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Stress resistance of polar hydro-terrestrial algae *Zygnema* spp. (Zygnematophyceae, Streptophyta)

Stresová odolnost polárních hydro-terestrických řas *Zygnema* spp. (Zygnematophyceae, Streptophyta)

Ph.D. Thesis

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Prague, 2014

Declaration

I hereby declare that I have written this thesis independently, using the listed references; or in cooperation with other paper co-authors. I have submitted neither this thesis, nor any of its parts, to acquire any other academic degree.

Prague, 4th September 2014

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Martina Pichrtová

This thesis is based on the following three papers:

I. PICHRTOVÁ, M., REMIAS, D., LEWIS, L. A. & HOLZINGER, A. (2013): Changes in phenolic compounds and cellular ultrastructure of Arctic and Antarctic strains of *Zygnema* (Zygnematophyceae, Streptophyta) after exposure to experimentally enhanced UV to PAR ratio. – Microbial Ecology 65 (1): 68–83.

II. PICHRTOVÁ, M., HÁJEK, T. & ELSTER, J. (2014): Osmotic stress and recovery in field populations of *Zygnema* sp. (Zygnematophyceae, Streptophyta) on Svalbard (High Arctic) subjected to natural desiccation. – FEMS Microbiology Ecology. DOI: 10.1111/1574-6941.12288.

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Authors‘ contributions:

Paper I. Daniel Remias, Andreas Holzinger and Martina Pichrtová designed the study. Martina Pichrtová cultivated the algae, performed the experiments and Daniel Remias helped with HPLC. Martina Pichrtová wrote the major part of the manuscript and performed statistical analyses. Andreas Holzinger was responsible for the transmission electron microscopy and Louise A. Lewis for molecular phylogeny and they both wrote the respective parts of the text. All co-authors participated in final improvements of the manuscript.

Paper II. Tomáš Hájek and Martina Pichrtová jointly planned the study, performed the experiments and the statistical analyses. Josef Elster and Tomáš Hájek organized and planned the field work. Martina Pichrtová took both light and fluorescence microscope images, created the figures and wrote the manuscript. Tomáš Hájek and Josef Elster contributed to improve the text of the manuscript.

Paper III. Martina Pichrtová planned the study together with Andreas Holzinger. Martina Pichrtová cultivated the algae, performed the desiccation experiments, light microscope observations and fluorescence measurements. Andreas Holzinger performed transmission electron microsopy investigations. Martina Pichrtová performed the molecular work and statistical analyses together with Jana Kulichová. Martina Pichrtová and Andreas Holzinger jointly wrote the manuscript and Jana Kulichová helped with the parts concerning molecular phylogeny.

On behalf of all the co-authors, we declare the keynote participation (as first author) of Martina Pichrtová in completing the research and writing the papers, as described above.

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Abstract

Filamentous green microalgae of the genus *Zygnema* belong to the most common primary producers in the polar hydro-terrestrial environment. In such unstable habitats, organisms are subject to various stress factors, e.g., freeze–thaw cycles, desiccation and high irradiation levels. However, the stress resistance mechanisms that enable *Zygnema* spp. to thrive in this extreme environment are only partially understood. Therefore, polar *Zygnema* spp. were examined under various stress conditions using both field samples and cultures. Moreover, molecular phylogeny methods were applied that provided first insights into the diversity of polar *Zygnema*. Sequencing of the chloroplast gene *rbc*L revealed several different *Zygnema* genotypes and, surprisingly, one *Zygnemopsis* sp. with vegetative *Zygnema* sp. morphology. First set of experiments examined the effects of UV exposure. It turned out that polar strains of *Zygnema* produce phenolic substances as UV screens. These substances are most likely stored in vacuoles and other vesicles at the cell periphery, providing protection for other organelles. In the next study, *Zygnema* spp. were investigated under natural conditions in the Arctic. At the end of summer, the cells gradually lose their typical vegetative appearance (with large vacuoles and stellate chloroplasts) and form pre-akinetes, which are stationary-phase-like cells filled with storage material and characterized by reduced chloroplast lobes and thickened cell walls. While all natural populations consisted of pre-akinetes regardless of their water status, significant differences were revealed in their osmotic stress tolerance. These results indicated that formation of pre-akinetes was not triggered by desiccation, but hardening during slow dehydration was required for the pre-akinetes to become stress-resistant. Subsequent laboratory experiments showed that the formation of pre-akinetes was induced by nitrogen starvation. In general, viability and recovery rate after experimental desiccation depended on pre-cultivation conditions and drying rate. Moreover, the pre-akinetes survived even rapid drying (at 10% relative air humidity) when hardened by mild dehydration stress. Presented thesis contributes to our understanding of algal stress resistance mechanisms in polar hydro-terrestrial environment. The results indicate that naturally hardened pre-akinetes play a key role in survival under extreme conditions, while the production of other types of specialized cells (e.g., zygospores) is largely suppressed. Moreover, desiccation-tolerant cells derived from disintegrated filaments can act as airborne propagules.

