amplicons were prepared for the Arctic NÅ and the Antarctic JC to compare passive and active biodiversity. These amplicons shared a total of 473 and 665 OTUs for JC and NÅ, respectively (Figure 5). Moreover, the RNA derived amplicons contained more unique OTUs (JC: 47,

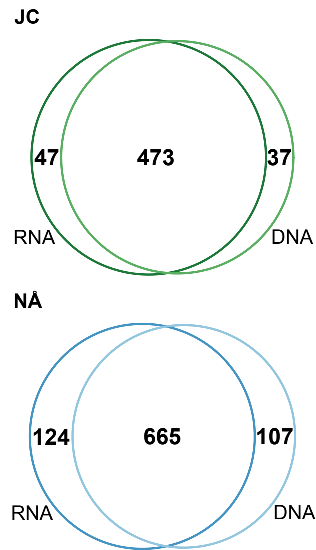

Figure 5: Comparison of DNA- and RNA-derived amplicons. OTUs shared between the DNA and RNA amplicons of sample JC (green shades) and NÅ (blue shades).

NÅ: 127) than the DNA derived ones (JC: 37, NÅ: 107). Concerning biodiversity indices, NÅ exhibited significant differences ($p < 0.001$) in the Shannon and Inverse Simpson index for the eukaryotic and prokaryotic communities between the DNA and RNA derived amplicons.

The BSC samples JC and NÅ were tested for differential abundance comparing DNA and RNA derived amplicons. NÅ exhibited changes in abundance, while JC did not. For NÅ, we observed an abundance increase in *Bacteroidetes* and *Cyanobacteria*, and a decrease in *Verrucomicrobia*, *Archaeplastida* and *Opisthokonta* (Figure 6). While *Bryophyta* became less abundant in the RNA derived dataset, uncultured *Cyanobacteria*, e.g. *Nostoc*, and two *Diatomea* (*Amphora*, *Stauroneis*) occurred more frequently.

4.5.4 Co-occurrence patterns

Testing the collected BSC samples for co-occurrences revealed a total of six clusters, three solely prokaryotic (II, III, VI) and three mixed ones (I, IV, V; Figure 7). A cluster was defined as a connected network of nodes with at least

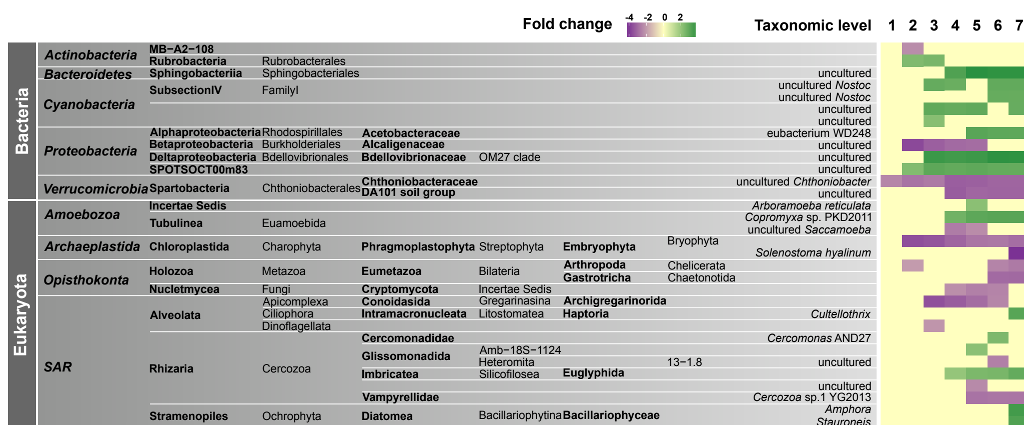

Figure 6: Differential abundance analysis. Differential abundance heat map of sample NÅ comparing DNA and RNA amplicons. The analysis was carried out at different taxonomic levels which were based on the classification according to the Silva database.

one node that has three edges. Cluster I is the biggest consisting of 40 nodes, 24 eukaryotic and 16 prokaryotic. The taxa with the most links are *Arboramoeba*, *Eustigmatophyceae*, *Hyphochytriomycetes*, *Malawimonadea*, *Polytrichopsida* (*Eukaryota*), *Bacteroidales*, *Chroococcales*, *Gloeobacterales*, *Desulfurellales*, *Desulfuromonadales*, *Ignavibacteriales* and *Rubrobacterales* (*Prokaryota*). Interestingly, cluster V contains four classes of eukaryotic algae, which appear to be co-occurring: *Chlorophyceae*, *Klebsormidiophyceae*, *Trebouxiophyceae* and *Xanthophyceae*.

4.5.5 Influence of environmental parameters

The relationship of OTU abundance and environmental parameters was investigated for the Arctic BSC samples. Precipitation and soil parameter values were retrieved from Borchhardt et al. (2017a) and fitted onto ordination (Figure 8). For the eukaryotic amplicons, a correlation with precipitation, carbon, nitrogen, sulfur and phosphorus (total and water-extractable) content was found (Figure 8a). Precipitation exhibited the highest correlation coefficient with $R^2 = 0.95$. Total phosphorus, sulfur and nitrogen also showed a high correlation with a coefficient of 0.85, 0.84 and 0.83, respectively. The

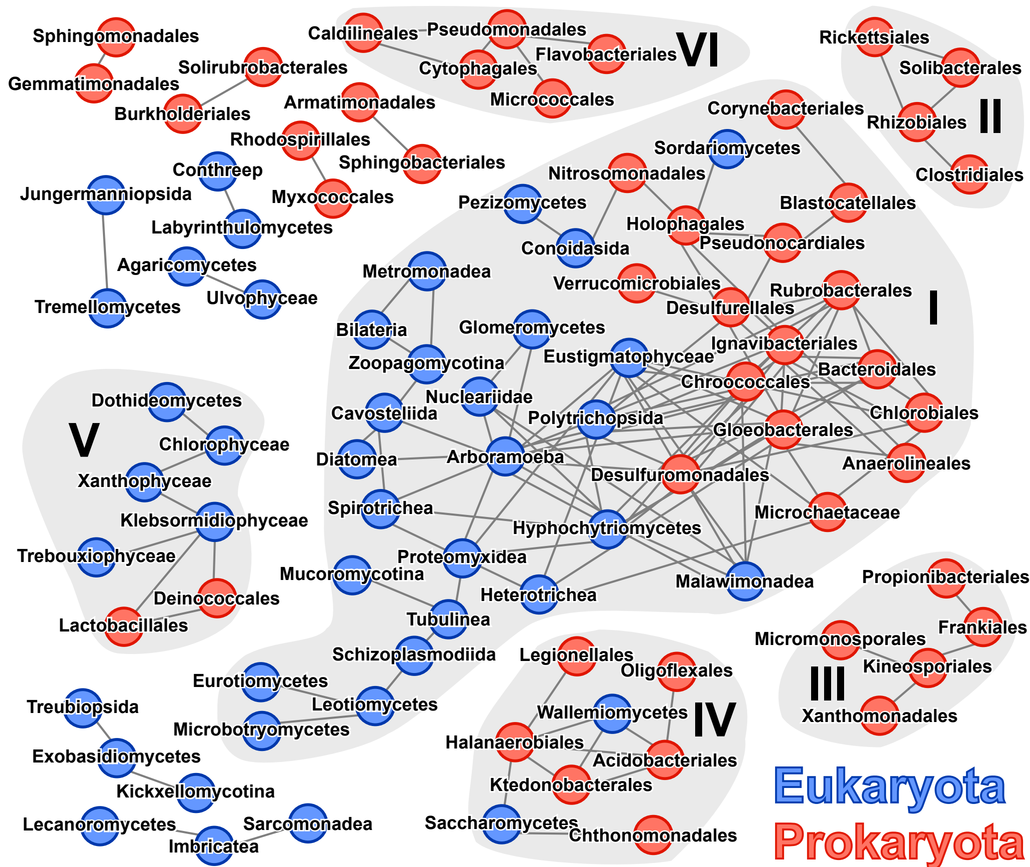

Figure 7: Co-occurrence analysis. Co-occurrence patterns between eukaryotic classes (if applicable, blue) and prokaryotic orders (red) based on a Spearman correlation coefficient of at least 0.75.

prokaryotic community composition was correlated to precipitation, carbon, nitrogen, sulfur and total phosphorus content again with precipitation showing the highest influence ($R^2 = 0.73$; Figure 8b). Total phosphorus exhibited the second highest correlation with $R^2 = 0.6$.

4.6 Discussion

4.6.1 BSCs in polar ecosystems

BSCs represent special microbiotas in polar environments as opposed to barren soil. Typical soil communities are dominated by *Opisthokonta*, *SAR*, *Acidobacteria*, *Bacteroidetes*, *Proteobacteria* and *Verrucomicrobia* (Faoro et al., 2010;

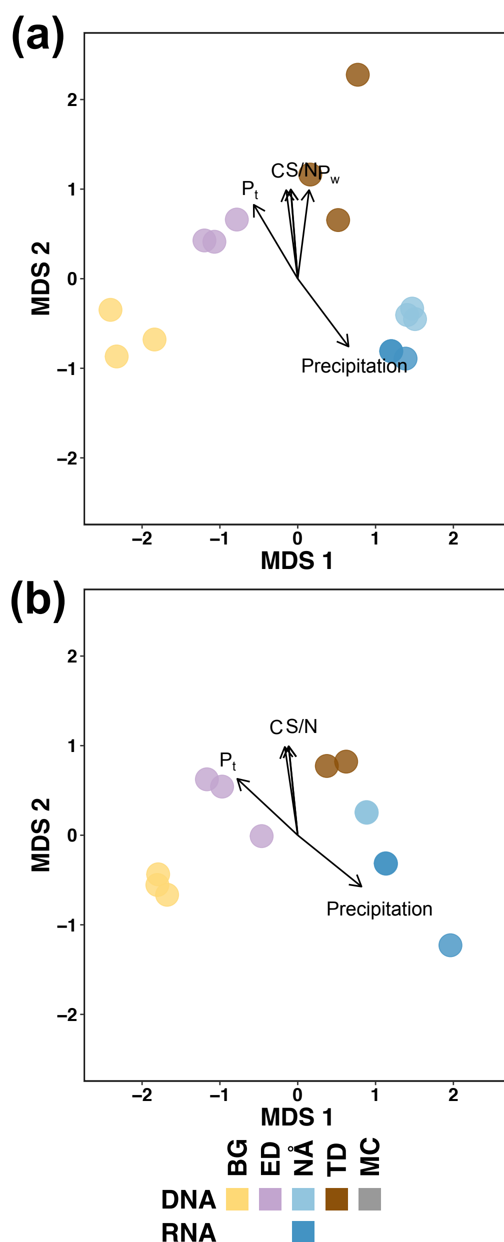

Figure 8: Environmental vector fitting. MDS plot based on Jaccard distance matrix derived from normalized relative abundance data. Vectors corresponding to environmental variables given in Table 2 (Precipitation, C = carbon, N = nitrogen, S = sulfur, P_t = total phosphorus, P_w = water-extractable phosphorus) were fitted onto the ordination. For eukaryotic dataset, the vectors representing precipitation**, C***, N/S***, P_t** and P_w** were fitted (a), while the prokaryotic amplicon data was explained by vectors representing precipitation***, C*, N/S* and P_t** (b). (Significance levels <0.001 ***; <0.01 **; <0.05 *)

Fierer, 2017; He et al., 2017). In Arctic desert soils, the prokaryotic ratios appear to be slightly shifted with *Actinobacteria* and *Chloroflexi* to be more abundant and *Acidobacteria* as well as *Verrucomicrobia* to be less dominant (McCann et al., 2016). In contrast, the bacterial community in Antarctic soil habitats is dominated by *Acidobacteria*, *Bacteroidetes*, *Planctomycetes*, *Proteobacteria* and *Verrucomicrobia* (Geyer et al., 2014). Geisen et al. (2015) reported a dominance of *SAR* in the eukaryotic community of Arctic peatland soils, while *Opisthokonta* were less abundant. All these microbiotas, in contrast to BSCs, feature little to no photosynthetic organisms. The data, presented in this study, shows a clear dominance of photosynthetically active organisms including *Archaeplastida*, algal groups within the *Alveolata* and *Stramenopiles*, as well as *Cyanobacteria* (Figure 3). Steven et al. (2013) analyzed BSCs, collected from the Canadian high Arctic, and also found that *Cyanobacteria* make up a major fraction of the prokaryotic community. These results highlight that BSCs play an essential role as primary producers in polar environments as higher vegetation is mostly absent (Pointing et al., 2015; Thomas et al., 2008b; Yoshitake et al., 2010).

Interestingly, the molecular analysis of the BSCs, collected from different sites at Svalbard and Livingston Island, revealed similar overall compositions and abundance on high taxonomic levels (Figure 3a/b). We argue that the BSCs, collected for this study, were all in a late successional stage, with *Bryophyta* to be one of the most abundant eukaryotic taxa, which is typical for cold deserts (Belnap, 2006). Furthermore, we detected a high abundance of *Cyanobacteria* which are generally regarded as the pioneers which form the basis for BSC development by stabilizing the soil (Belnap, 2006; Gundlapally and Garcia-Pichel, 2006). However, on lower levels the communities show taxonomic variation, e.g. *Zygnematophyceae* were only present in BG and NÅ, while *Trebouxiophyceae* were found in all samples (Figure 4). These differences may be explained by the varying microhabitat features that different organisms or taxonomic groups require (Fierer, 2017). For example, *Cyanobacteria* and algae often colonize the lower surface of quartz pebbles in deserts due to

the condense water, that accumulates there, as well as the enhanced protection against radiation (Belnap et al., 2001a).

When comparing the different biodiversity indices for *Eukaryota* and *Prokaryota* of the analyzed BSC samples, the diversity of *Prokaryota* appeared to be generally higher (Figure 2). Uyaguari-Diaz et al. (2016) compared different watersheds in British-Columbia assessing the biodiversity of the microbial communities. They calculated the Simpson index for the eukaryotic community, based on the markers 18S and the internal transcribed spacer, and prokaryotic diversity based on 16S and chaperonin-60. Astonishingly, the average index is approximately the same for both eukaryotic markers but the prokaryotic index for 16S is lower and for chaperonin-60 higher than the eukaryotic ones. Regarding amplicon length, the eukaryotic targets are the same, while the 16S target is shorter and the chaperonin-60 is longer than 18S and the internal transcribed spacer. Accordingly, the prokaryotic amplicon, used in our study, was longer than the eukaryotic sequence. If amplicon length influences diversity estimates, this observation could explain the higher prokaryotic indices. Moreover, the variability of the target region is also important as amplicons are clustered into OTUs based on global sequence similarity (Forster et al., 2016). Furthermore, the differences in cell size and evolutionary age between prokaryotes and eukaryotes could be responsible for these observations (Cooper, 2000).

The biodiversity in these communities is highly dependent on climate, chemical properties of the soil etc. (Belnap et al., 2001b). Borchhardt et al. (2017a) verified the influence of precipitation on microalgal communities at Svalbard which was also detected for the prokaryotic and eukaryotic communities in this study. Precipitation and water availability are key factors that affect microbiotas in a more quantitative than qualitative manner (Cruz-Martínez et al., 2012; Hoffmann, 1989; Thomas et al., 2008c; Zhang et al., 2016). In the Arctic, water availability is changing drastically throughout the season (Thomas et al., 2008b). In winter, most water is frozen and cannot be utilized by terrestrial organisms, while liquid water becomes available in summer as rain or meltwater runoffs (Thomas et al., 2008b). Thus, the sole

influence of precipitation is arguable. Additionally, the impact of carbon, nitrogen, sulfur and phosphorous content on the community composition of BSCs was confirmed. Previous studies confirmed the influence of these parameters on soil biocoenoses, while the ratio between carbon and nitrogen might also have an effect (Cong et al., 2015; Zarraonaindia et al., 2015). On the contrary, BSC organisms will also affect the elemental composition of the adjacent soil (Brankatschk et al., 2013; Evans and Lange, 2001). For example, *Cyanobacteria* fix nitrogen which will be either used by other organisms or deposited in the soil (Hoffmann, 1989).

4.6.2 Eukaryotic algae and Cyanobacteria

Eukaryotic algae often occur later during BSC development and in association with bryophytes, due to their high water-holding capacity, while *Cyanobacteria* often act as initial ecosystem engineers (Büdel et al., 2016). Thus, the dominance of *Bryophyta* in the collected BSCs may explain the presence and high diversity of eukaryotic algae. All taxonomic groups, typical for BSCs, were detected: *Klebsormidiophyceae*, *Zygnematophyceae*, *Chlorophyceae*, *Trebouxiophyceae*, *Ulvophyceae*, *Dinophyceae*, *Chrysophyceae*, *Diatomea*, *Eustigmatophyceae* and *Xanthophyceae* (Büdel et al., 2016; Karsten and Holzinger, 2014). When looking at individual samples, the occurrence of algal and cyanobacterial genera mostly differed from isolate to isolate. However, we found *Chloromonas* (*Chlorophyceae*), *Coccomyxa*, *Elliptochloris* (*Trebouxiophyceae*), *Spumella* (*Chrysophyceae*) and *Nostoc* (*Cyanobacteria*) to be present in all BSC. The genus *Chloromonas* is often associated with polar BSCs and contains certain snow alga species, e.g. *Chloromonas brevispina* (Büdel et al., 2016; Hoham, 1975; Pushkareva et al., 2016; Raymond, 2014). *Coccomyxa*, on the other hand, is a ubiquitous and versatile alga that can occur both terrestrial and planktonic (Darienko et al., 2015; Ettl and Gärtner, 2014). The green alga *Elliptochloris* commonly occupies cold terrestrial habitats and is a potential phycobiont for lichens (Borchhardt et al., 2017a; Ettl and Gärtner, 2014; Pushkareva et al., 2016). For the first time, we report the presence of the chrysophyte *Spumella*

in polar BSCs. *Spumella* is a nanoflagellate which can be found in marine, freshwater and terrestrial habitats (Stoeck et al., 2008). These mixotrophs are often bacterivorous and link bacterial production to higher trophic levels (Boenigk et al., 2005). The cyanobacterial genus *Nostoc* is commonly associated with BSCs but may also occupy freshwater environments (Dojani et al., 2014; Pushkareva et al., 2016; Rippka et al., 1979). Previous studies confirm that *Nostoc* also colonizes soils in the Arctic and Antarctica (Pushkareva et al., 2016; Zidarova, 2008). Overall, eukaryotic algae and *Cyanobacteria* are important primary producers and form the nutritional basis for heterotrophic organisms (Ettl and Gärtner, 2014; Nweze, 2009). Moreover, they enhance the growth and development of other autotrophs as well as heterotrophs (Belnap and Lange, 2001; Metting, 1981). A number of *Cyanobacteria*, e.g. *Nostoc* and *Oscillatoria*, possess the ability to fix dinitrogen from the air and make it subsequently available to other organisms in the BSCs (Metting, 1981). Furthermore, certain algae and *Cyanobacteria*, which are able to synthesize photoprotective pigments, are crucial for protection against excessive light and ultraviolet radiation (Belnap and Lange, 2001; Karsten and Holzinger, 2014). These organisms form an âĂIJumbrellaâĂİ on top of the BSC and, thus, shade other species without sunscreen pigmentation (Belnap and Lange, 2001; Karsten and Holzinger, 2014). The formation of the crust matrix is carried out by filamentous and mucilage-producing genera within the *Klebsormidiophyceae*, *Zygnematophyceae* and *Cyanobacteria* by glueing soil particles together (Belnap, 2006; Büdel, 2005; Büdel et al., 2016). This process increases soil stability and makes it less prone to wind and water erosion (Evans and Johansen, 1999). Some algal and cyanobacterial taxa establish symbioses with fungi to form lichens (Büdel et al., 2016; Ruprecht et al., 2014). A typical photobiont is the green alga *Trebouxia* which was detected in all samples except NÅ (Ruprecht et al., 2014). On the other hand, the fungal genera *Elasticomyces* and *Leciophysma* were detected in the samples BG and ED (data not shown) which are potential mycobionts (Selbmann et al., 2008; Wedin et al., 2009).