Abstrakt

Vláknité zelené řasy rodu *Zygnema* patří mezi nejhojnější primární producenty v polárním hydro-terestrickém prostředí. V těchto nestabilních habitatech jsou organismy vystaveny celé řadě stresových faktorů, jako například cyklickému zamrzání a tání, vysychání nebo nadměrné ozářenosti. Přesto existuje jen málo studií, které se zabývají výzkumem mechanismů stresové odolnosti, které těmto řasám umožňují přežít v extrémním prostředí. Předkládaná práce proto studuje polární zástupce rodu *Zygnema* v různých stresových podmínkách, a to s využitím jak přírodních vzorků, tak kultur. Kromě toho zde byly použity metody molekulární fylogenetiky, které přinášejí vůbec první poznatky o diverzitě těchto řas v polárních oblastech. Sekvenování chloroplastového genu *rbc*L odhalilo několik rozrůzněných linií v rámci rodu *Zygnema* a přineslo i překvapivý nález jednoho druhu *Zygnemopsis* sp., jehož morfologie je ve vegetativním stavu neodlišitelná od řas rodu *Zygnema*. První sada experimentů studovala vliv UV záření na vybrané polární kmeny rodu *Zygnema*. Ukázalo se, že zkoumané řasy produkují fenolické látky, které mají schopnost pohlcovat UV záření. Tyto látky jsou pravěpodobně uloženy ve vakuolách a dalších váčcích při okraji buňky, čímž chrání ostatní organely. Další studie se zabývala přírodními populacemi řas *Zygnema* spp. v Arktidě. Ke konci léta řasy postupně mění svoji morfologii od typických vakuolizovaných vegetativních buněk k tzv. pre-akinetám, které jsou charakteristické silnější buněčnou stěnou, nahromaděním zásobních látek a redukovanou strukturou chloroplastu. Všechny zkoumané populace byly tvořeny pre-akinetami, nezávisle na stupni vyschnutí lokality, významně se však lišily svojí odolností k osmotickému stresu. Tyto výsledky naznačily, že pomalé vysychání není faktorem, který spouští tvorbu pre-akinet, ale zato je důležité pro získání stresové odolnosti. Následné laboratorní experimenty ukázaly, že tvorba pre-akinet je indukována nedostatkem dusíku. Proporce přeživších buněk a rychlost obnovy fyziologického stavu po experimentálním vysušení obecně závisela na podmínkách předchozí kultivace a na rychlosti sušení. Navíc, pre-akinety, které prošly postupným, pomalým vysycháním, byly schopné přežít velmi rychlé vysušení (při 10% relativní vzdušné vlhkosti). Tato práce přináší nové poznatky o mechanismech stresové odolnosti u řas v polárním hydro-terestrickém prostředí. Získané výsledky naznačují, že „otužené“ pre-akinety hrají klíčovou roli v přežití extrémních podmínek, kde je tvorba jiného typu specializovaných buněk (např. zygospor) velmi vzácná. Kromě toho, buňky odolné k vyschnutí hrají po rozpadu vláken důležitou roli pro šíření vzduchem.

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1 Introduction

1.1 Polar regions

In recent decades, polar regions have been a focus of much research in various scientific fields. One of the main reasons for this is contemporary climate change and its expected severe impact on high-latitude ecosystems and indigenous people. Polar ecosystems are important components of biogeochemical processes, and any future change can have significant effects on a global scale. It has been shown that polar regions have already experienced the most rapid environmental changes of any regions in the world, including changes in temperature and precipitation as well as increases in UV-B radiation levels. Moreover, polar regions are particularly vulnerable to disturbances and have long recovery times (Robinson et al. 2003; ACIA 2005; Thomas et al. 2008).

Polar regions have always fascinated explorers, as they represent some of the world's most isolated and severe wilderness areas. However, living organisms can be found even in the most remote and improbable places on our planet, and polar regions are also home to many extremophiles. Therefore, many investigations have focused on the special adaptations and survival strategies of such organisms. Revealing the secrets of their success can help us understand stress resistance mechanisms in general and can be applied to other areas of research, e.g., cryobiology (Kang et al. 2007), astrobiology (Vishnivetskaya et al. 2003) and the search for potentially biotechnologically interesting compounds (Liu et al. 2013).

Polar regions can be climatically defined by the average summer isotherm of 10°C which roughly corresponds to the tree line. In the Arctic, this isotherm undulates around the Polar Circle. In the Antarctica, on the other hand, this boundary runs well north of the Polar Circle, at about 50°S. The Arctic (Fig. 1 a) to the North and the Antarctic (Fig. 1 b) to the South differ from each other in many aspects, e.g., geological history, geography, climate and biodiversity, as well as energy transport and balance. The Arctic is primarily covered by ocean and is very well interconnected with other regions of the northern hemisphere; these regions exchange matter and energy and continuously influence each other. The Antarctic, on the other hand, is dominated by a landmass. It is a continent that is very well-isolated from the rest of the world by the wind-driven Circumpolar ocean current; the coldest, highest, driest and windiest continent in the world. Together, the Arctic and Antarctic comprise the largest area with extreme environments on Earth, challenging living organisms with multiple environmental stresses (Robinson et al. 2003; Elster & Benson 2004; Thomas et al. 2008).