4.6.3 Activity patterns

Differences in the relative abundance of rDNA and rRNA in environmental samples are caused by various factors (Blazewicz et al., 2013; Charvet et al., 2012; Hansen et al., 2007). DNA based approaches will detect active and dormant organisms but dead as well (Hansen et al., 2007). Additionally, extracellular DNA is well preserved in polar ecosystems, due to the low temperatures, leading to an overestimate in biodiversity (Charvet et al., 2012; Nielsen et al., 2007). The relative abundance of rRNA, on the other hand, can be regarded as potential metabolic activity meaning the potential to perform protein synthesis (Blazewicz et al., 2013). The comparison between rDNA and rRNA abundances might be used as a measure for passive vs. active diversity (Blazewicz et al., 2013). However, certain limitations have to be considered, e.g. varying rDNA copy numbers across different taxa (Blazewicz et al., 2013; Prokopowich et al., 2003; Zhu et al., 2005).

RNA derived amplicons were prepared for JC and NÅ using both the eukaryotic and the prokaryotic marker. More unique OTUs were detected for the rRNA than the rDNA based datasets suggesting a higher diversity. Lanzén et al. (2013) calculated the OTU richness, based on rDNA and rRNA abundance, for two soda lakes at different depths and observed varying ratios for rDNA:rRNA. Thus, no general assumption regarding present and active OTU diversity can be made. Regarding the Shannon and Inverse Simpson indices, significant differences between the DNA and RNA derived libraries could be determined solely for NÅ. Curiously, the eukaryotic indices of the RNA derived dataset were significantly higher, while the prokaryotic indices were significantly lower. These observations are probably linked to seasonality. These seasonal changes in microbiota were reported both for prokaryotic and eukaryotic soil communities (Bass and Bischoff, 2001; Davey, 1991; Lara et al., 2011; Lipson and Schmidt, 2004). Davey (1991) studied the periodicity of microalgae and *Cyanobacteria* on Antarctic fellfield soils and found *Phormidium* and *Pinnularia* to be dominant for most of the year while, *Zygnema* and *Ulothrix* occurred only during austral summer.

Significant changes in rDNA to rRNA abundance were determined within the prokaryotic and eukaryotic communities in sample NÅ. The *Amoebozoa Copromyxa* exhibited a higher rRNA than rDNA abundance indicating that it is active probably due to the milder conditions during the summer at Svalbard. Seasonal patterns have been previously described for other *Tubulinea* by Mansano et al. (2013). *Bryophyta*, on the other hand, were less abundant in the RNA derived set of amplicons. As the samples were collected in August 2014, bryophytes should be active and growing because temperatures are above 0°C and, thus, liquid water is available (Førland et al., 2011; Prestø et al., 2014). However, the fact that samples were collected during growing season and the lower abundance of rRNA are not contradictory as *Bryophyta* exhibit growth and photosynthetic activity mostly at the apex and older annual segments are less active or even inactive (Clymo and Hayward, 1982; Longton, 1988). Hence, older segments are likely to contain less rRNA compared to rDNA and only the shoot tip features increased rRNA levels. *Archigregarinorida* also appeared to be less abundant in the RNA based dataset. The taxon is parasitic, occurs in aquatic and terrestrial habitats and can form cyst if exposed to unfavorable conditions (Rueckert et al., 2011). The reduced rRNA content compared to the rDNA suggests that the alveolates are in a resting stage. In contrast, the cercozoan taxon *Euglyphida* exhibited an increased abundance in rRNA. These findings indicate that the soil is not dried out as the bacterivorous amoeba strongly reacts to drought with reduced abundance (Harder et al., 2016). The same holds true for *Cyanobacteria*, e.g. *Nostoc*, which also exhibited a higher abundance in the RNA derived dataset. Elster et al. (2012) found Arctic *Nostoc* colonies to be photosynthetically active throughout the summer with no significant changes in the monitored physiological parameters despite the fluctuating water supply. Hence, the *Cyanobacteria* occurring in the BSC were metabolically active as we detected a higher rRNA than rDNA abundance. The taxon *Bdellovibrionaceae* also exhibited increased levels of rRNA. These organisms occupy both aquatic and terrestrial habitats and prey on other bacteria which is also assumed for Clade OM27 (Orsi et al., 2016;

Pineiro et al., 2004). Little is known about Clade OM27, thus, it is difficult to draw a conclusion regarding their ecology. Similarly, SPOTSOCT00m83 was more abundant in the RNA derived set of amplicons. However, ecological data on this taxon is rare. Yun et al. (2016) confirmed that SPOTSOCT00m83 can be found in soil. Another soil-dwelling taxon are the *Alcaligenaceae* which are quite diverse (Ghosh et al., 2011). The taxon showed lower rRNA to rDNA abundance which indicates suboptimal growth conditions at the time of sampling. *Chthoniobacterales* were also less abundant in the RNA derived dataset. Yun et al. (2016) found that *Chthoniobacterales* prefer more acidic soils (mean pH 5.59) compared to the pH at NÅ (6.3) (Borchhardt et al., 2017a).

4.6.4 Ecological Interactions

Co-occurrence patterns of different taxa or taxonomic modules enable an identification of ecological interactions between organisms, especially organisms that are poorly understood due to difficulties in cultivation (Stewart, 2012; Williams et al., 2014). Moreover, these patterns can be used to identify functional and ecological traits, and draw conclusions on the life-history strategies of the organisms (Williams et al., 2014). However, the unavailability of ecological data for certain taxonomic groups limits the interpretation to certain modules.

Several bacterial orders contributing to the sulfur cycle, one of the most important cycles linked to the carbon cycle, co-occur in the sampled BSCs (Zavarzin, 2010). Sulfate-reducing bacteria, such as *Desulfurellales* and *Desulfuromonadales*, form H_2S either by incomplete oxidation of organic acids or complete oxidation of unfermentable compounds produced by fermentation (Kleindienst et al., 2014; Timmers et al., 2015; Zavarzin, 2010). Reduced sulfur compounds (H_2S, sulfide, sulfite), on the other hand, are oxidized by, e.g., either the strictly anaerobic *Chlorobiales* or *Ignavibacteriales* (Gregersen et al., 2011; Iino et al., 2010; Ontiveros-Valencia et al., 2014; Zavarzin, 2010). The aforementioned bacterial orders co-occur within cluster I and are directly linked within the network underlining their collective participation in the sulfur

cycle. The cyanobacterial taxon *Microchaetaceae* is heterocystous and, thus, contributes to the nitrogen cycle by nitrogen fixation which is indirectly coupled to the sulfur cycle (Hauer et al., 2014; Hoffmann, 1989; Zavarzin, 2010). Furthermore, *Microchaetaceae* and the sulfur metabolising bacteria are linked to the cyanobacterial taxa *Chroococales* and *Gloeobacterales*, the stramenopile class *Eustigmatophyceae* and the bryophyte class *Polytrichopsida* which all contribute to the carbon fixation by oxygenous photosynthesis (Hoffmann, 1989; Zavarzin, 2010). The organic carbon cycle links all elemental cycles together and, thus, is of major importance (Zavarzin, 2010). Cluster I also contains a connection between *Polytrichopsida* and *Arboramoeba*. Davidova et al. (2016) described the association of testate amoeba with terrestrial bryophytes due to their ability to store water. This might be also true for *Arboramoeba* which co-occurred with the bryophyte class *Polytrichopsida* within the analyzed BSCs. Cluster V contains the algal classes *Chlorophyceae*, *Klebsormidiophyceae*, *Trebouxiophyceae* and *Xanthophyceae* which were found to be co-occurring in the Arctic and Antarctic BSCs. All of these microalgae are capable of photosynthesis which might cluster them together due to the link to the carbon cycle (de Morais et al., 2015; Zavarzin, 2010). However, another possible explanation might be their ability of synthesizing antibiotic, antiviral and antifungal secondary metabolites of which these organisms mutually benefit (de Morais et al., 2015; Sahayaraj et al., 2014). Nevertheless, co-occurrences can also depend on environmental factors and known biological interactions might be missing due to the parameters chosen in the analysis (Coutinho et al., 2015; Williams et al., 2014).

4.7 Conclusion

BSCs are key components of polar ecosystems that possess a huge diversity of different autotrophic, heterotrophic and saprotrophic organisms. Thus, these biocoenoses can be regarded as oases in polar deserts. This comprehensive survey shed light on the prokaryotic and eukaryotic diversity of different BSCs

collected from the Arctic Svalbard and Livingston Island which is part of the Antarctic Peninsula Region. Overall, the polar BSCs are dominated by photoautotrophs such as eukaryotic algae, Cyanobacteria, lichens and bryophytes. Nevertheless, a plethora of heterotrophic and saprotrophic, aerobic and anaerobic organisms was found alongside those primary producers. BSCs are indeed amalgamations of eukaryotic algae, lichens, bryophytes, autotrophic and heterotrophic bacteria as well as *Fungi* but also other protists, such as *Cercozoa* and *Amoebozoa*, and *Bilateria* appear to be important for these communities. In order to gain further insights into functionality, shotgun metagenomics, -transcriptomics, -proteomics and metabolomics should be combined. These integrated results would help to draw a detailed picture of how the organisms interact with each other and annual time series could reveal the seasonal development of BSCs.

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Chapter 5

Paper IV
Enhanced desiccation tolerance in mature cultures of the streptophytic green alga *Zygnema circumcarinatum* revealed by transcriptomics

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5.1 Abstract

Desiccation tolerance is commonly regarded as one of the key features for the colonization of terrestrial habitats by green algae and the evolution of land plants. Extensive studies, focused mostly on physiology, have been carried out assessing the desiccation tolerance and resilience of the streptophytic genera *Klebsormidium* and *Zygnema*. Here we present transcriptomic analyses of *Zygnema circumcarinatum* exposed to desiccation stress. Cultures of *Z. circumcarinatum*, grown in liquid medium or on agar plates, were desiccated at 86% relative air humidity until Y(II) ceased. In general, the response to dehydration was much more pronounced in *Z. circumcarinatum*, cultivated in liquid medium for one month, compared to filaments grown on agar plates for seven and twelve months. Cultivation on solid medium enables the alga to acclimate to dehydration much better and an increase in desiccation tolerance was clearly correlated to increased culture age. Moreover, gene expression analysis revealed that photosynthesis was strongly repressed upon desiccation treatment in the liquid culture while only minor effects were detected in filaments cultivated on agar plates for seven months. Otherwise, both samples showed an induction of stress protection mechanisms such as ROS scavenging (Early light-induced proteins, glutathione metabolism) and DNA repair as well as the expression of chaperones and aquaporins. Additionally, *Z. circumcarinatum*, cultivated in liquid medium, upregulated sucrose synthesizing enzymes and strongly induced membrane modifications in response to desiccation stress. These results corroborate the previously described hardening and associated desiccation tolerance in *Zygnema* in response to seasonal fluctuations in water availability.

5.2 Keywords

Desiccation tolerance, *gene expression*, *streptophytic algae*, *transcriptomics*, Zygnema

5.3 Abbreviations

ANOVA	Analysis of variance
BBM	Bold's basal medium
BLAST	Basic local alignment search tool
CTAB	Cetyl trimethylammonium bromide
DTT	Dithiothreitol
ERD	Early-response-to-dehydration protein
FDR	False discovery rate
GO	Gene ontology
HSD	Honestly significant difference
KEGG	Kyoto encyclopedia of genes and genomes
KO	KEGG orthology
OTU	Operational taxonomic unit
PAM	Pulse-amplitude modulated fluorometer
ROS	Reactive oxygen species
RuBisCo	Ribulose-1,5-bisphosphat-carboxylase/-oxygenase
UVR	Ultraviolet radiation
Y(II)	Effective quantum yield of photosystem II

5.4 Introduction

The colonization of terrestrial habitats by plants is accompanied by a number of abiotic stress factors such as high irradiance and dehydration (Borstlap, 2002; Holzinger and Pichrtová, 2016). Tolerating water stress is crucial for survival of land plants as well as terrestrial algae and has been addressed extensively (Borstlap, 2002; Dinakar and Bartels, 2013; Holzinger et al., 2014; Oliver, 2007). Most land plants are able to actively regulate their water status (homoiohydry) but lost the ability to tolerate desiccation. However, the so-called resurrection plants, such as *Craterostigma plantagineum*, possess desiccation tolerance (poikilohydry; Norwood et al., 2003) and are not able to ac-

tively regulate their water content (Holzinger and Karsten, 2013). Hence, *C. plantagineum* and other resurrection plants were used as models for desiccation stress tolerance (Bartels and Salamini, 2001; Dinakar and Bartels, 2013). Additionally, other higher plants (Basu et al., 2016; Ma et al., 2015; Oliver et al., 2011) and mosses were studied to unravel the mechanisms of desiccation response and resilience (Gao et al., 2015; Shinde et al., 2012).

Over the past years, substantial information about the impact of desiccation stress on streptophytic green algae, including *Zygnema* spp. and *Klebsormidium* spp., became available (for summary see Holzinger and Karsten, 2013; Holzinger and Pichrtová, 2016; Karsten and Holzinger, 2014). *Klebsormidium* generally occupies moist terrestrial habitats (Lokhorst, 1996) while *Zygnema* occurs in hydro-terrestrial environments, meaning in or in close vicinity to freshwater bodies or streams (Davey, 1991; Hawes, 1990). Upon decreasing air humidity, water is rapidly lost but these poikilohydric organisms have the ability to tolerate dehydration in the vegetative state to a certain degree. However, in *Zygnema* desiccation tolerance is strongly dependent on the physiological state of the cell. Stress tolerance increases during maturation of the algae (Herburger et al., 2015; Pichrtová et al., 2014) which is associated with the transition from vegetative cells to pre-akinetes and akinetes (McLean and Pessoney, 1971). The maturation process is accompanied by changes in the fatty acid composition which have recently been studied by Pichrtová et al. (2016a).

To shed light on the molecular mechanisms of desiccation response and tolerance in streptophyte green algae, transcriptomic profiling was performed for *Klebsormidium crenulatum* revealing reaction patterns similar to land plants when exposed to water stress (Holzinger et al., 2014). Dehydration in plants and algae is linked to a number of defense mechanisms, e.g. protection of the photosynthetic apparatus by the expression of early light-induced proteins (ELIPs), synthesis of low-molecular-weight osmolytes to maintain turgor pressure, induction of ROS scavenging and increase of the chaperone transcript pool (late embryogenesis abundant (LEA) and heat-shock proteins (Hsps))

(Fernández-Marín et al., 2013; Wang et al., 2004). The above listed defense systems were also induced in the basal streptophyte alga *K. crenulatum* upon harsh desiccation over silica gel as demonstrated by Holzinger et al. (2014).

In contrast to Klebsormidiophyceae, which are located closer to the basis of the Streptophyta (Becker and Marin, 2009), Zygnematophyceae are the sister lineage of the land plants (Wickett et al., 2014). This has been proven through several phylogenetic analyses but is also confirmed by the fact that zygnematophycean algae possess a modified plastid, the 'embryoplast', which played a key role in the development of the land plants (de Vries et al., 2016; Ruhfel et al., 2014; Wodniok et al., 2011; Zhong et al., 2013). Thus, the investigation of their water stress tolerance on a molecular level is particularly interesting from an evolutionary point of view. While the dehydration-induced changes in physiology of *Zygnema circumcarinatum* and *K. crenulatum* are similar, with a drastic reduction of Y(II), the kinetics of water loss are different (Herburger and Holzinger, 2015; Lajos et al., 2016). Herburger and Holzinger (2015) reported an elevated callose content in the cell walls of *K. crenulatum*, which enables shrinkage of the whole cell, compared to *Z. circumcarinatum* which forms rigid cellulosic secondary walls. In terms of water loss, *Z. circumcarinatum* reduces the protoplast volume more rapidly compared to *Klebsormidium* at a relative air humidity (RH) lower than 85%, however, this observation was reversed for higher RH (Lajos et al., 2016). To gain deeper insights into the mechanisms of desiccation tolerance in *Z. circumcarinatum* and study the differences to *K. crenulatum*, transcriptomic analyses were performed on algal cultures, either grown in liquid medium or on agar plates and, subsequently, subjected to dehydration. Furthermore, differently matured cultures (one, seven and twelve months) were used to investigate the influence of culture age, which is associated with pre-akinete formation in cultures older than seven months. In contrast to *Klebsormidium*, the genome of *Zygnema* has not been published yet. Thus, transcriptomic data also provide a valuable information resource and give insights into a plethora of molecular mechanisms.