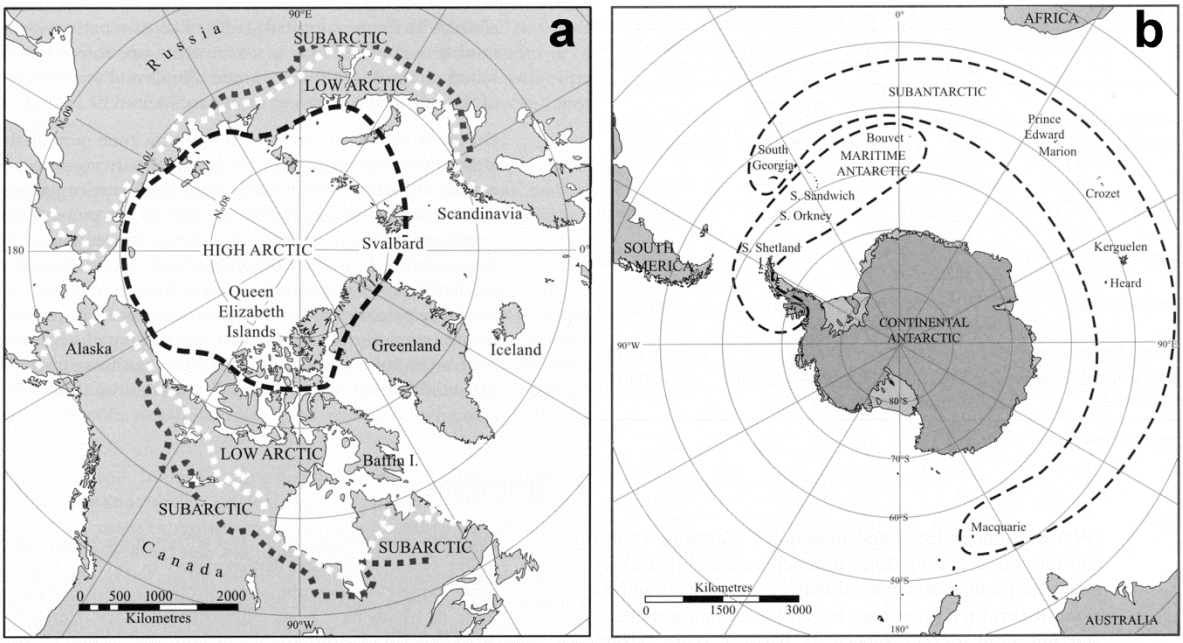

Figure 1. The Arctic (**a**) and Antarctica (**b**) with generally recognized biogeographical zones. From Thomas et al. (2008).

1.2 Extremes and stresses

How are "extremes" and "stresses" actually defined? Unfortunately, the answer is not straightforward and there are many definitions of these terms. "Extreme" can be perceived as something that differs considerably from "normal" from the anthropocentric point of view, which can be misleading because such environments can still be inhabited by well-adapted organisms. More precisely, extremes can be, for example, defined by physical factors that are near the limit required for cellular functioning (Rothschild & Mancinelli 2001).

Lichtenthaler (1996) defined stress as any unfavorable condition or substance that affects or blocks a plant's metabolism, growth or development. He discerned two types of stress with different effects: i) eustress, which is stimulating, activates reactions and increases physiological activity, and ii) distress, which is purely damaging. Subsequently, Lambers et al. (2008) defined stress as an environmental factor that reduces the rate of a physiological process under its maximal value. He described three timescales in the reaction to stress: stress response (the immediate detrimental effect), acclimation (morphological or physiological adjustment in response to changing conditions) and adaptation (a genetically fixed response). In addition, Elster (1999) distinguished acclimatization as a natural process and acclimation as a response induced under laboratory conditions.

1.3 Stress factors in polar regions

There are several more or less interrelated stress factors that are typical of polar regions. Low temperatures are primarily associated with Arctic and Antarctic habitats. In fact, the lowest temperature ever recorded on Earth (-89.2°C) occurred in the Antarctic station Vostok in 1983. In general, in polar regions, frost-free periods last at most a few months (Figs 2 a, 3), and diurnal fluctuations in temperature causing repeated freeze–thaw cycles can occur at any time (Davey 1991a). However, the soil temperature at sites covered with snow does not drop far below zero, even during the winter (Davey 1991b; Hawes 1989), and larger lakes can even retain liquid water year-round (Hawes 1988). Low temperatures have a profound impact on living organisms. With decreasing temperatures, membrane fluidity also decreases, and in accordance with the Arrhenius equation, the rate of metabolic reactions slows down. This slowdown also affects repair processes, which explains why other stress factors are more harmful at low temperatures (Roos & Vincent, 1998). Nevertheless, biochemical processes can continue even at subzero temperatures, as long as the intracellular water remains liquid (Elster & Benson 2004).