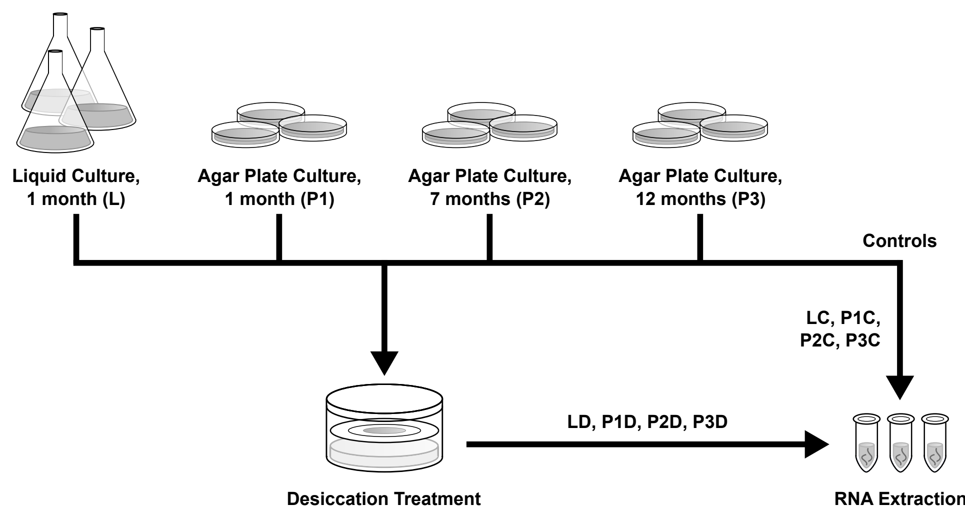

Figure 1: Experimental Setup. Four different culture conditions were chosen: one month old culture grown in liquid medium (L), one month old culture grown on solid medium (P1), seven months old culture grown on solid medium (P2), 12 months old culture grown on solid medium (P3). Samples were taken as controls and for desiccation treatment and RNA was extracted subsequently.

5.5 Results

5.5.1 Physiological response to desiccation

All samples were desiccated over KCl and the Y(II) was monitored. An experimental overview is given in Figure 1. For all samples dehydration stress was applied until Y(II) dropped to zero. Hence, the physiological state of all filaments is comparable. The biomass of the liquid culture (L) and the 12 months old agar plates (P3) maintained photosynthesis the longest until approximately 390 min and 360 min, respectively (Figure 2A/D). Y(II) of the filaments, cultivated for one month on solid medium (P1), drops first at about 90 min (Figure 2B) while Y(II) of the 7 months old culture (P2) reaches zero at approximately 220 min (Figure 2C). Table 1 displays the desiccation time and observed water loss for all samples. When comparing the time required for the cells cultivated on agar plates to reduce Y(II) to zero, increased age is clearly correlated ($r = 0.98$) with a prolonged activity. All three cultures grown on

agar (P1, P2, P3) are differing significantly in desiccation time ($p < 0.001$). No significant difference has been observed between P3 and L, however, P2 and P1 differ significantly from L. Concerning water loss, no significant differences were detected between L, P2 and P3. Sample P1 differs significantly in water content reduction from L ($p < 0.001$), P2 ($p < 0.01$) and P3 ($p < 0.01$).

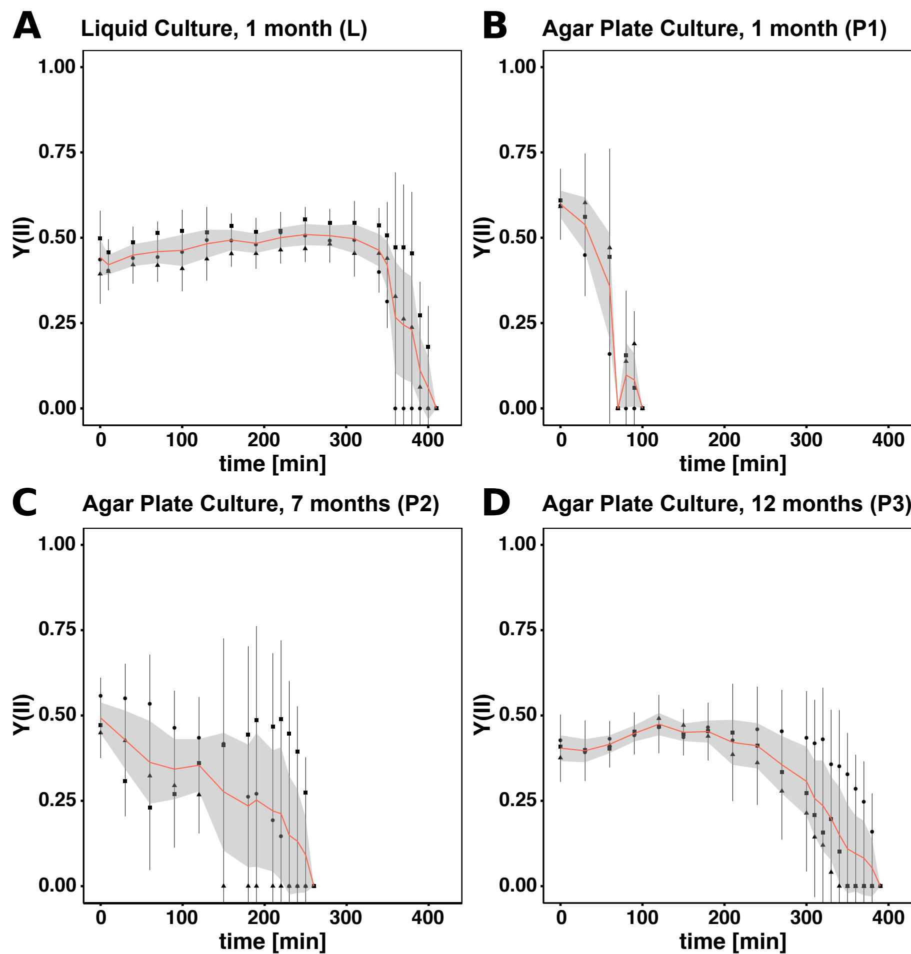

Figure 2: Y(II) measured over the course of desiccation for all samples. Each triplicate was measured three times at different positions of the filter. The bar indicates standard deviation per replicate while square, triangle and circle indicate the mean value. The mean of all replicates is displayed in red giving the lower and upper Gaussian confidence limit in light grey. A) One month old culture grown in liquid medium (L). B) One month old culture grown on solid medium (P1). C) Seven months old culture grown on solid medium (P2). D) 12 months old culture grown on solid medium (P3).

Table 1: Physiological results of desiccation stress experiment. Desiccation time means the time that elapsed from the start of the desiccation treatment until Y(II) dropped to zero. (n=3 if not indicated otherwise, *n=2)

Sample ID	Cultivation	Desiccation time [min]	Water loss [%]
L	liquid medium, 1 mo	390 ± 26.5	93.7 ± 2
P1	solid medium, 1 mo	90 ± 17.3	81 ± 3.4*
P2	solid medium, 7 mo	223 ± 40.4	92.8 ± 3.2
P3	solid medium, 12 mo	360 ± 26.5	89 ± 0.01

5.5.2 Sequencing outcome and reference library

The triplicates for P3 were pooled for sequencing due to low RNA quantities. Hence, this group was excluded from analysis of differential gene expression. Furthermore, one replicate of P2D exhibited a low extraction yield and, thus, was not sequenced. The sequencing results for all libraries are summarized in supplementary Table S1. For the reference, which was pooled from all samples, 13,241 Mbp were obtained, and for all samples a total of 85,721 Mbp was sequenced.

Figure 3A gives an overview of the bioinformatic pipeline used for raw read processing, assembly and further analysis. The assembly of the quality filtered and trimmed reference reads yielded a total of 135,572 contigs with an N50 of 950 bp, and a smallest and largest contig size of 224 bp and 24,724 bp, respectively. To assess the completeness of the established reference transcriptome, Benchmarking Universal Single-Copy Orthologs (BUSCO) were used. As displayed in Figure 3B, we found 76% to be complete, 8% to be fragmented while only 16% were missing. Compared to a variety of other

transcriptomes, these values can be regarded as excellent (Simão et al., 2015). Annotating the assembly against the Swiss-Prot database resulted in 28,427 (21%) hits with an e-value smaller than E-10 (Figure 3C). Moreover, all contigs were tested for homology to amino acid sequences, retrieved from complete streptophyte and chlorophyte genomes, resulting overall in higher annotation rates for streptophytic than for chlorophytic sequences (Figure 3D, Figure S2 in the supporting information). For all examined e-values ranging from E-3 to E-20, all assembled contigs shared most sequences with *Physcomitrella patens* and *Klebsormidium flaccidum* (Figure S2). The homology comparison of the assembly to all streptophytic and all chlorophytic sequences showed that our assembly shared 21,262 with both groups while 10,672 and 615 sequences were exclusively aligned to streptophytic and chlorophytic proteins, respectively (Figure 3D). In order to evaluate the coverage of metabolic networks, the assigned KO numbers (5.9% of all contigs) were mapped onto the KEGG metabolic pathways map (ko01100, Supplemental Figure S3). In general, we observed a very good coverage with the most important pathways (e.g. carbohydrate metabolism, amino acid metabolism, fatty acid metabolism, nucleotide metabolism, respiration) being complete. The annotation rate for GO terms was 28% of all contigs.

5.5.3 rRNA analysis

The *Z. circumcarinatum* culture used in this study was only recently established (Herburger et al., 2015). It is unialgal but neither axenic nor characterized with respect to possible contamination by heterotrophic eukaryotes. The rRNA reads, that were filtered out during quality control, were used to investigate the presence of putative contaminations in the algal culture (Supplemental Figure S4). The largest fraction of all rRNA reads could be annotated as *Zygnema* sp. However, a large number of different bacterial rRNA species as well as a few eukaryotic rRNAs, with most of them mapping to the genus *Naegleria* sp., was detected. We also observed a large number of putative rRNAs which could not be annotated at all (up to 20% for P3C). Overall, rRNA reads

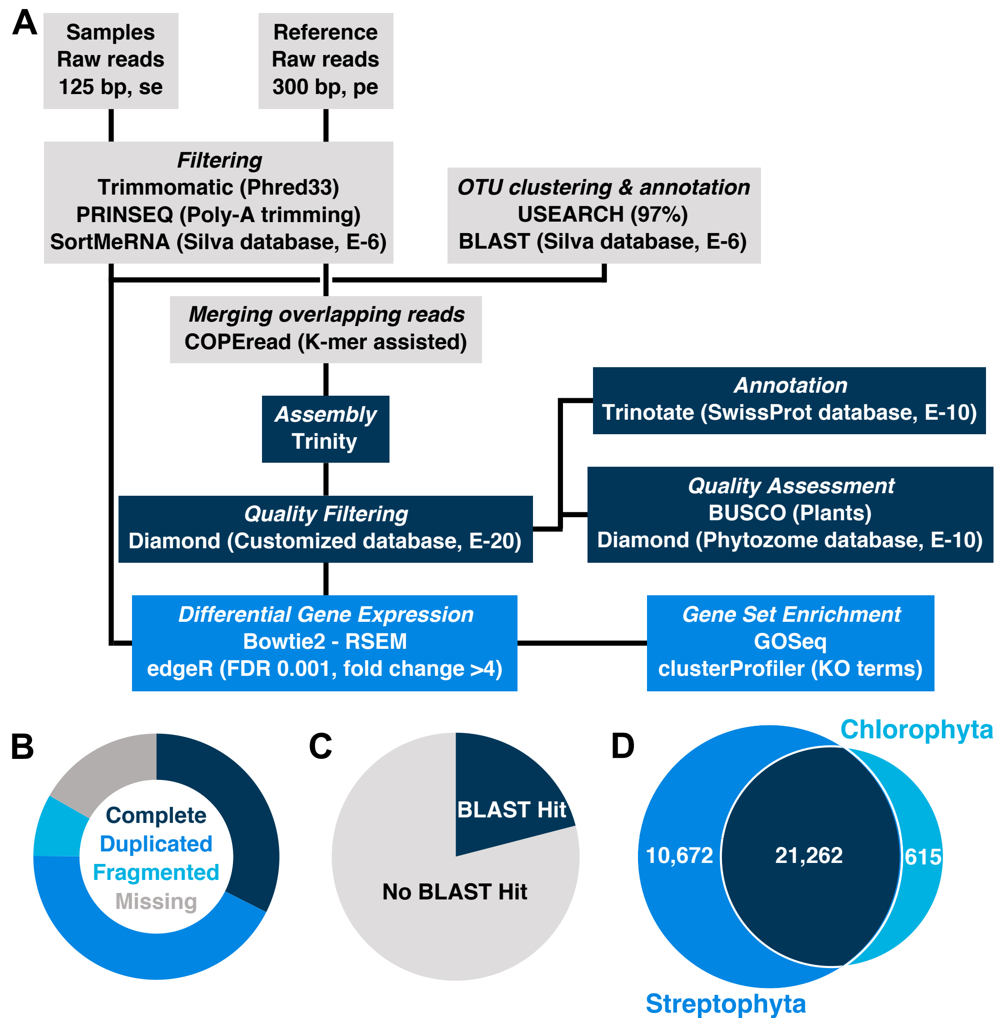

Figure 3: A) Bioinformatic Pipeline. Raw Reads of all samples (controls and treated) as well as the reference (pool of all samples) were filtered using Trimmomatic, PRINSEQ and SortMeRNA. The remaining paired-end reads of the reference were merged, if possible, using COPEread and subsequently assembled with Trinity. The filtered rRNA reads of the samples were clustered and annotated using USEARCH and BLAST. The assembly was further quality filtered against a customized database containg sequences originating from *Physcomitrella*, *Klebsormidium* and *Naegleria*. After quality assessment with BUSCO and Diamond the contigs were annotated with Trinotate. The sample reads were mapped onto the contigs using Bowtie2, read counts were calculated with RSEM and differential gene expression was performed with edgeR. Finally, GOSeq and clusterProfiler were used for gene set enrichment analyses. More details on the procedure can be found in the material and methods

Figure 3 (Continued): section. B) The donut chart displays the results of the BUSCO analysis which was carried out to assess the completeness of the assembly. The categories complete, duplicated, fragmented and missing are represented by 310, 408, 78 identified and 160 not identified orthologs, respectively. C) In total, 28,427 contigs of the assembly could be annotated at E-10 while 107,145 could not. D) Venn-diagram depicting the number of contigs mapping to sequences of selected Streptophyta (blue), Chlorophyta (turquoise) or both (dark blue) at E-10.

related to *Zygnema* sp. or with no significant similarity to any organism in BLAST analyses constituted about 90% of the rRNA except for the P1C and P1D. For these samples a large contamination with bacterial (up to 40%) and eukaryotic (*Naegleria* sp., up to 17%, among others) sequences was observed. Hence, P1C and P1D were excluded from analyses if not indicated otherwise.

5.5.4 Expression analysis

Differential expression analysis was carried out for control versus desiccated samples of the liquid culture (LC to LD) and the seven months old solid culture (P2C to P2D). The analysis was performed without P1C and P1D as well as P3C and P3D due to high levels of contamination and low RNA extraction yields, respectively. A total of 2,886 (2.1%) transcripts exhibited differential expression (FDR less or equal to 0.001) upon desiccation treatment compared to the corresponding control in at least one of the two group comparisons (L, P2). For the liquid culture (L), we observed the strongest reaction with 2,494 contigs regulated while only 849 were differentially expressed in group P2 (Figure 4A, supplementary Table S5). Between 30% and 54% of the upregulated and downregulated contigs in those groups were successfully annotated. The largest part of the annotated sequences showed similarities to proteins of Viridiplantae. Regarding the overlap of genes responsive to desiccation in both groups, a total of 457 contigs were regulated; 317 were induced while 150 were repressed (Figure 4B). A total of 2,037 and 392 transcripts exhibited differential expression solely in group L and P2, respectively. To assess the correlation

of the replicates of each sample, a principal component analysis was performed and sample correlations were visualized as a heatmap (Supplementary Figure S6). We found that the individual replicates cluster closer according to the sample affiliation than randomly when looking at the first two principal components (Figure S6A). The same is true for the Pearson correlation matrix, where clustering of the associated replicates as well as the controls and treated samples was observed (Figure S6B).

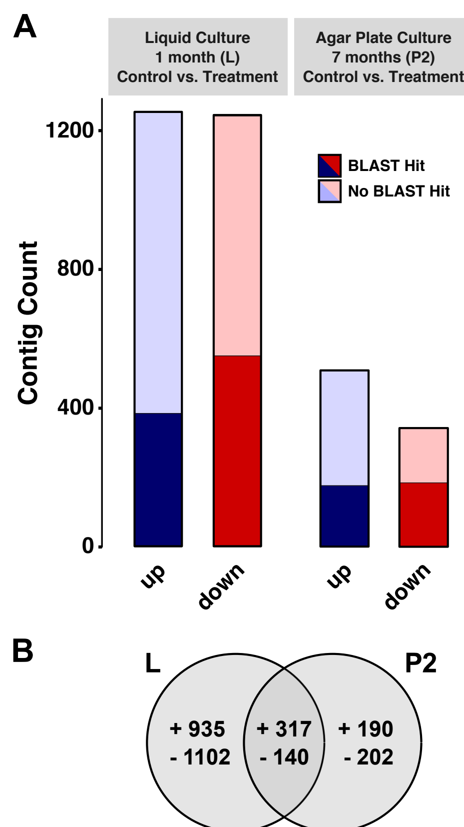

Figure 4: A) The total of up- (dark, light blue) and downregulated (dark, light red) contigs of *Z. circumcarinatum* under desiccation in group L and P2. Only contigs with a FDR of less than or equal to 0.001 were considered. All contigs were annotated against the Swiss-Prot database using BLASTx with an e-value of less than or equal to E-10. B) Overview of total up- and downregulated contigs of *Z. circumcarinatum* upon desiccation in group L and P2. Only contigs with a FDR of less than or equal to 0.001 were considered. The Venn diagram displays the number of regulated genes shared between both groups.

Table 2: Enriched KEGG pathways.