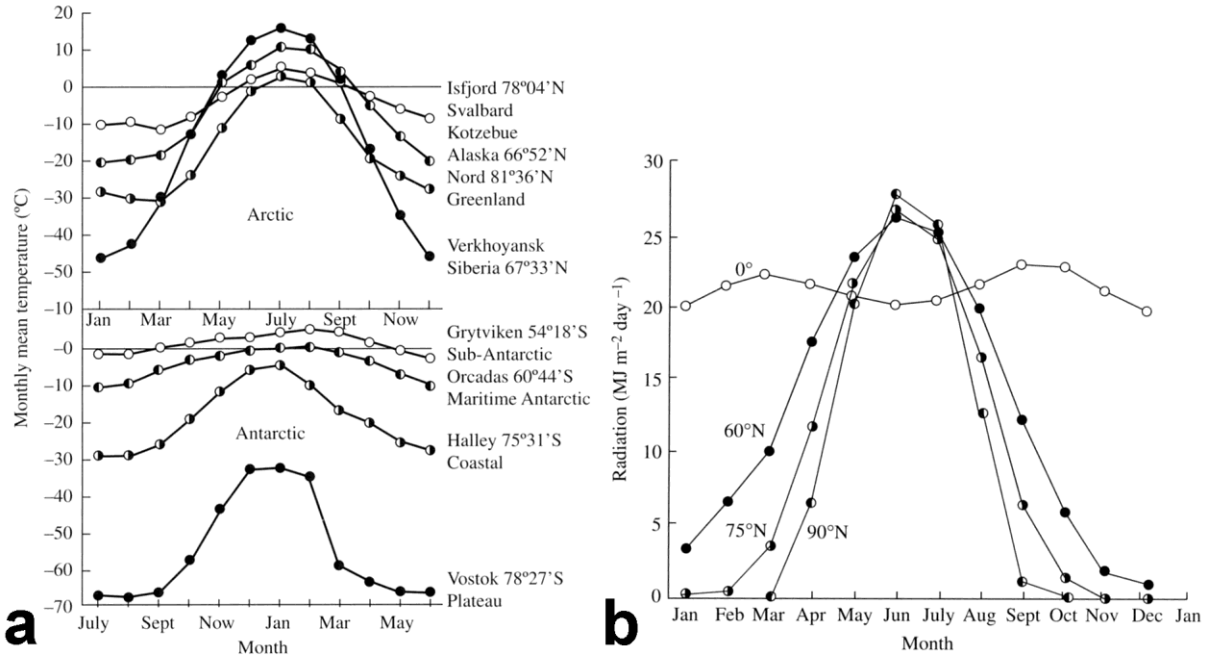

Figure 2. a Mean monthly temperature at different locations in the Arctic and Antarctic, from Thomas et al. (2008); **b** estimates of total direct radiation, with corrections for variations in atmospheric turbidity on the 15th day of each month at sea level at various latitudes north, from Thomas et al. (2008), after Hutchinson.

The presence of liquid water is essential for maintaining the three-dimensional structures of proteins and other molecules, and only fully hydrated cells can be physiologically functional (Holzinger & Karsten 2013). Freezing leads to the loss of water in liquid form, and extracellular freezing causes intracellular solute concentrations to increase. Moreover, intracellular freezing disrupts membranes and mechanically damages cells (Hawes 1990). Besides, polar regions are generally characterized by arid climates, and the lack of liquid water in the environment is an important stress factor even in frost-free periods (Robinson et al. 2003; ACIA 2005). Thus, freezing, direct desiccation and salt stress are closely related because they all lead to osmotic dehydration and lower intracellular water potential (Bisson & Kirst 1995). The physiological effects of these stresses are similar, as are their resistance mechanisms, and it has even been hypothesized that acclimation during exposure to an individual stress can result in resistance to other stresses (Morison & Sheath 1985; Pearson & Davison 1994; Welsh 2000; Vilumbrales et al. 2013). In addition, the use of solutions of a defined osmotic potential was even proposed as the most straightforward method for investigating desiccation tolerance (Pearson & Davison 1994; Hoppert et al. 2004). Nevertheless, the abovementioned stresses are complex, and components other than osmotic dehydration play a role in their effects (e.g., ionic component of salt stress, matric component of direct desiccation, mechanical injury from ice crystals). This is reflected by their differential effects on physiology and survival in comparable experiments; there are many such examples in phycological research (Gilmour et al. 1984; Davey 1989; Pearson & Davison 1994; Stibal & Elster 2005; Šabacká & Elster 2006; Knowles & Castenholz 2008; Affenzeller et al. 2009; Darehshouri & Lütz-Meindl 2010; Gustavs et al. 2010; Souffreau et al. 2010; Gao et al. 2014).

Solar radiation, despite being an essential factor for photosynthesis, can also be stressful. Around midsummer, the daily energy income reaching the Earth's surface at the poles is higher than that on the equator (Fig. 2 b), despite the fact that the overall annual sum of energy per unit surface is low (Thomas et al. 2008). The maximum incident photosynthetically active radiation (PAR) flux measured in the Arctic and Antarctic reaches approximately 1500 µmol m^{-2} s^{-1} (Davey 1991b; Stibal et al. 2007; Becker et al. 2010). High PAR flux can lead to photoinhibition or even photodamage (Franklin & Forster 1997). The damaging effect of high irradiance is even more pronounced when it is coupled with desiccation stress. In this case, the photosynthetic apparatus can be damaged, as photochemical and biochemical reactions become blocked, but the continuing chlorophyll excitation results in the formation of reactive oxygen species (ROS); (Büdel 2011).

Photoinhibition is accelerated by various stress factors that limit CO_2 fixation and thus suppress repair of photodamaged photosystem II (PSII); (Takahashi & Murata 2008).