Group	Regulation	KEGG ID	Pathway
L	up	ko00500	Starch and sucrose metabolism
	down	ko00195	Photosynthesis
		ko00630	Glyoxylate and dicarboxylate metabolism

5.5.5 Gene set enrichment analyses

In order to identify desiccation related metabolic pathways, a KEGG pathway enrichment analysis based on KO annotations was performed for the up- and downregulated transcripts in both analysed groups. A total of 16,910 contigs could be annotated with KO terms. Significantly enriched pathways were found for the liquid culture dehydration treatment (L). Among upregulated transcripts, we found the "starch and sucrose metabolism" to be enriched while "photosynthesis" and "glyoxylate and dicarboxylate metabolism" were enriched in the downregulated contigs (Table 2).

The concept of GO categorization enables the comparison of homologous genes in different organisms (Ashburner et al., 2000). One or multiple GO terms, belonging to one of the three root categories, are assigned to each protein, similar parent categories are grouped and, subsequently, tested for enrichment in one sample compared to another (Ashburner et al., 2000; Young et al.,

Table 3: Outcome of GO enrichment analysis displaying solely root category distribution (CC = "Cellular component", MF = "Molecular function", BP = "Biological process"). Detailed information is included in supplemental Table S7.

Group	Regulation	CC	MF	BP
L	up	4	16	60
	down	26	31	50
P2	up	8	8	39
	down	0	2	4

2010). For example, the large subunit of RuBisCo was assigned the "biological process" photosynthesis, the "molecular function" ribulose-bisphosphate carboxylase activity and the "cellular component" chloroplast. In our study, GO terms could be assigned to 26,879 sequences forming the basis for a GO enrichment analysis. Enriched root categories for each group and directed regulation are summarized in Table 3 featuring more enriched GO terms for group L, with 80 and 107 up- and downregulated, respectively, than for group P2 with 55 and 6 terms up- and downregulated, respectively. Most enriched GO terms belonged to the root category "biological process" while "molecular function" and "cellular component" were represented to a lesser extent. In Figure 5, a network of enriched non-redundant GO terms in up- and downregulated gene sets is displayed with major categories highlighted in light grey. Both "photosynthesis" and "stress response" were enriched in up- and downregulated contigs while "carbohydrate metabolism", "lipid metabolism" and "transport" appeared upregulated and "signaling" downregulated. Lists of all enriched GO terms are included in the supplemental Table S7.

5.5.6 Individual analysis of desiccation responsive genes

Based on gene set enrichment analyses, we studied individual differentially expressed transcripts responsive to the applied desiccation treatment (Supplemental Table S5). Our main focus lies on photosynthesis, carbohydrate and lipid metabolism, transporter proteins and signaling as well as stress protection. Selected genes, exhibiting differential expression, and the detected fold changes (all given in $\log_2$ hereinafter) are displayed in Table 4.

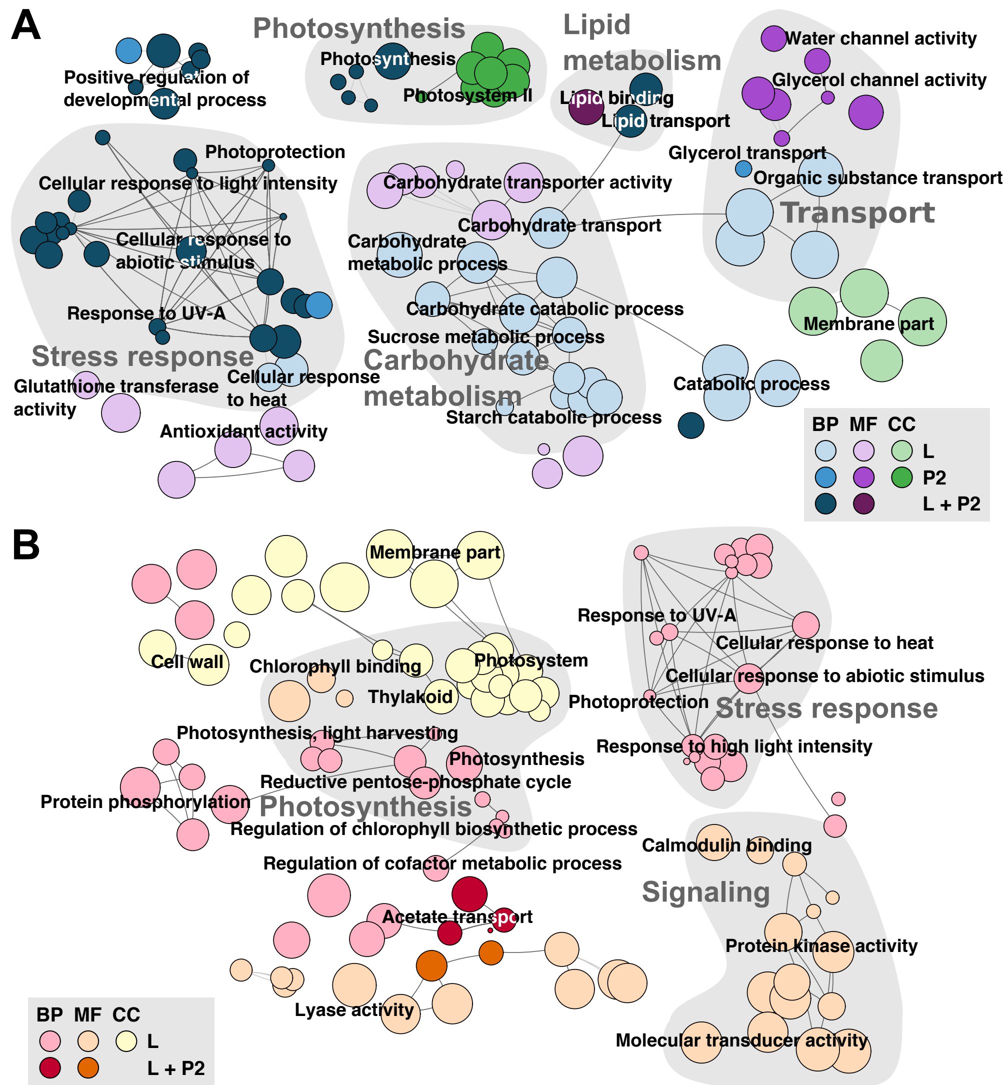

Figure 5: GO Network displaying all enriched categories in both groups L and P2 as well as A) up- and B) downregulation. The root categories are "Biological Process" (BP; blue or red), "Molecular Function" (MF; violet or orange) and "Cellular Component" (CC; green or yellow). Edges depict shared terms. Highlighted in grey are selected groups such as photosynthesis, lipid metabolism, transport, carbohydrate metabolism, stress response and signaling.

Table 4: Selection of contigs showing differential expression in response to desiccation stress (The complete list can be found in Table S5). Contigs are divided into the following groups: Photosynthesis and photorespiration, carbohydrate metabolism, lipid metabolism, transporter proteins, signaling, stress protection. Selected contigs are displayed with ID, annotation, e-value and fold change (log_2 transformed) for group L and P2.

Contig ID	Annotation	E-value	L	P2
	Photosynthesis and photorespiration			
TR14384|c0_g2 _i1	Photosystem I subunit II	2.18E-85	-3.2	-
TR18990|c0_g9_i1	Photosystem I subunit IV	5.94E-22	-2.7	-4.7
TR1369|c1_g1_i1	Photosystem I subunit III	8.25E-76	-2.5	-
TR59163|c0_g5_i2	Photosystem I subunit V	1.36E-28	-3.0	-
TR21504|c0_g2_i1	Photosystem I subunit VI	8.11E-40	-2.3	-
TR16905|c0_g2_i1	Photosystem I subunit X	1.42E-34	-2.3	-
TR33976|c0_g1_i1	Photosystem II oxygen-evolving enhancer protein 1	3.57E-135	-3.0	-
TR48275|c1_g1_i1	Photosystem II oxygen-evolving enhancer protein 3	3.9E-49	-3.4	-
TR20185|c2_g1_i1	Photosystem II 22kDa protein (PsbS)	1.08E-83	-4.6	-4.1
TR24382|c0_g1_i1	Photosystem II protein (PsbY)	4.85E-13	-3.4	-
TR71565|c0_g1_i1	Photosystem II protein (Psb27)	2.46E-36	-2.5	-
TR64030|c0_g1_i1	Light-harvesting chlorophyll-protein complex I subunit A4	4.67E-116	-2.9	-
TR37377|c0_g1_i2	Photosystem I light harvesting complex protein 5	2E-93	-3.0	-

Table 4 (Continued).

Contig ID	Annotation	E-value	L	P2
TR62754|c5_g48_i1	Photosystem II light harvesting complex protein 2.2	5.38E-82	-6.2	-
TR12320|c6_g1_i1	Light-harvesting chlorophyll B-binding protein 3	1.83E-96	-3.5	-
TR37376|c0_g1_i2	Light harvesting complex photosystem II	5.2E-121	-3.8	-3.9
TR25593|c4_g1_i1	Light harvesting complex of photosystem II 5	1.57E-117	-2.7	-
TR1329|c0_g2_i1	Light harvesting complex photosystem II subunit 6	3.79E-92	-2.8	-
TR75181|c0_g1_i1	ATPase delta chain	6.28E-52	-2.3	-
TR3752|c0_g5_i1	ATPase subunit b'	2E-37	-2.7	-4.3
TR4441|c0_g1_i2	Plastocyanin	-3.75E-37	-2.5	-
TR31328|c0_g1_i1	Chlorophyllide a oxygenase	0	-2.1	-
TR68443|c0_g1_i3	Magnesium chelatase subunit	0	-4.5	-4.4
TR8034|c11_g29_i1	Early light-induced protein, chloroplastic (ELI)	1.83E-15	-	12.2
TR58021|c0_g5_i1	Early light-induced protein 1, chloroplastic (ELIP1)	1.43E-21	3.5	5.2
TR4192|c1_g15_i1	High molecular mass early light-inducible protein, chloroplastic (HV58)	1.86E-21	5.2	4.3
TR4440|c0_g2_i1	Low molecular mass early light-inducible protein, chloroplastic (HV60)	4.1E-16	-3.4	-

Table 4 (Continued).

Contig ID	Annotation	E-value	L	P2
TR73556|c0_g9_i2	(S)-2-Hydroxy-acid oxidase	0	-2.5	-3.5
TR29652|c0_g1_i1	Serine-glyoxylate transaminase	0	-3.4	-3.6
TR68913|c1_g1_i2	Glycine dehydrogenase	0	-2.6	-4.2
TR54933|c0_g2_i1	Glutamate-glyoxylate aminotransferase	0	-3.0	-
TR48225|c1_g1_i1	Glycerate dehydrogenase	0	-3.5	-
Carbohydrate metabolism				
TR23256|c0_g1_i1	Glycogen phosphorylase	0	8.3	-
TR75230|c1_g1_i1	alpha-Amylase	1.95E-175	4.3	-
TR70181|c1_g1_i2	beta-Amylase	0	2.7	-
TR24697|c0_g3_i4	Isoamylase	6.66E-40	2.6	-
TR25586|c0_g1_i1	4-alpha-Glucanotransferase	1.47E-45	2.2	-
TR45454|c0_g2_i1	Sucrose-phosphatase	6.91E-108	3.6	-
TR61067|c0_g1_i2	Sucrose synthase	0	3.0	-
Lipid metabolism				
TR39622|c0_g1_i1	Lysophospholipid acyltransferase	4.12E-110	2.0	-
TR53082|c0_g1_i1	Diacylglycerol kinase	2.68E-160	2.0	-
TR16611|c0_g1_i2	alpha-Galactosidase	1.91E-154	2.3	3.5
TR42973|c1_g1_i1	Sulfoquinovosyltransferase	4.73E-12	3.7	-
TR31318|c0_g1_i1	Phospholipase D1/2	0	3.7	-
TR28615|c2_g10_i1	Phosphoethanolamine N-methyltransferase	5.8E-102	2.9	3.2
TR41908|c1_g1_i4	Phosphatidylserine synthase 2	1.62E-177	-	3.8
TR13652|c0_g1_i13	2-Acylglycerol O-acyltransferase 1	2.54E-89	-	3.5

Table 4 (Continued).

Contig ID	Annotation	E-value	L	P2
	Transporter proteins			
TR43432|c0_g2_i2	Probable aquaporin TIP1-2	8.15E-17	13.3	13.5
TR43432|c0_g3_i1	Aquaporin TIP2-1	2.25E-32	2.9	2.7
TR61568|c0_g2_i1	Aquaporin TIP2-3	2.22E-33	4.1	-
TR34049|c0_g1_i2	Plastidic glucose transporter 2	2.02E-138	4.2	3.6
TR23238|c0_g1_i2	Sucrose transport protein 3	2.09E-147	2.7	-
TR31|c0_g1_i1	Glucose-6-phosphate/phosphate translocator 1	1.22E-164	2.1	-
TR40733|c1_g1_i1	Sugar transport protein 13	8.24E-177	5.8	5.5
TR41946|c0_g1_i8	Sugar-transport protein ERD6-like 16	3.33E-71	9.0	-
	Signaling			
TR52105|c7_g5_i3	Leucine-rich repeat receptor-like serine/threonine-protein kinase BAM2	2.56E-18	3.4	-
TR58701|c1_g2_i3	Leucine-rich repeat receptor-like serine/threonine-protein kinase FLS2	7.92E-65	-5.4	-6.7
TR34848|c0_g2_i1	Calcium-dependent protein kinase 17	1.25E-81	3.4	-
TR6882|c0_g1_i1	Calcium-dependent protein kinase 20	5.18E-76	-6.1	-
	Stress protection			
TR10757|c0_g3_i1	Chaperone protein ClpB1	7.43E-16	3.4	-

Table 4 (Continued).

Contig ID	Annotation	E-value	L	P2
TR35960|c0_g2_i2	Proteasome assembly chaperone 2	4.89E-28	11.2	-
TR41947|c0_g1_i3	Chaperone protein DnaJ	1.06E-10	3.9	-
TR75210|c0_g1_i2	Molecular chaperone Hsp31	1.57E-10	2.5	4.2
TR39621|c0_g1_i2	Glutathione S-transferase	4.85E-53	2.9	5.0
TR14048|c0_g1_i1	Peroxisomal catalase	0	3.7	6.7
TR58823|c0_g2_i1	Peroxiredoxin	6.92E-54	2.3	3.4
TR57779|c0_g1_i1	Peptide methionine sulfoxide reductase	4.35E-79	2.5	3.0
TR35953|c0_g2_i4	(Chloroquine-resistance transporter)-like transporter 3	7.29E-81	9.0	-
TR50557|c0_g2_i12	Nijmegen breakage syndrome 1 protein	3.14E-13	9.5	9.7
TR35997|c1_g1_i8	DNA-damage-repair/toleration protein	1.42E-42	3.0	-
TR49464|c0_g1_i1	Late embryogenesis abundant protein 4 (LEA4; AT3G53040)	8 .1E-19	5.1	3.6
TR39628|c0_g2_i1	Late embryogenesis abundant protein 4 (LEA4; AT2G18340)	4.6E-24	5.0	-
TR69744|c2_g23_i1	Late embryogenesis abundant protein 4 (LEA4; AT4G36600)	1.1E-14	5.3	3.4
TR60896|c0_g1_i1	Late embryogenesis abundant protein 5 (LEA5; AT2G40170)	6.5E-27	-	9.8

Photosynthesis and photorespiration

Transcriptomic analysis of photosynthetic processes revealed a strong downregulation of components of both PSs in group L. A repression of transcripts encoding parts of PS I and PS II with fold changes of -2.3 to -3.4 was observed. The strongest downregulated transcript was the PS II 22kDa protein (PsbS) with a fold change of -4.6. In group P2, only PS I subunit IV and PS II 22kDa protein exhibited fold changes of -4.7 and -4.1, respectively. Furthermore, several contigs coding for light-harvesting complexes as well as some proton transporting ATPase subunits and plastocyanin, which is part of the electron transport chain, were downregulated in group L. Detected fold changes lay in the range of -2.3 to -3.8 while the PS II light harvesting complex protein 2.2 displayed a rather strong repression of -6.2. In addition, the putative chlorophyllide a oxygenase and the magnesium chelatase subunit, which are both part of the chlorophyll metabolism, were repressed 2.1- and 4.5-fold, respectively. In contrast, group P2 exhibited a weaker downregulation. Upon desiccation, the light harvesting complex PS II, ATPase subunit b' and magnesium chelatase subunit were repressed 3.9-, 4.3- and 4.4-fold, respectively. Rather striking is the plethora of ELIPs that showed differential expression during dehydration. Desiccation of the liquid culture caused the transcription level of ELIP1 and the chloroplastic high molecular mass ELIP to increase while the chloroplastic low molecular mass ELIP was repressed. The solid culture of *Z. circumcarinatum* (P2) showed an upregulation of the chloroplastic ELIP (ELI), ELIP1 and the chloroplastic high molecular mass ELIP. The chloroplastic high molecular mass ELIP exhibits the strongest induction with a fold change of 12.2.

Desiccation also caused a repression of enzymes involved in photorespiration in both comparisons. The transcript pools of the (S)-2-hydroxy-acid oxidase, serine-glyoxylate transaminase and glycine dehydrogenase showed a decline for L and P2 while group L also exhibits a downregulation of glutamate-glyoxylate aminotransferase and glycerate dehydrogenase. The detected fold changes ranged from -2.5 to -4.2.

Carbohydrate metabolism

Starch degradation and sucrose formation were induced during dehydration in group L while group P2 shows only minor effects. A variety of starch consuming enzymes were upregulated such as glycogen phosphorylase, alpha- and beta-amylase, isoamylase, 4-alpha-glucanotransferase as well as the sucrose synthesizing enzymes sucrose-phosphatase and sucrose-synthase. The strongest induction was observed for glycogen phosphorylase with an 8.3-fold upregulation while the others exhibit fold changes between 2.2 and 4.3.

Lipid metabolism

Investigating both the glycerolipid and glycerophospholipid metabolism, an enhanced gene expression of certain enzymes was found. Group L showed an increased transcript level for the lysophospholipid acyltransferase, diacylglycerol kinase, alpha-galactosidase, sulfoquinovosyltransferase, phosphoethanolamine N-methyltransferase and phospholipase D1/2. The response of group P2 was less pronounced with an upregulation of alpha-galactosidase, phosphoethanolamine N-methyltransferase, phosphatidylserine synthase 2 and 2-acylglycerol O-acyltransferase 1. Transcript levels were increased between 2- and 3.8-fold.

Transporter and signaling

The upregulation of the contig TR43432|c0_g2_i2, which was annotated to be an aquaporin (AQP) of type TIP (tonoplast intrinsic protein), was most pronounced considering all differentially expressed transcripts. Additionally, TIP2-1 in both groups L and P2 was enhanced and TIP2-3 only in group L. Moreover, the expression of various putative sugar transporters (plastidic glucose transporter 2, sucrose transport protein 3, glucose-6-phosphate/phosphate translocator 1, sugar transport protein 13, sugar-transport protein ERD6-like 16) was strongly induced in the liquid sample and to a lesser extent also in group P2. The sugar-transport protein ERD6-like 16 exhibited a fold-change

of 9 while the induction of other sugar transport proteins lay in the range of 2.1- to 5.8-fold. A complex regulation of signaling pathways in group L was identified. Mainly transcripts, which were showing similarities to the family of serine/threonine-protein kinases but also other kinases and transcription factors, were differentially expressed. Leucine-rich repeat receptor and receptor-like serine/threonine-protein kinases and calcium-dependent protein kinases (CPK), such as the leucine-rich repeat receptor-like serine/threonine-protein kinases BAM2 and FLS2 as well as the calcium-dependent protein kinases 17 and 20, were the most prominent transcripts. Overall, more signaling related contigs were repressed than upregulated.

Stress response

An upregulation of other stress protection associated genes was discovered. Chaperone and Hsp encoding genes, such as the chaperone protein ClpB1, proteasome assembly chaperone 2, the chaperone protein DnaJ and the molecular chaperone Hsp31, appeared to be highly transcribed during desiccation, primarily in group L, while genes involved in ROS scavenging, such as the glutathione-S-transferase, peroxisomal catalase, peroxiredoxin, peptide methionine sulfoxide reductase, and (chloroquine-resistance transporter)-like transporter 3, were induced in both groups. For the desiccated liquid culture, the proteasome assembly chaperone 2 exhibits the largest change of 11.2-fold. Transcripts similar to the Nijmegen breakage syndrome 1 protein and DNA-damage-repair/toleration protein, both involved DNA repair, also showed an enhanced expression. Astonishingly, the Nijmegen breakage syndrome 1 protein was upregulated 9.5- and 9.7-fold in group L and P2, respectively. Furthermore, a large number of LEA proteins showed high induction, consisting only of LEA4 proteins in group L while group P2 showed the upregulation of both LEA4 and LEA5 proteins. The LEA5 gene exhibited an 9.8-fold increase in expression. The BLAST analysis of several Chlorophyta and Streptophyta (Figure 6) reveals LEA group distribution among Viridiplantae species. We observed that all studied streptophyte genomes contained at least LEA proteins

from group LEA2, LEA4 and LEA5 while chlorophytes mostly only possessed LEA2 proteins with some exceptions (in our example, *D. salina*). Overall, photosynthesis appears to be repressed in group L while several ELIPs are upregulated in both groups. Desiccation stress also causes an upregulation of transcripts involved in the lipid metabolism and transport in L and P2. Furthermore, group L exhibits an induction of the carbohydrate metabolism. Both groups increase the transcript pool of ROS scavenging and chaperone proteins.

5.6 Discussion

5.6.1 Photosynthesis and photorespiration

Dehydration has an extremely negative influence on photosynthesis as water is crucial for structural integrity and functionality of the algal cell and also acts as an electron donor in the electron transport chain (Fernández-Marín et al., 2013). Herburger et al. (2015) observed a complete loss of photosynthetic activity in *Z. circumcarinatum* during prolonged desiccation. However, older cultures appeared to be more tolerant towards dehydration as photosynthesis was maintained longer compared to younger cells (Herburger et al., 2015). These findings are in agreement with our observations for *Z. circumcarinatum* cultivated on agar (Figure 2B-D). Culture P1 abandoned photosynthetic activity first, then the effective quantum yield of P2 dropped while P3 resisted the longest. According to Herburger et al. (2015), this increased tolerance is likely caused by the formation of pre-akinetes which can be regarded as a stress tolerant resting stage (Pichrtová et al., 2016b). Typical features of pre-akinetes are hardened cell walls, accumulation of starch and lipid bodies in the cytoplasm as well as reduced growth and physiological activity (Herburger et al., 2015; Pichrtová et al., 2016b). Compared to the agar cultures, filaments grown in liquid medium appeared to maintain photosynthesis as long as P3. However, this effect is clearly linked to the clinging water which could not completely

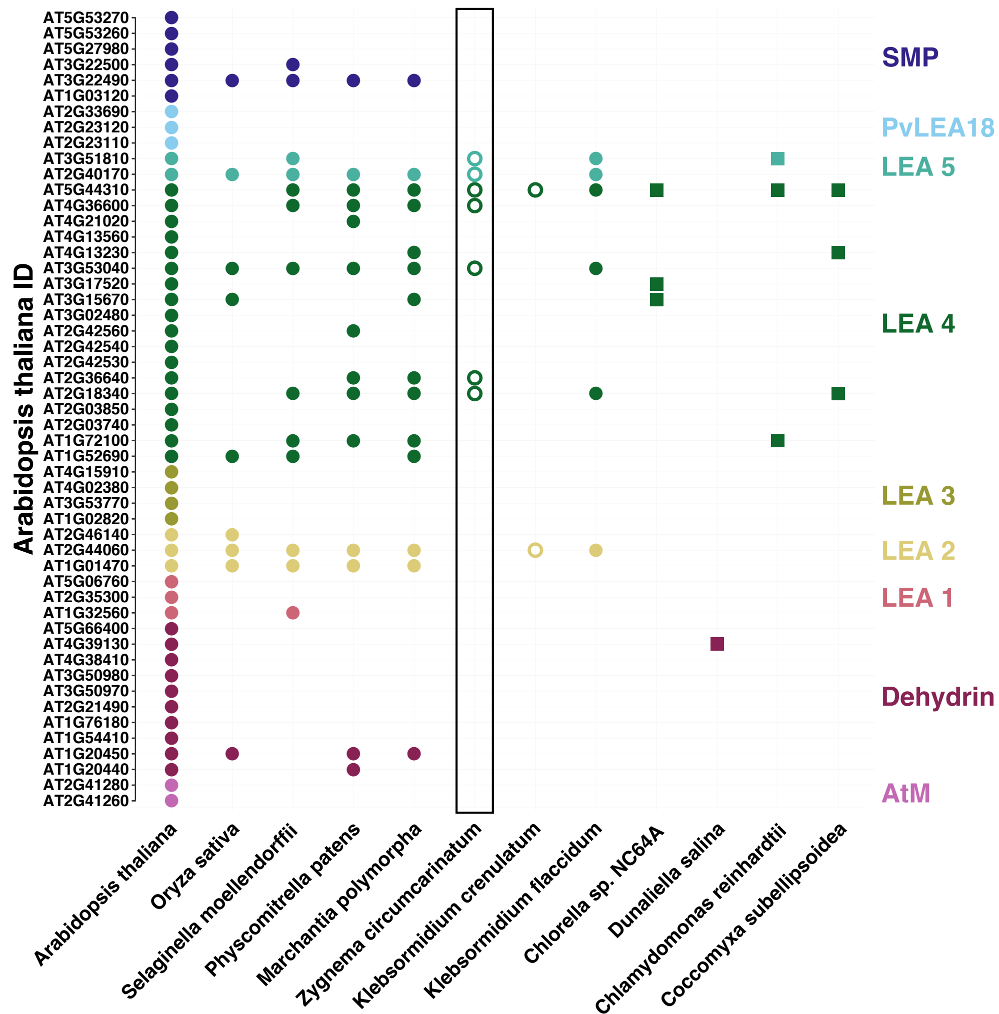

Figure 6: LEA proteins found in *A. thaliana* and described by Hundertmark and Hincha (2008). Annotations were retrieved from Phytozome v11.0 for *A. thaliana*, *C. reinhardtii*, *C. subellipsoidea*, *D. salina*, *M. polymorpha*, *P. patens* and *S. moellendorffii*. The same is true for the transcripts of *O. sativa* which were annotated using diamond BLASTx with E-9. The transcripts of *Chlorella* sp. NC64A, *K. crenulatum* and *K. flaccidum* were downloaded from the JGI Genome Portal, Holzinger et al. (2014) and the *Klebsormidium flaccidum* genome project, respectively, and processed accordingly. Points indicate streptophytes while squares stand for chlorophytes. Solid and hollow symbols represent sequences derived from genomes or only transcriptomes, respectively.

removed by blotting. During the desiccation treatment, the excess water had to evaporate before the algal biomass could be desiccated effectively.

The dehydration of *Z. circumcarinatum*, cultivated in liquid medium for one month, led to a strong repression of transcripts encoding components of PS I and II as well as light-harvesting proteins indicating photoinhibition. In contrast, *K. crenulatum* (Holzinger et al., 2014) showed an upregulation of genes related to photosynthesis in response to desiccation. Holzinger et al. (2014) suggest that this mechanism serves as a preparation to rapidly resume photosynthesis upon rehydration. Similar results were obtained for *Trebouxia gelatinosa* (Carniel et al., 2016). However, Carniel et al. (2016) compared a couple of different transcriptomic studies on desiccation of Viriplantae detecting a repression of photosynthetic transcripts in *Syntrichya ruralis* (desiccation tolerant moss), *C. plantagineum* (resurrection plant), *Haberlea rhodopensis* (resurrection plant) and *Xerophyta humilis* (resurrection plant). Moreover, the resurrection plant *Myrothamnus flabellifolia* also exhibited a downregulation of photosynthesis genes when desiccated (Ma et al., 2015). Surprisingly, mainly genes, encoding parts of PS I and II, the electron transport chain and the ATP synthase, were repressed in *Myrothamnus flabellifolia* (Ma et al., 2015). This is also true for *Z. circumcarinatum*, as demonstrated in the present study. Ma et al. (2015) argue that thereby excitation energy and, thus, ROS production likely are reduced. Moreover, the level of transcripts, involved in chlorophyll biosynthesis, decreased. Similar findings were obtained for desiccated *Vitis vinifera* (grapevine) leaves (Salman et al., 2016) and salt stressed *Oryza sativa* (rice) seedlings (Turan and Tripathy, 2015). The observed impairment of the chlorophyll biosynthesis is probably occurring to avoid ROS formation (Farrant et al., 2003). As an additional protection, the expression of ELIPs was enhanced in group L and P2. ELIPs are photoprotectants and belong to the chlorophyll a/b-binding (CAB) superfamily, respond to abiotic stress, mainly to high light and UVR, and are located in the thylakoid membrane (Hayami et al., 2015; Hutin et al., 2003; Norén et al., 2003). Similar reactions were observed for other green algae, for example, *Chlamydomonas reinhardtii* and

Dunaliella bardawil, both of which increased the ELIP transcript pool in response to high light stress which is also common for various higher plants such as *Arabidopsis* (Lers et al., 1991; Teramoto et al., 2004). Less studies have been dedicated to the relationship of cold stress and ELIP expression in algae. Król et al. (1997) reported an induction of ELIPs in *Dunaliella salina* when exposed to low temperatures. *Spirogyra varians* (Zygnematales) also exhibits an accumulation of ELIP-like transcripts when cultivated at 4°C (Han and Kim, 2013). Nevertheless, the accumulation of ELIPs is also commonly associated with desiccation stress (Dinakar and Bartels, 2013; Ma et al., 2015; Zeng et al., 2002). These proteins protect the thylakoid membranes against photooxidative damage by scavenging free chlorophyll molecules and act as sinks for excitation energy (Heddad et al., 2012; Zeng et al., 2002). Paradoxically, some contigs of *Zygnema*, exhibiting similarities to ELIPs, are negatively regulated. However, Holzinger et al. (2014) also found a complex regulation of ELIP-related transcripts for *K. crenulatum* hinting at a multigenetic family with several ELIPs being responsive to desiccation stress (Hutin et al., 2003; Marraccini et al., 2012; Zeng et al., 2002).

A decrease in photorespiratory transcripts upon desiccation was detected of both groups of *Z. circumcarinatum* suggesting a repression of photorespiration. In contrast, photorespiration can function as a protection of the photosynthetic apparatus against photoinhibition (Wingler et al., 1999). Especially, during drought stress when carbon dioxide fixation and, thus, the consumption of electrons is reduced (Wingler et al., 1999). However, protection of the PSs by photorespiration is not essential during desiccation (Wingler et al., 2000) Furthermore, dehydration is reported to reduce photorespiratory activity in plants which may be caused by the decreased photosynthetic activity (Levitt, 1980).

5.6.2 Carbohydrate metabolism

A common protective mechanism against water stress is the accumulation of low-molecular-weight osmolytes which can be sugars, polyols and proteins (Di-

nakar and Bartels, 2013; Fernández-Marín et al., 2013; Hincha et al., 1996; Holzinger and Pichrtová, 2016; Ma et al., 2015). By increasing the amount of osmoprotectants in the cell, a negative osmotic potential is achieved, membranes are stabilized and protein protection is enhanced (Bisson and Kirst, 1995). Nagao et al. (2008) found that *K. flaccidum* accumulates the osmolyte sucrose during cold acclimation which contributes to a higher freezing tolerance. Moreover, the alga *Chlorella vulgaris* exhibits an increase in sucrose and raffinose content in response to cold shock treatment (Salerno and Pontis, 1989). However, sucrose is also typically formed upon desiccation stress in plants and algae (Cruz de Carvalho et al., 2014; Dinakar and Bartels, 2013; Holzinger and Pichrtová, 2016; Ramanjulu and Bartels, 2002; Sadowsky et al., 2016). Sadowsky et al. (2016) reported increased sucrose levels in an Antarctic *Trebouxia* strain to counteract desiccation. Our data indicate a metabolic shift towards sucrose as starch degrading as well as sucrose biosynthetic enzymes were upregulated in dehydrated filaments. The KEGG enrichment analysis also clearly indicated an enhancement of the "starch and sucrose metabolism". A similar strategy is pursued by *K. crenulatum* inducing transcripts encoding sucrose synthase and the sucrose phosphate synthase (Holzinger et al., 2014). The authors suggest that raffinose family oligosaccharides also function as osmoprotectants because several enzymes, belonging to the galactinol/raffinose metabolism, exhibited a higher expression in desiccated cells (Holzinger et al., 2014). *Z. circumcarinatum* did not show this expression pattern in response to water stress. However, the sucrose phosphate synthase of *Z. cirumcarinatum* contains a conserved phosphorylation site (results not shown) which is typically found in angiosperms and known to become modified upon osmotic stress (Winter and Huber, 2000). Hence, the sucrose metabolism of *Z. circumcarinatum* is most likely not only regulated by transcription but also posttranslational modifications.

Callose is an important polysaccharide found to be involved in response to different abiotic stress factors, e.g. in drought stress in plants, such as *Gossypium hirsutum* L. (cotton; McNairn, 1972), but also algae such as *K.*

crenulatum (Herburger and Holzinger, 2015). Complementary, the desiccation transcriptome of *Klebsormidium* revealed an upregulation of the callose synthase complex confirming the significance of this carbohydrate during dehydration events (Holzinger et al., 2014). Albeit the occurrence of this enzyme in the *Zygnema* transcriptome no differential expression was detected. These results are in agreement with Herburger and Holzinger (2015) who reported a stable callose content throughout desiccation for 2.5 h. As the callose synthase is located in the plasma membrane and the protoplast is retracting from the cell wall upon dehydration callose incorporation is prevented (Herburger and Holzinger, 2015).

5.6.3 Lipid metabolism and membranes

The fact, that low temperatures, dehydration etc. initially target biomembranes, highlights the importance of membrane modification upon water stress to preserve integrity and fluidity (Dinakar and Bartels, 2013; Holzinger et al., 2014; Perlikowski et al., 2016; Valledor et al., 2013). For example, decreased temperatures cause membrane modifications in the green alga *C. reinhardtii* (Valledor et al., 2013; Wang et al., 2017). Gasulla et al. (2013) observed similar tendencies in *C. plantagineum* induced by desiccation treatment. Monogalactosyldiacylglycerol was removed from the thylakoid membranes and either transformed to digalactosyldiacylglycerol or hydrolyzed to form diacylglycerol (Gasulla et al., 2013). In contrast, our results indicate the conversion from digalactosyldiacylglycerol to monogalactosyldiacylglycerol to be amplified as the putative alpha-galactosidase is upregulated in group L. However, parts of the glycero- and glycerophospholipid metabolism are enhanced during desiccation suggesting other membrane modifications. Similarly, the lichen phycobiont *Asterochloris erici* exhibited elevated levels of phosphatidic acid upon desiccation indicating that phospholipase D is involved in stress protection mechanisms (Gasulla et al., 2016). *Z. circumcarinatum* induced phospholipase D1/2 during dehydration stress confirming that phospholipase D1/2 is part of the stress response.

5.6.4 Transporter proteins and signaling

Major intrinsic proteins (MIPs), or AQPs, establish channels for passive transportation of small uncharged substances, such as water or glycerol, across the membrane (Anderberg et al., 2011, 2012; Barkla et al., 1999). AQPs in embryophytes comprise seven groups: GlpF-like intrinsic proteins (GIPs), hybrid intrinsic proteins (HIPs), X intrinsic proteins (XIPs), small basic intrinsic proteins (SIPs), nodulin-26 like intrinsic proteins (NIPs), plasma membrane intrinsic proteins (PIPs) and TIPs (Danielson and Johanson, 2008). Anderberg et al. (2011) studied different chlorophytes and found AQPs of these groups only in Trebouxiophyceae (PIP, GIP). *Z. circumcarinatum*, a charophyte alga, is more closely related to land plants and expresses TIPs, NIPs and SIPs while only TIPs appeared to play an important role in desiccation. Both groups, L and P2, showed a strong induction of TIPs during water stress which was also observed for other AQPs in the plants *Arabidopsis thaliana* and *C. plantagineum* (Dinakar and Bartels, 2013; Ramanjulu and Bartels, 2002) as well as the alga *T. gelatinosa* (Carniel et al., 2016). Carniel et al. (2016) argue that AQPs are protection against damage during rehydration by increasing the permeability of biomembranes to water.