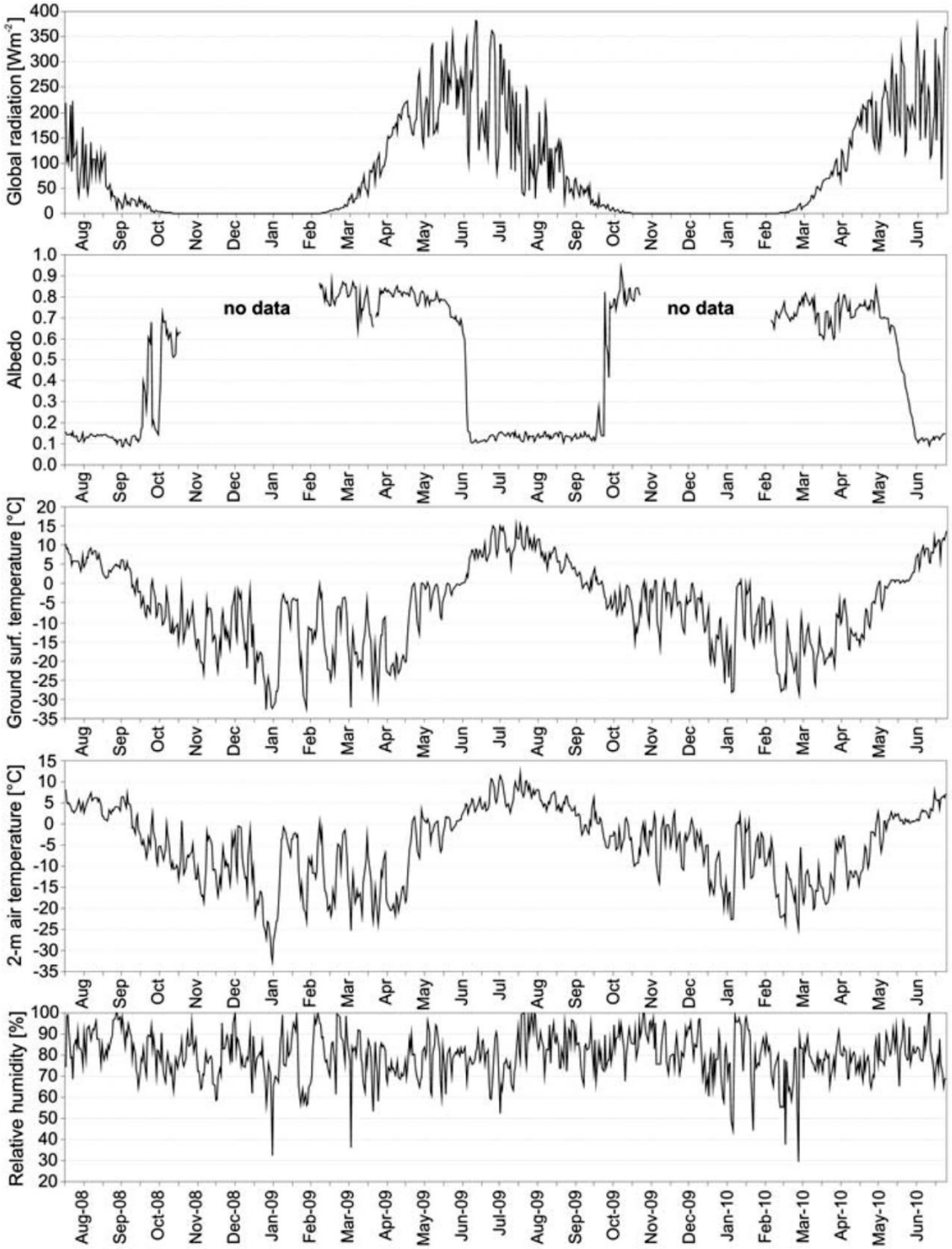

Figure 3. Various meteorological parameters measured in Petuniabukta, Svalbard, in the period from August 2008 to June 2010. Analysis of the albedo was used to estimate snow cover occurrence near the study site. Notably, ground surface temperature was measured by a near-infrared temperature sensor located at a height of 1.5 m above ground, i.e. temperature on the snow surface is given, not soil temperature beneath. From Láska et al. (2012).

Even more injurious are the effects of ultraviolet radiation. UV radiation is differentiated into four wavebands: vacuum UV (less than 200 nm) and UV C (200-280 nm) that do not reach the earth surface, UV B (280-315 nm) that is mostly attenuated by ozone layer and UV A (315-400 nm) that reaches the earth relatively unattenuated (Cockell & Knowland 1999). UV radiation is naturally low at higher latitudes due to the lower elevation of the sun. However, anthropogenic depletion of stratospheric ozone causes high UV-B events to occur regularly in the springtime in both the Arctic and Antarctic (Wessel et al. 1998). The increase in UV-B irradiance is also expected to interact with climate change. For example, it is believed that climate change will affect the dissolved organic carbon content in freshwater habitats, leading to deeper underwater UV penetration (Perin & Lean 2004). UV radiation damages DNA and other biomolecules either directly or indirectly via the production of ROS, inhibits various metabolic processes (Björn et al. 1999; Perin & Lean 2004) and causes visible ultrastructural changes (Holzinger & Lütz 2006). Increased UV radiation will not only affect the physiology and distribution of individual species, but it will also have consequences for entire communities and ecosystems (Vincent & Roy 1993; van Donk et al. 2001; Wong et al. 2007).

In addition, several other stress factors are also characteristic of polar habitats. In contrast to high irradiance in the summer, the winter period brings up to 5 months of complete darkness (Figs 2 b, 3). The temperature and light availability also determine the length of the vegetation season, which is very short in polar regions. Nutrient cycling is relatively slow in polar habitats (Robinson et al. 2003) and nutrient availability is usually a limiting factor (Davey & Rothery 1992; Arnold et al. 2003), although exceptions occur, such as in bird rookeries (González Garraza et al. 2011). Moreover, the unpredictability and instability of the polar environment can be regarded as a separate stress factor. Finally, it should be noted that in addition to abiotic stress factors, several biotic stress factors also affect organisms in polar regions, e.g., competition and grazing pressure (Rautio et al. 2011).

1.4 Microalgae in polar regions

One of the most apparent consequences of extreme climatic conditions in polar regions is the low biodiversity and ground cover of vascular plants (Robinson et al. 2003; ACIA 2005). On the other hand, microalgae, along with Cyanobacteria and lichens, have adapted to a wide range of extreme environments on Earth, including the Arctic and Antarctic. These organisms

thrive in various habitats, both marine and terrestrial, which differ in their environmental characteristics and dominant stress factors, which in turn governs their biodiversity. Microalgae represent the most important primary producers, form and stabilize the soil and participate in primary succession following deglaciation (Elster 2002; Elster et al. 2002; Elster & Benson 2004). Standing at the base of the food web, polar microalgae also play a key role in global biogeochemical cycling (Lyon & Mock 2014). In this thesis, I will focus on polar non-marine non-lichenized eukaryotic algae (microalgae) and their resistance to various stress factors, above all, desiccation and UV radiation.