As mentioned above, the formation of sugars and other osmolytes is increased during water stress, however, these molecules also need to be distributed within the cell (Jarzyniak and Jasinski, 2014). Thus, desiccation tolerance is dependent on sugar transportation within the cell. Liu et al. (2016) reported an increased drought tolerance in *A. thaliana* associated with the expression of the hexose facilitator AtSWEET4. Similar results, indicating desiccation induced expression of sugar transporters, were obtained analyzing *Caragana korshinskii* (leguminous shrub; Liu et al., 2016) and *Saccharum* spp. (sugar cane; Zhang et al., 2016).

The signaling in plants during desiccation generally involves several hormones such as abscisic acid, cytokinin and ethylene (Campo et al., 2014; Holzinger and Becker, 2015; van de Poel et al., 2016; Zhou et al., 2014) as well as calcium-dependent and serine/threonine-protein kinases (Campo et al.,

2014; Ramanjulu and Bartels, 2002). Especially abscisic acid plays a major role in the desiccation stress response linking host and plastid signaling which is considered a key step in the land plant evolution (de Vries et al., 2016). In contrast to *K. crenulatum* (Holzinger and Becker, 2015), the up-regulation of hormone specific signaling transcripts was not evident in *Zygnema*. However, we found an intricate expression pattern of threonine/serine- and calcium-dependent protein kinases in response to dehydration in *Z. circumcarinatum*. Other studies obtained desiccation induced signaling networks with similar complexity, e.g. for *K. crenulatum* and *V. vinifera* (Holzinger et al., 2014; Salman et al., 2016).

5.6.5 Stress protection

The formation of ROS, occurring during different abiotic stress conditions, is extremely harmful making ROS scavenging crucial for cell survival (e.g. Cruz de Carvalho, 2008; Cruz de Carvalho et al., 2012; Dong et al., 2016; Han et al., 2012; Heinrich et al., 2016; Kranner et al., 2005). For example, high light stress triggers the expression of ROS scavenging enzymes in the green alga *C. reinhardtii* and the marine diatom *Thalasiosira pseudonana* (Dong et al., 2016; Erickson et al., 2015). Similarly, the dinoflagellate *Symbiodinium* involves a number of ROS defense proteins and antioxidants when exposed to heat stress (Gierz et al., 2017). The same holds true for cold stress which induces the accumulation of antioxidants in *S. varians* to counteract ROS formation (Han et al., 2012). Our data suggests ROS generation upon desiccation as *Z. circumcarinatum* expresses a multitude of enzymes related to ROS protection: the glutathione-S-transferase conjugates glutathione (GSH) to hydrophobic molecules (Rezaei et al., 2013), the peroxisomal catalase acts directly on ROS (Gorrini et al., 2013), peroxiredoxin catalyzes the reduction of peroxides (Dayer et al., 2008), the peptide methionine sulfoxide reductase reduces methionine sulfoxide back to methionine (Weissbach et al., 2002) and (chloroquine-resistance transporter)-like transporter 3 transports GSH (Noctor et al., 2011). Interestingly, the induction of the glutathione-S-

transferase, peroxisomal catalase, peroxiredoxin and peptide methionine sulfoxide reductase was higher in group P2 than L suggesting a more pronounced ROS stress response of filaments grown on agar plates than filaments from a liquid cultivation. An upregulation of peroxiredoxin and the peptide methionine sulfoxide reductase was also detected in *T. pseudonana* exposed to high light stress (Dong et al., 2016). Hence, both enzymes are certainly involved in a general ROS coping mechanism. Moreover, group L strongly increases the transcript pool of the (chloroquine-resistance transporter)-like transporter 3. These transporter proteins play a major role in GSH homeostasis in *A. thaliana* as they connect the plastid and the cytosolic thiol pool (Maughan et al., 2010). Thus, (chloroquine-resistance transporter)-like transporters are essential to counteract ROS stress (Maughan et al., 2010). Furthermore, DNA damage, which is also linked to ROS formation (Cruz de Carvalho, 2008; Heinrich et al., 2015), was addressed by a strong upregulation of repair enzymes in desiccation stressed *Z. circumcarinatum*. For example, the Nijmegen breakage syndrome 1 protein was highly induced with fold changes of 9.5 and 9.7 in group L and P2, respectively, suggesting a higher risk of DNA damages associated with desiccation (Akutsu et al., 2007; Cruz de Carvalho, 2008).

Abiotic stress generally leads to aggregation and conformational changes in protein which is lethal to the cell (Wang et al., 2004). In response, plants and algae express chaperones and Hsps which are assisting the refolding of proteins and protect them from aggregation (Al-Whaibi, 2011; Mitra et al., 2013; Schulz-Raffelt et al., 2007; van de Poel et al., 2016; Wang et al., 2004). For example, *C. reinhardtii* raises the transcription level of Hsp90A when heat shocked at 40°C (Schulz-Raffelt et al., 2007). Kobayashi et al. (2014) found an increased expression of small Hsps in the red alga *Cyanidioschyzon merolae* and *C. reinhardtii* in response to heat stress. Heinrich et al. (2012b) and Dong et al. (2016) reported that the brown macroalga *Saccharina latissima* and the diatom *T. pseudonana*, respectively, induce various chaperones and Hsps when treated with high light intensities. Similarily, cold stress triggers the expression of two chaperones in the Antarctic diatom *Chaetoceros neogracile* (Park

et al., 2007). The same holds true for desiccation which causes an increased expression of certain chaperones and Hsps in *Selaginella lepidophylla* (lycophyte, Carniel et al., 2016), *Physcomitrella patens* (moss, Wang et al., 2009), *Pyropia orbicularis* (red alga, López-Cristoffanini et al., 2015) and *Asterochloris erici* (green alga, Gasulla et al., 2013). *Z. circumcarinatum* showed an induction of chaperones and Hsps as well, which is probably a preparation for protein refolding upon rehydration (Carniel et al., 2016). Clp proteins are chaperones capable of refolding protein complexes and are induced in response to various stress factors (Lee et al., 2006). In *O. sativa*, a number of Clp proteins showed an increased transcription during drought stress (Hu et al., 2009) while the cytosolic chaperone ClpB1 of *A. thaliana* is involved in chloroplast development and acclimation to increased temperatures (Lee et al., 2006). However, *Z. circumcarinatum* probably raised the transcription level of the chaperone ClpB1 in response to desiccation stress to enable remodeling of aggregated and misfolded proteins. Another important group of stress induced proteins are the co-chaperones DnaJ (Wang et al., 2014). Wang et al. (2014) reported an increase in drought tolerance in transgenic tobacco resulting from an overexpression of the chloroplast-targeted chaperone DnaJ. Furthermore, an induction of DnaJ in *Saccharum* in response to desiccation was demonstrated by de Andrade et al. (2015). The applied desiccation treatment caused *Z. circumcarinatum* to induce the co-chaperone as part of its stress response. Interestingly, DnaJ expression is also triggered by high light exposure in *S. latissimi* and *T. pseudonana* (Dong et al., 2016; Heinrich et al., 2012b). Furthermore, our data shows an upregulation of the transcription factor Hsp31 upon water stress which is in agreement with the findings in *O. sativa* by Wang et al. (2011). Another important component of the protein quality control are proteasomes which selectively eliminate dysfunctional proteins (Hanssum et al., 2014). In response to changing conditions and environmental stress, the demand of proteasomes increases immensely forcing the cell to increase the proteasome pool (Hanssum et al., 2014). The assembly process is promoted by proteasome assembly chaperones such as proteasome assembly chaperone

2 (Le Tallec et al., 2007). The desiccated filaments of *Z. circumcarinatum* will most likely accumulate a number of misfolded proteins which need to be degraded. To be able to assemble proteasomes, the alga requires adequate chaperones. Additionally, a number of putative LEA proteins are upregulated. LEA proteins are proposed as water stress specific chaperones (Goyal et al., 2005; Hatanaka et al., 2014; Shinde et al., 2012) which can be grouped depending on their sequence motifs/patterns (Hundertmark and Hincha, 2008). The drought induced expression of LEA proteins has been already reported for several species, e.g. *M. flabellifolia* (Ma et al., 2015), *P. patens* (Shinde et al., 2012) and *K. crenulatum* (Holzinger et al., 2014), confirming our findings. An interesting aspect is the distribution of these chaperones across Viridiplantae which reveals evolutionary relationships of the different subfamilies. Based on the classification by Hundertmark and Hincha (2008), the *Z. circumcarinatum* transcriptome covered sequences belonging to LEA4 and LEA5 (Figure 6). LEA4 is likely the group, which emerged first, as it shows the biggest diversity of all and is present in all investigated algae and plants except *D. salina.* LEA5 was probably also emerging early because it was found in all streptophyta and *C. reinhardtii.* All analyzed streptophyta genomes featured LEA2 proteins but this group is missing in *Z. circumcarinatum.* The absence of LEA2 is likely linked to the missing induction in response to any condition tested. Finally, the LEA protein group SMP probably evolved during the development of mosses from streptophytic green algae as all embryophyta possess these proteins.

5.6.6 Conclusion

In this study, the molecular response of the conjugating green alga *Z. circumcarinatum* to desiccation stress was investigated using differential gene expression analysis. To assess the effect of hardening, the impact of dehydration on a young liquid culture and a seven months old agar culture of *Zygnema* was analyzed. Our results found a 3-fold stronger transcriptional response of filaments grown in liquid medium compared to older filaments cultivated on agar. These findings are a clear indication of a pre-acclimation to low water

availability of the algal culture grown on agar for seven months. In agreement with earlier observations, photosynthesis related genes are highly repressed in group L while the response of group P2 is much less pronounced. Furthermore, water withdrawal causes membrane modifications and the expression of several transporters such as aquaporins and carbohydrate transporter proteins. Desiccation also induces the accumulation of sucrose, a common osmolyte, to counteract the rapid loss of water. Finally, a number of stress related molecules are produced, e.g. ELIPs, chaperones such as LEA proteins, proteins involved in ROS scavenging and DNA repair proteins. Overall, we conclude that culture age and conditions highly influence the physiological state of the algal filaments and the acclimation to water stress. However, it is difficult to mimic natural conditions in a laboratory environment as natural habitats are influenced by stochastic parameters such as weather and soil quality. Thus, future experiments shall include the transcriptomic analysis of field samples collected in different seasons with different water availability.

5.7 Material and methods

5.7.1 Algal strain and cultivation

Zygnema sp. (SAG 2419), previously isolated from a sandy shore near the river Saalach in Salzburg, Austria, was used for the experiments. The alga was either cultivated for one month in liquid Waris-H medium (McFadden and Melkonian, 1986) or on 1.5% agar plates, containing BBM medium, as previously described by Herburger et al. (2015). The algal strain clusters in phylogenetic analysis close to *Zygnema circumcarinatum* (Herburger and Holzinger, 2015), thus, we use this species name in the present study. However, an unambiguous morphological determination of the species was not possible as zygospores were not detected.

5.7.2 Desiccation experiment

Four different cultures of *Z. circumcarinatum* were prepared: a one month old liquid culture (L) and one month (P1), seven months (P2) and twelve months old cultures (P3) grown on agar plates (solid medium; Figure 1). The liquid culture of *Z. circumcarinatum* was harvested by centrifugation (300 xg) and blotted onto cellulose membrane filters (pore size 0.45 mm, Sigma Aldrich, St. Louis, MO, USA) while the biomass, grown on agar plates, was transferred directly to the filters without any blotting. The triplicates of all samples were placed into separate desiccation chambers, which were previously described (Karsten et al., 2014), and desiccated over a saturated KCl solution at a RH of approximately 86% according to Pichrtová et al. (2014). Y(II) was monitored during the desiccation event, using a portable PAM (Model 2500, Heinz Walz, Effeltrich, Germany), and samples were taken when Y(II) reached zero. The water loss of the biomass was determined prior to and after incubation in the desiccation chamber. The reduction of the water content was determined according to the following formula:

$$\text{Reduction [\%]} = 100 - \frac{\text{Desiccated biomass}}{\text{Fresh biomass}} \cdot 100$$

Both for Y(II) and water loss, differences between each group were assessed in R by a one-way ANOVA ($p < 0.01$) followed by Tukey's post-hoc test (HSD, $p < 0.01$). Correlation coefficients were calculated according to Pearson (1895).

5.7.3 RNA isolation and sequencing

Extraction of RNA was performed as previously described by Holzinger et al. (2014) with some modifications. Six filters from each cultivation (L, P1, P2, P3), containing either control samples (C, n = 3) or samples, subjected to desiccation (D, n = 3), were treated with 450 µl of LifeGuard^{TM} Soil Preservation solution (MO BIO Laboratories, Carlsbad, CA USA) and frozen in liquid nitrogen. Subsequently, 2% CTAB buffer (in 100 mM Tris, 50 mM EDTA, pH=8) was added and the samples were ground in a mortar as previously described by Heinrich et al. (2012a). Furthermore, 20 µl DTT were added to the

mixture followed by a centrifugation step. The supernatant was mixed with ethanol and, finally, RNA was extracted using the peqGold Plant RNA Kit (peqlab/VWR International, Erlangen, Germany) according to the manufacturer's instructions. RNA samples were further purified as described by Rippin et al. (2016). After DNase I treatment (Thermo Fisher Scientific, Waltham, MA, USA), the RNeasy MinElute Cleanup kit (Qiagen, Hilden, Germany) was utilized for concentration and clean-up. After assessing RNA quantity using the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) the RNA content of one replicate of P2D and all replicates of P3C and P3D, was insufficient for sequencing. Thus, the P2D replicate was excluded and the triplicates of each of the samples P3C and P3D were pooled in equal amounts. Finally, a reference was pooled from all samples in equal amounts.

After RNA quality control with the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA), two expression libraries for the reference and one for each of the sample replicates and the two triplicate pools were prepared by Eurofins Genomics (Ebersberg, Germany). Furthermore, mRNA was enriched using oligo-(dT) beads followed by fragmentation, random-primed cDNA synthesis and Illumina compatible adaptor ligation. The two normalized reference libraries were sequenced on one lane of an Illumina MiSeq, 300 bp paired end mode, and all sample libraries together on three different lanes of an Illumina HiSeq 2500, 125 bp single mode, using the MiSeq Control Software 2.5.0.5 or HiSeq Control Software 2.2.38, RTA 1.18.54 or RT 1.18.61 and bcl2fastq-1.8.4 (Illumina, San Diego, CA, USA).

5.7.4 Bioinformatic analyses

Raw reads of the reference were first quality trimmed and filtered using Trimmomatic 0.35 (Bolger et al., 2014) and PRINSEQ lite 0.20.4 (Schmieder and Edwards, 2011). Subsequently, rRNA sequence reads were separated by SortMeRNA 2.1 (Kopylova et al., 2012) employing the SILVA SSU NR Ref 119 and LSU Ref 119. Before assembly, COPEread (Liu et al., 2012) was uti-

lized to stitch overlapping reads together. The assembly of the reference was done with Trinity 2.0.6 (Grabherr et al., 2011) and the quality was assessed with scripts from the Trinity package and BUSCO plants 1.1b (Simão et al., 2015). An additional quality filtering step was carried out annotating all contigs with Diamond 0.8.24 (Buchfink et al., 2015) against a custom-made database which contained the protein sequences of *Physcomitrella patens* (Phytozome database version 12), *Klebsormidium flaccidum* (Hori et al., 2014) and *Naegleria gruberi* (Fritz-Laylin et al., 2010). Contigs scoring an e-value higher than E-20 against *N. gruberi* were removed. Control and treatment reads were subjected to trimming with Trimmomatic 0.35, rRNA filtering with SortMeRNA 2.1 and were subsequently mapped to the reference assembly using Bowtie2 2.2.9 (Langmead and Salzberg, 2012) estimating the abundance with RSEM (Li and Dewey, 2011). For differential gene expression analysis, the R package edgeR (Robinson et al., 2010) was employed, analyzing LC, LD, P2C and P2D and proceeding only with differentially expressed genes possessing an FDR (Benjamini and Hochberg, 1995) smaller than 0.001 and a fold change of at least 4. Contig annotation was performed with the Trinotate pipeline 3.0.0 (http://trinotate.github.io/), including TransDecoder 2.1 (http://transdecoder.github.io/), NCBI BLAST+ 2.3.0 (Altschul et al., 1990), HMMER 3.1b (Finn et al., 2011), SignalP 4.1 (Petersen et al., 2011), TMHMM 2.0c (Krogh et al., 2001), RNAmmer 1.2 (Lagesen et al., 2007) as well as the databases Swiss-Prot and PFAM 3.1b2. The e-value cutoff was set to E-10. Two gene set enrichment analyses were carried out in R, GO term enrichment with GoSeq 1.26.0 (Young et al., 2010) and KEGG pathway enrichment with clusterProfiler 3.2.11 (Yu et al., 2012), setting the FDR in both cases to 0.001. Diamond 0.8.24 was used for annotations against the Phytozome database version 12 and the *Chlorella* sp. NC64A genome (Blanc et al., 2010), the *Klebsormidium crenulatum* transcriptome (Holzinger et al., 2014) and the *Klebsormidium flaccidum* genome. Filtered rRNA reads were clustered into OTUs utilizing USEARCH 5.2.2 (Edgar, 2013) and annotated against the Silva SSU database 123.1 by feeding them into the QIIME 1.9.1 (Caporaso et al.,

2010) script assign_taxonomy.py (e-value cutoff E-6). All raw reads and assembled contigs were submitted to the SRA database (SRP117803) and will become available upon publication of the study.

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5.9 Disclosure

The authors declare that they do not have a conflict of interest.

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Chapter 6

Discussion

The work presented here contributes greatly to the understanding of terrestrial ecosystems in polar deserts and alpine regions. BSC communities cover most of the ground in these environments and occupy important ecological functions (Borchhardt et al., 2017a,b; Williams et al., 2017). However, climate change is a major threat to these communities and, thus, to the whole ecosystem (Colesie et al., 2014; Frey et al., 2013; Pushkareva et al., 2016). In order to predict future scenarios, BSCs need to be studied in more detail, especially in the context of changing environmental conditions, disturbance and climate change induced succession (Bowker et al., 2014; Frey et al., 2013).

This thesis comprises results from methodology to biodiversity, ecofunctionality and response patterns of BSCs to abiotic stress. The main focus is on terrestrial microalgae and Cyanobacteria, inhabiting the crust, but the broader biodiversity was also assessed using molecular techniques. At first, different protocols were tested and compared in terms of suitability for the isolation of high-quality nucleic acids, which form the basis for any molecular study. Within the project, the biodiversity of different BSCs, collected from the Arctic and Antarctica, was analyzed using metatranscriptomics and metabarcoding. Subsequently, those results were compared to microalgae and Cyanobacteria identified based on morphology by Borchhardt et al. (2017a) and Jung et al. (in preparation). The results retrieved from the biodiversity survey were also used to conclude on ecological functions. Finally, an

alpine isolate of *Zygnema circumcarinatum*, a hydro-terrestrial, mat-forming alga, was exposed to harsh desiccation stress and the response pattern was monitored using transcriptomics.

6.1 Methodology

Studying the ecology of BSCs with classical cultivation based approaches has serious limitations as most microorganisms cannot be cultured (Chandra and Yadav, 2015; Van Elsas et al., 2014; Wang et al., 2012). However, this *microbial dark matter* can still be analyzed by omitting the cultivation step and using molecular techniques directly on environmental samples (Filée et al., 2005; Van Elsas et al., 2014). The basis for a successful molecular analysis, e.g. metagenomics, -transcriptomics or -proteomics, are pure and high-quality biomolecules (Chen et al., 2009; Heinrich, 2018; Renella et al., 2014; Sánchez et al., 2016; Van Elsas et al., 2014). Due to the chemical properties of soil, the extraction of appropriate amount of high-grade DNA, RNA and proteins for metagenomics, -transcriptomics and -proteomics, respectively, is rendered difficult (Chandra and Yadav, 2015; Chen et al., 2009; Renella et al., 2014; Van Elsas et al., 2014; Wang et al., 2012).

Soil can contain high amounts of humic substances, yellow to dark brown colored compounds which are formed during the breakdown of organic material (Chandra and Yadav, 2015; Wang et al., 2012). Humics can be divided into three groups: humic acids, which are only soluble in alkali, fulvic acids which are soluble at any pH and humin, which is insoluble (Chandra and Yadav, 2015; Wang et al., 2012). Fulvic acids are polymerase chain reaction (PCR) inhibitors, while humic acids interfere with almost all molecular methods such as enzymatic reactions (e.g. restriction enzymes), PCR reactions, transformation, nucleic acid measurements and hybridizations (Chandra and Yadav, 2015; Van Elsas et al., 2014; Wang et al., 2012). Unfortunately, humic and fulvic acids possess properties similar to nucleic acids and are coextracted during DNA and RNA extraction (Chandra and Yadav, 2015; Wang et al.,

2012). Hence, extraction protocols need to be adapted and optimized to reduce the amount of these polyelectrolytes or even remove them (Chandra and Yadav, 2015; Van Elsas et al., 2014; Wang et al., 2012). In Chapter 2, three different approaches to extract RNA were tested on four different BSC samples: an extraction using a self-prepared buffer with cetyl trimethylammonium bromide (CTAB) and polyvinylpolypyrrolidone (PVPP) and two commercial kits. The results indicate that the CTAB containing buffer performed better than the commercial kits in terms of reliability. Furthermore, the protocol is cheaper than the kits and the nucleic acids show no degradation. CTAB and PVPP are both known to chelate humic substances which helps to remove them during the extraction (Chandra and Yadav, 2015; Subbiah and Mishra, 2008). Another advantage of this CTAB protocol is the scalability. Commercial kits usually allow only small sample amounts and soil samples generally yield only small amounts of RNA (Van Elsas et al., 2014; Wang et al., 2012). Moreover, the extensive purification necessary to remove humic substances further decreases the yield (Wang et al., 2012). By processing a larger amount of sample it is easier to obtain the quantity of RNA required for NGS technologies (Wang et al., 2012). The CTAB based protocol provides another benefit as in addition to RNA, DNA is extracted which can be purified and used for metabarcoding or metagenomic approaches.

The extracted and purified nucleic acids are either used in shotgun metagenomics and metatranscriptomics or as a template for amplicon sequencing (Urich et al., 2008, 2014; Zimmermann et al., 2015). All three of these techniques are able to provide information about the biodiversity in the sample and often yield higher taxon numbers than classical morphology based methodologies (Hultman et al., 2015; Zimmermann et al., 2015). The results displayed and discussed in Chapter 3 and Chapter 4 confirm this assumption. Nevertheless, these methods also exhibit certain pitfalls (Taberlet et al., 2012).

Metabarcoding is limited in a sense that different barcodes have to be used for different groups, e.g. Archaea, Bacteria and different phyla within the eukaryotes (Taberlet et al., 2012). Hence, the relative abundance cannot be

compared across different groups (Taberlet et al., 2012). Designing primers fitting the marker gene is complicated and primers tend to be biased towards certain taxa (Taberlet et al., 2012). Furthermore, the PCR can cause substitutions, insertions deletions and even chimera formation during amplification (Cline et al., 1996; Van Elsas et al., 2014). Similarly, sequencing can introduce errors which also holds true for metatranscriptomics and -genomics (Glenn, 2011). Errors introduced during PCR and sequencing, that cause overestimation and spurious OTUs, can be minimized by using a mock community as described in Chapter 4. Finally, the identification of the sequence is dependent on the eligibility of the chosen barcode(s) and the reference database used (Taberlet et al., 2012). These databases, e.g. SILVA and PR2 database, need to contain high-quality references that are taxonomically verified and curated (Guillou et al., 2013; Quast et al., 2013; Taberlet et al., 2012).

Many of the above mentioned problems, such as PCR and primer bias, can be avoided using metagenomics and -transcriptomics since the total DNA or RNA, respectively, are sequenced (Urich et al., 2008). In addition to insights into biodiversity, these techniques also provide information about the functionality of the community (Urich et al., 2008). When comparing the results from Chapter 3 and 4, the algal and cyanobacterial genus richness within the same BSC sample is higher for the metatranscriptomic approach than for the metabarcoding one. This can be explained by the longer sequences in the metatranscriptome (assembled 16S and 18S rRNA gene) compared to the barcode (16S rRNA gene V3-V4 region; 18S rRNA gene V4 region). Longer sequences and, thus, more information give a better taxonomic resolution (Wu et al., 2015).

A major drawback of molecular identification of organisms in environmental samples is that once the sample is processed the organisms cannot be studied further. Culture isolates, on the other side, allow researchers to analyze organisms' physiology and response mechanisms to biotic and abiotic stress (Iñiguez et al., 2017). The combination of physiological measurements and transcriptomic analysis is a powerful approach to shed light on the underlying cellular

processes involved in counteracting stressors such as radiation or desiccation (Holzinger et al., 2014; Iñiguez et al., 2017). In Chapter 5, the molecular response of *Zygnema circumcarinatum* to desiccation was studied which revealed adaptive strategies in terms of resilience and acclimation. However, this methodology completely neglects the interactions with other organisms which might associate with the streptophyte (Chi et al., 2017; Poonguzhali et al., 2007; Williams, 2007).

6.2 Biodiversity

BSCs are complex communities which accommodate a number of micro- and macroorganisms leading an either autotrophic, heterotrophic or saprotrophic lifestyle (Belnap et al., 2001a, 2016; Darby and Neher, 2016). The autotrophic fraction of BSCs include Cyanobacteria, eukaryotic microalgae, bryophytes and lichens which are the main primary producers in arid and semi-arid regions (Andersen, 1992; Kallio and Kärenlampi, 1975; Prestø et al., 2014; Whitton and Potts, 2012). These organisms are highly diverse and may occupy additional ecological functions that will be discussed in the following paragraphs.

The molecular analyses in Chapter 3 and Chapter 4 revealed that Cyanobacteria are present in all sampled BSCs. However, the degree of diversity differs among the communities with the sample from Ny-Ålesund, Svalbard, exhibiting the highest genus richness and the sample from Breinosa Gruve 7, Svalbard, the lowest. Surprisingly, the genus *Microcoleus* was only found in two BSCs, although it is commonly regarded as a keystone species in the development of biocrusts (Couradeau et al., 2016). The absence of *Microcoleus* could be due to successional shifts as previously reported by Couradeau et al. (2016). The cyanobacterium typically initiates crust formation and may later be replaced by other organisms (Couradeau et al., 2016). Metabarcoding (Chapter 4) revealed a high relative abundance of bryophytes indicating a late developmental stage of the crusts (Belnap, 2006; Garcia-Pichel et al., 2016). Another explanation might be that *Microcoleus* does not play major role in biocrust formation

in polar ecosystems and is substituted by *Nostoc*, another BSC engineer that appears to be more abundant in both Polar Regions compared to *Microcoleus* (Büdel et al., 2016; Matuła et al., 2007; Pushkareva et al., 2016; Zidarova, 2008). This hypothesis is supported by the fact that species of *Nostoc* were found in all biocrusts analyzed in Chapter 3 and Chapter 4. The genus is ubiquitously distributed and capable of forming heterocysts and, therefore, fixing nitrogen (Büdel et al., 2016). Four out of five samples contained the genera *Leptolyngbya* and *Phormidium*, typical representatives of polar Cyanobacteria (Matuła et al., 2007; Pushkareva et al., 2016; Zidarova, 2008). Both organisms have been reported to synthesize EPS which can enhance crust development and protect the soil surface against erosion (Büdel et al., 2016; De Philippis et al., 2005; Vicente-García et al., 2004).

Streptophytic algae belonging to the taxa Klebsormidiophyceae and Zygnematophyceae are commonly associated with BSCs (Büdel et al., 2016). Especially, the genus *Klebsormidium* (Klebsormidiophyceae) is often found in crust communities in both the Arctic and Antarctica (Borchhardt et al., 2017b; Büdel et al., 2016; Pushkareva et al., 2016; Zidarova, 2008). The results of the metatranscriptomic analysis (Chapter 3) and the amplicon sequencing (Chapter 4) confirm that *Klebsormidium* is present in four out of five BSCs. This filamentous alga is involved in crust formation by weaving through the soil and, thus, stabilizing it (Büdel et al., 2016). Other filamentous streptophytes identified in Chapter 3 are *Zygnema* and *Mougeotia* (Zygnematophyceae; results not shown) (Herburger et al., 2014; Pichrtová et al., 2013). The former was earlier isolated from alpine and polar habitats and identified in both an Arctic and an Antarctic BSC that were analyzed in Chapter 3 (Herburger et al., 2014; Pichrtová et al., 2013; Zidarova, 2008). *Zygnema* is able to produce phenolics in response to UVR stress, often beneficial to other, more sensitive organisms within the biocrust (Belnap and Lange, 2001; Pichrtová et al., 2013). Other zygnematophycean algae that could be detected with the molecular techniques were *Cosmarium*, *Cylindrocystis*, *Netrium* and *Penium*. These genera are likely contributing to the crust stabilization as they are known to form biofilms and

excrete mucilage (Büdel et al., 2016; Domozych, 2014; Kiemle et al., 2007). For the streptophytes *Cosmarium* and *Cylindrocystis*, previous records exist that confirm their presence in terrestrial habitats in both Polar Regions, which is in agreement with our results (Pushkareva et al., 2016; Zidarova, 2008). The Chlorophyceae found in biocrusts are mostly unicellular and comprise the biggest biodiversity among BSC algae (Büdel et al., 2016). A similar trend was observed for the metatranscriptomic analysis (Chapter 3) showing that Chlorophyceae possessed the highest genus richness among eukaryotic algae. In contrast, metabarcoding (Chapter 4) revealed a moderate biodiversity for Chlorophyceae suggesting that Chlorophyceae are indeed diverse but probably only present in low abundances. At least four out of five samples contained the genera *Bracteacoccus*, *Chlamydomonas* and *Chloromonas* which have previously been reported for terrestrial habitats in the Polar Regions (Borchhardt et al., 2017b; Matuła et al., 2007; Pushkareva et al., 2016). The trebouxiophyceaen algae *Coccomyxa*, *Dictyochloropsis*, *Elliptochloris* and *Leptosira*, that could be identified in all samples by means of metatranscriptomics (Chapter 3) and metabarcoding (Chapter 4), are also commonly associated with polar BSC communities (Borchhardt et al., 2017b; Matuła et al., 2007; Pushkareva et al., 2016). Furthermore, the genera *Asterochloris*, *Chloroidium* and *Trebouxia*, typical lichen photobionts, were found in some of the sampled BSCs and, therefore, could indicate the presence of lichens (Büdel et al., 2016). Astonishingly, the molecular analysis indicated that the chrysophyte *Spumella* inhabited all isolated biocrusts. *Spumella* is an ubiquitously distributed nanoflagellate that can be either autotrophic or heterotrophic (Boenigk et al., 2005; Stoeck et al., 2008). Mixotrophy gives an competitive advantage in low-light and dark environments helping the protist to survive the polar night (McMinn and Martin, 2013). The second most diverse group of BSC algae are diatoms (Büdel et al., 2016). All samples contained at least one genus, however, the biocrust isolated from Ny-Ålesund exhibited the highest genus richness (Chapter 4). *Pinnularia*, a genus commonly associated with polar BSCs, was found in four out of five samples (Borchhardt et al., 2017b; Bowker et al., 2016; Zidarova, 2008). Rep-

resentatives of the algal classes Eustigmatophyceae and Xanthophyceae have previously been found in polar soils (Borchhardt et al., 2017b; Büdel et al., 2016; Matuła et al., 2007; Pushkareva et al., 2016; Zidarova, 2008). Typical genera are *Eustigmatos* (Eustigmatophyceae), *Botrydiopsis*, and *Heterococcus* (Xanthophyceae), all of which were present in the amplicon dataset (Chapter 4) (Büdel et al., 2016).

In general, all of the BSC isolates showed a dominance of bryophytes (Chapter 4) indicating that all samples were in a late successional stage (Garcia-Pichel et al., 2016; Lan et al., 2013). Apart from primary production, mosses also stabilize the soil surface, increase water infiltration and present microhabitats for other BSC microorganisms (Seppelt et al., 2016). Among Bryophyta, the three genera *Brachythecium*, *Pohlia* and *Andreae* showed the highest relative abundance in the eukaryotic community. These mosses have previously been observed in the both Polar Regions (Engelskjøn, 1987; Prestø et al., 2014; Putzke et al., 2015; Putzke and Pereira, 2001). The genus *Andreae*, for instance, has been found to form associations with the lichen *Usnea* in Antarctica (Putzke and Pereira, 2001).

The molecular dataset in Chapter 4 also detected a number of OTUs belonging to Fungi, non-cyanobacterial prokaryotes and microfaunal organisms. Apart from non-lichenized Fungi, the genera *Elasticomyces* and *Leciophysma*, potential mycobionts, were found in the analyzed Arctic isolates (Selbmann et al., 2008; Wedin et al., 2009). The most dominant prokaryotic phyla, besides Cyanobacteria, were Acidobacteria, Actinobacteria, Bacteroidetes, Gemmatimonadetes, Proteobacteria and Verrucomicrobia. Acidobacteria and Bacteroidetes, for instance, are able to produce EPS which support the adhesion of soil particles (Gundlapally and Garcia-Pichel, 2006; Kielak et al., 2016). Interestingly, the metabarcoding dataset also contained a diverse microfauna, e.g. the tardigrade *Isohypsibius* was present in the isolate from Ny-Ålesund. This particular BSC was rich in bryophytes, a typical habitat of *Isohypsibius* (Manicardi, 1989). Generally, tardigrades are extremophiles, able to withstand extreme temperatures and high levels radiation (Sloan et al., 2017). Hence,

these organisms are well equipped for the survival in hostile environments such as the Arctic and Antarctica.

Overall, the generated datasets give new insights into polar biocrusts as the community was studied as a whole for the first time. Despite the disadvantages, molecular surveys reveal a bigger part of the BSC diversity than cultivation based approaches. Furthermore, the newly established sequences are a valuable resource for future endeavors.

6.3 Abiotic stressors

Polar and alpine climate zones exhibit a number of extreme conditions forcing all life forms to adapt in order to survive (Martin, 2001; Thomas et al., 2008). Terrestrial organisms, such as BSC communities, have to cope with low temperatures, temporary to permanent snow and ice cover, occasionally elevated levels of radiation, high seasonality and low water availability leading to desiccation (Belnap et al., 2016; Martin, 2001; Thomas et al., 2008). In the following section, adaptive strategies of photoautotrophs, mainly to desiccation, will be discussed and contextualized.

In Chapter 5, the desiccation tolerance of the streptophytic alga *Zygnema circumcarinatum*, isolated from the shore of the river Salaach in the Alps, was assessed using transcriptomics. Prolonged desiccation stress caused a decrease of the effective quantum yield of photosystem II, an effect that has been observed in *Entransia*, *Hormidiella* and *Klebsormidium* (Klebsormidiophyceae, Streptophyta) (Herburger et al., 2016; Holzinger et al., 2014). In general, photosynthesis is prone to interference caused by abiotic factors such as cold stress, high radiation and dehydration (Fernández-Marín et al., 2013; Heinrich et al., 2015; Mao et al., 2017). Low temperatures and high UVR can inhibit CO_2 assimilation and increase the formation of ROS (Heinrich et al., 2015; Mao et al., 2017). The phaeophyte *Saccharina latissima*, for instance responds with an increased expression of photosynthetic components to counteract high light and UVR (Heinrich et al., 2015). Similarly, *Klebsormidium*

crenulatum reacts with an upregulation of genes involved in photosynthesis when exposed to desiccation (Holzinger et al., 2014). In contrast, *Zygnema* reduces the transcript pool of photosynthetic modules such as photosystem I and II as well as the light-harvesting complexes (Chapter 5). The resurrection plant *Myrothamnus flabellifolia* uses the same mechanism, probably to reduce excitation energy and the associated ROS production (Ma et al., 2015). Furthermore, the plant induces the expression of ELIPs when desiccated (Ma et al., 2015). These proteins are photoprotectants that are mainly upregulated in response to high light and UVR stress (Norén et al., 2003). For example, the chlorophytes *Chlamydomonas reinhardtii* and *Dunaliella bardawil* exhibited an accumulation of ELIPs when light stressed (Lers et al., 1991; Teramoto et al., 2004). Han and Kim (2013) exposed the streptophyte *Spirogyra varians* to low temperatures and observed a similar reaction. Since desiccation affects the photosynthetic apparatus negatively, it is not surprising that *Zygnema* also induces the expression of ELIPs (Chapter 5).