1.4.1 Non-marine microalgal habitats

Elster (1999) distinguished two types of extreme environment in polar regions: i) stable, where organisms live near the limit of their physiological potential but can still be very well-adapted to the environment, and ii) marginal, unstable habitats where organisms have to deal with seasonal and diurnal fluctuations across extremes. In addition, Elster (2002) classified polar non-marine habitats into three groups with respect to the availability of water: i) lacustrine, with liquid water available year-round, such as lakes; ii) hydro-terrestrial, with liquid water available almost throughout the summer; and iii) terrestrial, which are only temporarily or periodically wetted.

Lakes in polar regions are quite stable habitats because they usually do not freeze solid during the winter (Elster 2002). Nevertheless, in such habitats, other stress factors can determine the survival of organisms. For example, *Spirogyra* populations experience high mortality during the winter due to the anoxic conditions that develop under the ice (Hawes 1988). Hydro-terrestrial environments include wetlands, shallow lakes and pools, snow-fed streams and rivers, seepages, springs and so on. Moreover, snow and ice microalgae, including inhabitants of cryoconite holes and supraglacial pools, are also included in this category (Elster 2002). The terrestrial environment is home to soil and aerophytic microalgae that grow on various types of substrates including rocks, bones, wood and so on (Elster 2002). Endolithic microbial communities can also be found in McMurdo Dry Valleys, regions characterized by an extremely cold and dry climate, even by Antarctic standards (Omelon 2008). Soil crusts, i.e., surface aggregates of algae, Cyanobacteria, fungi, lichens and other microorganisms, also belong to this category (Elster 2002).

There are many publications that describe microalgal biodiversity in various environments in Svalbard (Kaštovská et al. 2005; Kim et al. 2008, 2011; Richter et al. 2009),

the Canadian Arctic (Elster & Svoboda 1996; Sheath et al. 1996; Elster et al. 1997) and the Antarctic (Marshall & Chalmers 1997; Mataloni & Tell 2002; Kopalová et al. 2011; Skácelová et al. 2013). Nevertheless, our knowledge of the ecophysiological performance and stress resistance mechanisms of polar microalgae is still very fragmentary.

1.4.2 Mechanisms of algal stress resistance

As microalgae are able to survive the harsh physical conditions of the polar environment, some special adaptations can be expected. However, the individual stress factors listed above are naturally present in other environments as well. For example, if we also take into account deep oceans, cold temperature ecosystems cover 70% of the Earth's surface (Morgan-Kiss et al. 2006). Therefore, the mechanisms of stress resistance are general and are not exclusively characteristic of polar algae. Throughout this thesis, the word "resistance" is used as a general term describing the ability of an organism to survive in a stressful environment.

The mechanisms of stress resistance can be divided into several groups. However, every classification is highly artificial and differs among various authors. Lambers et al. (2008) distinguished two main types of stress resistance: avoidance and tolerance. Elster & Benson (2004) distinguished avoidance and protection, which also includes partnerships with other organisms. Recent reviews describing the mechanisms of UV and desiccation tolerance define three main categories: avoidance, protection and repair (Holzinger & Karsten 2013; Karsten & Holzinger 2014), whereas other publications describing UV tolerance usually distinguish screening and quenching as two separate categories aside from avoidance and repair (Vincent & Roy 1993; Perin & Lean 2004). Sometimes, the categories even overlap. For example, the production of resting stages is usually considered to be an "avoidance" strategy, but the resting cell itself is resistant due to its thick cell wall, adjusted biochemical composition and so on, i.e., mechanisms usually considered to represent "protection" strategies.

Here, I present a brief, inexhaustive overview of some stress resistance mechanisms and strategies that are most important for polar microalgae and most relevant for the topic of this thesis without attempting to group them into larger categories:

Self-protection

Many microorganisms protect themselves by aggregating into extensive macroscopical multilayered structures, such as mats and crusts. Such growth patterns can protect the microorganisms from multiple stresses at the same time. The microorganisms are usually

embedded in mucilage or in an extracellular matrix formed by hydrophilic polysaccharides, which can retain water around the cells and protect the entire community from freezing and desiccation stress (de los Ríos et al. 2004; Knowles & Castenholz 2008). The multilayered structure of mats and crusts also provides protection from excessive radiation. For example, the upper layers of the littoral alga *Ulva* sp. bleach to effectively protect the physiologically active subcanopy from UV-B (Bischof et al. 2002). The importance of self-shading for mat- or crust-forming microalgae is also revealed by their low-light requirements for photosynthesis under experimental conditions (Karsten & Rindi 2010; Karsten et al. 2013) and their photosensitivity during desiccation (Davey 1989; Gray et al. 2007).

Formation of resistant cells

Forming specialized resistant stages is a widely used strategy for surviving periods of unfavorable conditions. Moreover, resting cells are important for dispersal in time and space (Rengefors et al. 1998). Agrawal (2009) distinguished so-called dormant cells, which require a period of dormancy before germination, from cells that are resistant but not dormant and thus can germinate immediately after formation. In some species, resistant cells are the result of sexual reproduction, e.g., zygospores in Zygnematophyceae (Stancheva et al. 2012) and *Chlamydomonas* (VanWinkle-Swift & Rickoll 1997) and oospores in *Chara* (Agrawal 2009). Alternatively, resistant cells can form directly from vegetative cells, e.g., hypnoblasts in snow algae (Remias 2012), aplanospores in Zygnematophyceae (Stancheva et al. 2014) and akinetes.

Akinetes are formed from vegetative cells during the gradual process that usually accompanies prolonged cultivation, and thus, they can also be described as “senescent” or “stationary-phase” cells. Akinetes possess thickened cell walls and large accumulations of storage products (Fritsch 1935; Pickett-Heaps 1975; Coleman 1983). Akinetes are not dormant (Agrawal 2009), but their physiological activity is diminished, which can be accompanied by structural reductions in their chloroplasts (Morison & Sheath 1985). The formation of akinetes is induced by starvation (McLean & Pessoney 1971; Nagao et al. 1999), desiccation (Morison & Sheath 1985; Nagao et al. 1999), salt stress (Meindl et al. 1989) or exposure of aging cultures to high temperatures (Hall & Walmsley 1991). Akinetes are markedly resistant to various stresses, e.g., desiccation (McLean & Pessoney 1971), UV radiation (Agrawal & Singh 2000) and freezing (Nagao et al. 1999). Similar strategies are well-known in other organisms, e.g., bacteria, where starved and metabolically less active cells are more stress-resistant than growing cells (Siegele & Kolter 1992).

UV screening

Algae are able to synthesize various substances that effectively screen UV radiation. In general, such substances possess aromatic conjugated systems, but biochemically, the UV screens belong to several different groups (Cockell & Knowland 1999). The screening effectiveness is determined not only by the content of the screening compounds, but also by other factors, for example their spatial location or shape of the cells (Xiong et al. 1999). These substances usually accumulate in cell walls or at the cell periphery to provide effective protection for chloroplasts and other organelles in the central region of the cell (Remias et al. 2011; Aigner et al. 2013).

Most widespread among various algal classes are mycosporine-like amino acids (MAAs). These substances are colorless and water-soluble, with peak absorbances between 310 and 360 nm (Cockell & Knowland 1999; Shick & Dunlap 2002). MAAs can be found, for example, in Cyanobacteria, dinoflagellates, prymnesiophytes (Carreto & Carignan 2011), red algae (Hoyer et al. 2002) and aeroterrestrial microalgae from the class Trebouxiophyceae (Karsten et al. 2005). Their accumulation in response to UV-B exposure has been proven many times (Karsten et al. 1999, 2005, 2007; Kitzing et al. 2013) and is believed to be species-dependent and MAA-specific (Xiong et al. 1999). Besides acting as passive UV screens, MAAs also function as antioxidants (Coba et al. 2009; Carreto & Carignan 2011) and are involved in osmotic regulation (Shick & Dunlap 2002; Oren 2007).

Another large group of UV-screening compounds are phenolics. Above all, phenolics are widespread in higher plants where they have various functions. In algae, phenolics are restricted to only a few groups. Phaeophyceae contain phlorotannins, which are stored in special vesicles termed physodes (Fig. 4 a); (Pavia et al. 1997; Schoenwaelder 2008; Holzinger et al. 2011). Siphonous green algae produce coumarins, which are involved not only in UV screening but also in the formation of plugs at the site of injury (Pérez-Rodríguez et al. 2001). Moreover, phenolics were also detected in some Zygnematophyceae, e.g., tannins in *Spirogyra* and purpurogallins in *Mesotaenium* (Fig. 4 c); (Remias et al. 2012). In contrast to MAAs, phenolics do not contain nitrogen (Fig. 4 c), which makes their synthesis advantageous in extreme habitats with nutrient deficiency (Figueroa et al. 2009; Aigner et al. 2013).

Algaenans (sporopollenin-like substances) are part of the cell walls of many algae. These substances were detected in the cell walls of zygnematophycean zygospores (Hull et al. 1985; Poulíčková et al. 2007) and *Coleochaete* zygotes (Delwiche et al. 1989) and in the vegetative

cell walls of various other microalgae (Xiong et al. 1997). Apart from UV screening, algaenans are also involved in desiccation resistance (Versteegh & Blokker 2004).

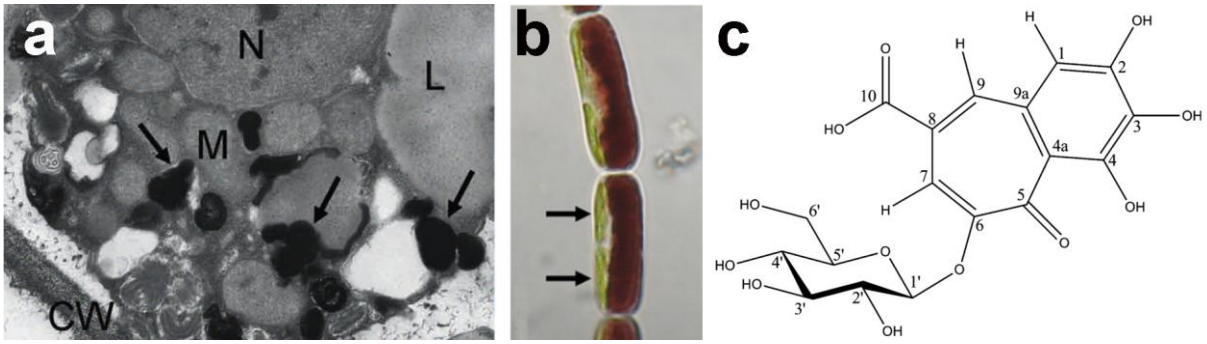

Figure 4. Phenolics in algae. **a** Physodes, phlorotannin containing vesicles (denoted by arrows) in a tissue of *Saccharina latissima* (Phaeophyceae), from Holzinger et al. (2011); **b** dark brown vacuoles of *Ancylonema nordenskioeldii* containing putative phenolic pigment, parietal chloroplasts denoted by arrows, from Remias et al. (2011); **c** chemical structure of purpurogallin carboxylic acid-6-O-b-D-glucopyranoside isolated from *Mesotaenium berggrenii*, Remias et al. (2012).