Upon desiccation, *Z. circumcarinatum* showed an upregulation of transcripts involved in sucrose production. Carbohydrates, such as sucrose and trehalose, are commonly synthesized as osmolytes to counteract water stress (Alpert, 2005; Fernández-Marín et al., 2013; Potts, 1994). The accumulation of osmoprotectants generates a negative osmotic potential and enhances membrane stabilisation and protein protection (Bisson and Kirst, 1995). The Antarctic chlorophyte *Trebouxia*, for example, exhibited increased sucrose concentrations in response to desiccation stress (Sadowsky et al., 2016). Holzinger et al. (2014) studied the transcriptomic response of *K. crenulatum* to water deprivation and observed, similarly to *Zygnema*, an induction of sucrose forming enzymes. Sucrose is also accumulated at low temperatures by the green algae *Klebsormidium flaccidum* and *Chlorella vulgaris* because of its cryoprotectant properties (Nagao et al., 2008; Rütten and Santarius, 1992; Salerno and Pontis, 1989).

Biological membranes are prone to stressors such as low temperature, increased radiation and drought as they alter membrane fluidity and integrity

(Al-Rashed et al., 2016; Hernando et al., 2005; Jung et al., 2014; Park et al., 1997; Perlikowski et al., 2016; Tsuji, 2016). Cold stress, for instance, induces membrane modifications in the chlorophyte *C. reinhardtii* (Valledor et al., 2013; Wang et al., 2017). Al-Rashed et al. (2016) observed that UVR causes lipid perioxidation in the cyanobacterium *Spirulina platensis* leading to membrane damage. The cyanobacterium *Nostoc* increases the amount of unsaturated fatty acids in its cytoplasmic membrane to protect it from desiccation (Potts, 1994). Holzinger et al. (2014) found that desiccation treatment causes an upregulation of genes involved in lipid metabolism in the green alga *K. crenulatum*, probably as a way to change the membrane composition. A similar reaction was detected for *Z. circumcarinatum* suggesting that desiccation causes a restructuring of the biomembranes (Chapter 5). Furthermore, a strong induction of aquaporins, channels for passive transportation of small uncharged molecules, was observed (Anderberg et al., 2011). Carniel et al. (2016) detected the same pattern in the chlorophyte *Trebouxia gelatinosa* and theorizes that these channels increase the permeability of membranes to protect them from damage upon rehydration.

Zygnema increases the transcript pool of enzymes involved in the protection against ROS when desiccated. Similarly, the alga *T. gelatinosa* induces ROS scavenging enzymes to counteract water deprivation (Carniel et al., 2016). The occurrence of ROS is linked to different abiotic stressors such as cold, high light as well as drought, and is extremely harmful to the cell as they cause biomolecules to denature (Cruz de Carvalho, 2008; Cruz de Carvalho et al., 2012; Dong et al., 2016; Han et al., 2012; Heinrich et al., 2015; Rajeev et al., 2013). As a protection mechanism against elevated levels of irradiance, the algae *C. reinhardtii* and *S. latissima* express scavengers that render these toxic radicals harmless (Erickson et al., 2015; Heinrich et al., 2012). The same holds true for *S. varians* when exposed to low temperatures (Han et al., 2012). ROS may also introduce modifications to the DNA, forcing the organism to protect it and/or counteract these effects (Cruz de Carvalho, 2008; Heinrich

et al., 2015). Upon desiccation, *Zygnema* exhibits a strong upregulation of DNA repair enzymes.

Chaperones, such as HSPs, play an important role in the protection and refolding of proteins during abiotic stress (Al-Whaibi, 2011; van de Poel et al., 2016; Wang et al., 2004). For instance, treatment with high irradiance causes the induction of various chaperones and HSPs in the kelp *S. latissima* and the diatom *Thalassiosira pseudonana* (Dong et al., 2016; Heinrich et al., 2012). The same holds true for the diatom *Chaetoceros neogracile* when exposed to low temperatures, as shown previously by Park et al. (2007). Moreover, the moss *Physcomitrella patens* and the microalga *Asterochloris erici* exhibit an increased expression of chaperones upon desiccation (Gasulla et al., 2013; Wang et al., 2009a). This increase in chaperone and HSP transcripts was also observed for *Z. circumcarinatum*, probably to enable remodeling of aggregated and misfolded proteins (Chapter 5). Additionally, the expression of several LEA proteins, which are typically associated with dehydration, was induced (Alpert, 2005; Goyal et al., 2005; Shinde et al., 2012). The streptophyte *K. crenulatum* also increases the expression of these chaperones when desiccated (Holzinger et al., 2014).

The desiccation transcriptome of *Zygnema circumcarinatum* provides an important sequence reference for studying the evolution of land plants as Zygnematophyceae are their sister lineage. Moreover, the datasets shed light on the molecular mechanisms underlying the ecophysiological plasticity of streptophytic green algae, especially in response to seasonal fluctuations of water availability.

6.4 Conclusion

BSCs are complex microecosystems and key players in polar and alpine environments. These agglomerations of different autotrophic, heterotrophic and saprotrophic micro- and macroorganisms are highly diverse and fulfill important ecological functions, e.g. primary production, nitrogen fixation and pro-

tection against erosion. To fully comprehend their biodiversity and the interactions between the different parts of these communities, reliable methodologies need to be available allowing to study BSCs in full depth. Molecular methods show great potential due to the fact that they circumvent cultivation of individual organisms. However, they depend on various factors, such as the availability of high-quality biomolecules (e.g. DNA and RNA), among others. During the course of this project, a nucleic acid extraction protocol, based on a CTAB containing buffer, has proven to be the most successful and was therefore used for all studies. Biodiversity assessment was carried out by means of metabarcoding and metatranscriptomics. The comparison with morphological data revealed that the integration of cultivation based and molecular approaches produces a more comprehensive picture. Furthermore, cultivation enables researchers to investigate individual BSC organisms in terms of growth, physiology and stress response patterns. Desiccation, especially, is a major stress factor that BSCs colonizing arid and semi-arid ecosystems are exposed to. Cell-biological and physiological studies combined with transcriptomics are able to shed light on molecular patterns and deliver explanations for adaptive mechanisms. In general, the integration of modern omic techniques with ecological and physiological methods is an essential step for future BSC research.

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Summary

The Polar Regions and alpine zones are extremely hostile environments due to prevailing low temperatures, high levels of solar radiation and extreme seasonality. Water is almost exclusively present as ice and snow, thus, unavailable for organisms. These conditions restrict the vegetational cover to be sparse or completely absent. However, the soil surface is not barren but mostly covered by biological soil crusts (BSCs). These crusts are complex aggregations of different organisms, such as Cyanobacteria and other prokaryotes, eukaryotic microalgae, bryophytes, Fungi, lichens and a versatile microfauna, occurring in varying portions. Biocrusts are integral parts of cold ecosystems as they contribute extensively to primary production and nitrogen fixation. Furthermore, BSCs are important ecosystem engineers that prevent soil loss, change hydrological patterns, albedo, and increase soil fertility. Global change is a major threat to these communities as it leads to alterations in biodiversity amplified by an invasion of non-indigenous species. Hence, studying and monitoring BSCs is essential to recognize shifts in community structures early on and predict future scenarios.

Reliable methodologies are the basis for sound results and conclusions. Extracting nucleic acids from BSC samples successfully is highly dependent on, for instance, soil properties. Soil often contains high amounts of humics, which can interfere with enzymatic reactions and should be removed. In this thesis, it was found that an extraction method based on a cetyl trimethylammonium bromide performed superior to commercial kits. All BSC isolates processed with this protocol yielded high-quality nucleic acids. Thus, this procedure and variations of it were used in the course of this project.

Biodiversity assessment is crucial to unravel the complexity of communities such as biocrusts. However, cultivating and examining individual organisms has several limitations and will only reveal a small fraction of the actual biodiversity. Molecular methods may overcome these limitations to a certain degree as total DNA or RNA is extracted from the sample and analyzed. In this thesis, five different BSCs were studied, four from Svalbard, Norway, in the Arctic and one from Livingston Island, a part of the Antarctic Peninsula. All five BSCs were in a late successional stage with bryophytes being most dominant. Moreover, Cyanobacteria exhibited a high relative abundance, while microalgae appeared to be diverse but only present at a low relative abundance. The genera *Chloromonas* (Chlorophyceae), *Coccomyxa*, *Dictyochloropsis*, *Elliptochloris*, *Leptosira* (Trebouxiophyceae), *Spumella* (Chrysophyceae) and *Nostoc* (Cyanobacteria) were detected in all samples suggesting an ubiquitous distribution. Especially, *Nostoc* may be an important keystone species in Polar Regions which may be further investigated in future studies. Furthermore, the presence of sulfur Bacteria indicates that BSCs are involved in sulfur cycling. Overall, the combination of cultivation based and molecular approaches provides a more comprehensive picture of BSC communities.

As mentioned above, BSC organisms have to cope with extreme conditions such as water scarcity, which can cause desiccation. The ability to tolerate water deprivation and adapt to it was studied in an alpine strain of *Zygnema circumcarinatum* (Zygnematophyceae). Similar to land plants, *Zygnema* increases the transcript pool of early light-induced proteins, aquaporins, reactive oxygen species scavengers and chaperones such as late embryogenesis abundant proteins. Additionally, transcriptional changes in the carbohydrate and lipid metabolism suggest accumulation of sucrose and membrane modifications, respectively.

In conclusion, the integration of physiological, cell-biological and omic approaches is a promising way to get extensive insights into the biodiversity and ecology of biological soil crusts. The outcome can be merged to shed light on

present community patterns and functionality, and help to generate predictions models for future developments.

Zusammenfassung

Die Polargebiete und Bergregionen sind außerordentlich unwirtliche Habitate, die durch niedrige Temperaturen und starke Sonneneinstrahlung geprägt und extremen saisonalen Schwankungen unterworfen sind. Da Wasser fast ausschließlich in festem Aggregatzustand vorliegt, ist es für die vorkommenden Organismen kaum oder gar nicht verfügbar. Diese Umstände bedingen, dass höhere Pflanzen größtenteils abwesend sind. Allerdings ist der Boden nicht kahl, sondern zu einem hohen Prozentsatz mit biologischen Bodenkrusten (BBK) bedeckt. BBK sind komplexe Zusammenschlüsse aus verschiedenen Organismen, wie Cyanobakterien und anderen Prokaryoten, eukaryotischen Mikroalgen, Bryophyten, Pilzen, Flechten sowie einer vielseitigen Mikrofauna. In kalten Ökosystemen sind Biokrusten ein integraler Bestandteil, da sie sowohl zur Primärproduktion als auch zur Stickstofffixierung beitragen. Zusätzlich formen BBK die Lebensräume, die sie besiedeln, indem sie den Bodenabtrag minimieren, das hydrologische Profil und das Lichtrückstrahlvermögen verändern und die Produktivität der Böden steigern. Klimaveränderungen sind eine ernstzunehmende Bedrohung für diese Gemeinschaften. Durch Veränderungen der Temperatur, der Niederschlagsmenge und -frequenz können gebietsfremde Arten in diese Lebensräume vordringen, was Verschiebungen in Biodiversität und Abundanz zur Folge hat. Daher wurden im Rahmen dieses Projektes BBK untersucht und damit eine Grundlage geschaffen, um Veränderungen frühzeitig zu erkennen und zukünftige Entwicklungen vorhersagen zu können.

Reproduzierbare und effiziente Methoden sind die Voraussetzung, um verlässliche Aussagen über die Artenzusammensetzung und Funktion von Bodenkrusten zu erzielen. Das erfolgreiche Extrahieren von Nukleinsäuren aus

BBK-Proben wird von bestimmten Bodeneigenschaften erschwert. Erdproben können große Mengen Huminstoffe enthalten, welche ähnliche chemische Eigenschaften wie Nukleinsäuren haben. Diese stören enzymatische Reaktionen und sollten entfernt werden. Das in der Arbeit vorgestellte auf Cetyltrimethylammoniumbromid basierende Protokoll erzielte im Vergleich zu den kommerziell erhältlichen Kits die besten Ergebnisse. Mit Hilfe dieses Protokolls konnten aus allen untersuchten BBK qualitativ hochwertige Nukleinsäuren gewonnen werden. Daher wurde dieses Verfahren mit einigen Variationen im weiteren Verlauf des Projektes verwendet.

Biodiversitätsuntersuchungen sind unabdingbar um komplexe Gemeinschaften wie Biokrusten zu analysieren. Klassischerweise werden Einzelorganismen isoliert, kultiviert und anschließend bestimmt. Diese Methode hat mehrere Nachteile, wie beispielsweise die Kultivierbarkeit von Organismen, und kann nur einen geringen Teil der eigentlichen Vielfalt enthüllen. Im Rahmen dieses Projektes wurden molekulare Methoden zur Bestimmung der Biodiversität verwendet. Dafür wurde die Gesamt-DNA oder -RNA einer Probe extrahiert und untersucht, was die Anzahl der identifizierten Organismen signifikant erhöht verglichen mit klassichen Methoden. Fünf verschiedene BBK wurden analysiert, vier vom arktischen Spitzbergen, Norwegen, und eine von der Insel Livingston, welche zur antarktischen Halbinsel gehört. Alle fünf untersuchten Biokrusten befanden sich in einer späten Entwicklungsphase, was durch die Dominanz der Bryophyten belegt wird. Auch Cyanobakterien zeigten eine besonders hohe relative Abundanz, wohingegen Mikroalgen eine hohe Diversität zeigten, aber nur eine geringe relative Abundanz. Die Gattungen *Chloromonas* (Chlorophyceae), *Coccomyxa*, *Dictyochloropsis*, *Elliptochloris*, *Leptosira* (Trebouxiophyceae), *Spumella* (Chrysophyceae) und *Nostoc* (Cyanobakterien) konnten in allen Proben nachgewiesen werden, was auf eine ubiquitäre Verbreitung schließen lässt. Besonders *Nostoc* könnte eine Schlüsselrolle für BBK in den Polargebieten spielen, was in kommenden Studien untersucht werden sollte. Außerdem ließen sich Schwefelbakterien nachweisen. Daher liegt die Vermutung nahe, dass BBK am Schwefelkreislauf

beteiligt sind. Als Fazit aus diesem Teil lässt sich festhalten, dass eine Kombination von Kultivierung und molekularen Techniken ein umfassenderes Bild von Biokrusten ergibt.

Wie bereits erwähnt müssen Organismen, die in BBK vorkommen, mit extremen Bedingungen zurechtkommen, wie z.B. Wasserknappheit, welche zu Austrocknung führen kann. Ein alpiner Stamm von *Zygnema circumcarinatum* (Zygnematophyceae) wurde einem Trockenstressexperiment unterzogen, um die Mechanismen zu identifizieren, die streptophytischen Grünalgen erlauben, Wasserentzug zu tolerieren und sich daran anzupassen. Es wurde gezeigt, dass *Zygnema*, ähnlich wie Land-pflanzen, die Transkriptanzahl von ELI*-Proteinen, Aquaporinen, Enzymen, die an dem Abbau von Radikalen beteiligt sind, und Chaperonen, wie z.B. den LEA**-Proteinen, erhöht. Zusätzlich konnten transkriptionelle Veränderungen im Zucker- und Lipidstoffwechsel gemessen werden, die auf eine Anreicherung von Saccharose und Membranmodifikationen hinweisen.

Als erweitertet Fazit ergibt sich, dass das Kombinieren von physiologischen und zellbiologischen Methoden sowie Omic-Techniken tiefe Einblicke in die Biodiversität und Ökologie von biologischen Bodenkrusten ermöglicht. Die Ergebnisse dieser Arbeit erlauben Rückschlüsse auf die gegenwärtigen Gemeinschaftsstrukturen und Funktionalität der BBK. Außerdem helfen sie dabei Modelle zu entwickeln, die Prognosen in Bezug auf zukünftige Entwicklungen zulassen.

*Early light-induced

**Late embryogenesis abundant

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Anteil an Teilpublikationen

Paper I

Die Studie wurde von mir mitgeplant, zwei von vier Proben wurden von mir isoliert, alle Vorversuche wurden von mir vorgenommen, alle unterschiedlichen Protokolle wurden von mir getestet und, wenn nötig, modifiziert, die Qualitätskontrolle (Nanodrop, Gelelektrophorese) wurde von mir durchgeführt, die Ergebnisse wurden von mir wissenschaftlich ausgewertet und das Manuskript wurde von mir geschrieben und editiert.

Paper II

Die Studie wurde von mir mitgeplant, alle RNA-Proben wurden von mir extrahiert und aufgereinigt, die Qualitätskontrolle (Nanodrop, Gelelektrophorese) wurde von mir durchgeführt, die Sequenzierrohdaten wurden von mir gefiltert und die Sequenzen assembliert, das *R*-Script für die bioinformatische Auswertung wurde von mir geschrieben und das Manuskript sowie die enthaltenen Graphen wurden von mir erstellt und editiert.

Paper III

Die Studie wurde von mir mitgeplant, alle Vorversuche wurden von mir durchgeführt, mehr als die Hälfte der Nukleinsäureproben wurden von mir gewonnen, ich habe bei der Qualitätskontrolle (Bioanalyzer, qPCR) assistiert, die Sequenzierrohdaten wurden von mir gefiltert und die Sequenzen assembliert, das

bash- und das *R*-Script für die bioinformatische Auswertung wurden von mir geschrieben und das Manuskript sowie die enthaltenen Graphen wurden von mir erstellt und editiert.

Paper IV

Alle RNA-Proben wurden von mir aufgereinigt, die Sequenzierrohdaten wurden von mir gefiltert und die Sequenzen assembliert, das *R*-Script für die bioinformatische Auswertung wurde von mir geschrieben und das Manuskript sowie die enthaltenen Graphen wurden von mir erstellt und editiert.

Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen - noch nicht veröffentlicht worden ist, sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Burkhard Becker betreut worden.

Köln, den 16. Februar 2018

Martin Rippin