Scavenging of reactive oxygen species

ROS are normal byproducts of aerobic metabolism (Apel & Hirt 2004). However, their production is enhanced by various abiotic stress conditions, e.g., under UV radiation (White & Jahnke 2002), desiccation (Kranner et al. 2008) and salt stress (Darehshouri & Lütz-Meindl 2010). ROS are highly reactive molecules or ions that produce oxidative damage in DNA, proteins and lipids. Their formation can be partly prevented by dissipating excess light energy in the xanthophyll cycle as heat (Kranner et al. 2008). In addition, to fight against ROS, algae produce a wide range of antioxidants such as ascorbic acid, glutathione, tocopherol and carotenoids. Moreover, several antioxidative enzymes (e.g., superoxide dismutase, peroxidase and catalase) are also involved in ROS scavenging (Apel & Hirt 2004; Kranner et al. 2008). For example, the activity of the antioxidative enzyme superoxide dismutase increases with increasing salinity in the Antarctic ice alga *Chlamydomonas* sp. (Kan et al. 2012) and after UV exposure in *Chaetomorpha linum* (Bischof et al. 2006). Similarly, secondary carotenoids accumulate in cytoplasmic oil droplets in *Haematococcus pluvialis* in response to various unfavorable environmental conditions (Lemoine & Schoefs 2010). In addition to their antioxidant activity, secondary carotenoids act as respiratory substrates and protect cells from excessive PAR irradiance (Bidigare et al. 1993). Moreover, a 13Z-cis isomer of a secondary carotenoid astaxanthin has an additional absorption shoulder near the UV-A region and can thus also be counted among the UV screens (Remias & Lütz 2007).

Accumulation of osmotically active compounds

Preventing water loss and maintaining positive turgor (or constant volume in wall-less cells) is essential for normal cellular function. To maintain homeostasis under stress conditions, algae synthesize and accumulate various substances that reduce cellular osmotic potential and increase the water-holding capacity (Bisson & Kirst 1995). Osmolytes, which are chemically diverse, include inorganic ions, sugars (e.g., sucrose, trehalose and raffinose), polyols (e.g., glycerol and sorbitol), amino acids (proline) and other substances (dimethylsulfoniopropionate); (Bisson & Kirst 1995). The accumulation of osmolytes in response to stress is an important part of the acclimation process. For example, sugars accumulate during cold acclimation in *Klebsormidium flaccidum* (Nagao et al. 2008), and the content of polyols increases with increasing salinity in the investigated Trebouxiophyceae species (Gustavs et al. 2010).

Some organic osmolytes play complex roles in cellular protection. These osmolytes are termed compatible solutes because they are highly soluble, can accumulate to high concentrations and do not interact with cellular functions. Such compounds protect proteins and stabilize membranes (Bisson & Kirst 1995; Yancey 2005). Some disaccharides, notably trehalose, are able to structurally replace water and bind to macromolecules and membranes, thus preserving their structural integrity and biological activity in the dehydrated state. This process is termed vitrification and is involved in both desiccation and freezing tolerance (Welsh 2000; Yancey 2005; Clarke et al. 2013). Furthermore, sugar alcohols can also act as antioxidants, cryoprotectants, heat protectants or rapidly available respiratory substrates (Welsh 2000; Yancey 2005), and they can even be used as chemotaxonomic markers (Gustavs et al. 2011).

Other important mechanisms and protective substances

Algae that are capable of active movement can easily avoid stress conditions via migration. This strategy does not apply exclusively to small flagellates; for example, even the filamentous alga *Spirogyra* is capable of phototactic gliding movements (Kim et al. 2005).

At low temperatures, it is necessary to maintain the appropriate reaction rates of enzyme-catalyzed reactions (Morgan-Kiss et al. 2006; Lyon & Mock 2014). Alternatively, low reaction rates can be compensated for by higher concentrations of the enzyme (Thomas et al. 2008). The maintenance of membrane fluidity is achieved by the incorporation of polyunsaturated, short, branched or cyclic fatty acids (Morgan-Kiss et al. 2006; Lyon & Mock 2014). To counteract the damaging effects of growing ice crystals, ice-binding proteins are

produced extracellularly, which causes structural modifications of ice and inhibits its recrystallization, thus maintaining external liquid water (Raymond et al. 2009).

Finally, if all of the avoidance and protection mechanisms fail to prevent damage, repair mechanisms must become involved. DNA damage caused by UV radiation is repaired via two processes: photoreactivation (Sutherland 1981) and excision repair (Britt 1995). In addition, the degradation of D1 protein of PS II, which causes chronic photoinhibition, must be compensated for by its de novo synthesis (Aro et al. 1993).

1.4.3 Survival strategies of hydro-terrestrial microalgae

The distinction between aquatic and terrestrial habitats is not always clear in polar regions, which is why the category "hydro-terrestrial" was introduced (Elster 2002). These habitats usually have a supply of liquid water during the vegetation season. However, they are shallow and freeze solid during the winter, which is why they are described as extreme and unstable. This section presents an overview of eukaryotic microalgae of the polar hydro-terrestrial environment, their ecophysiology and stress resistance, in light of the general mechanisms listed in the previous chapter.

Shallow lakes and ponds that seasonally dry out or freeze solid are quite abundant in the Arctic. Compared to large lakes, ponds are usually richer in nutrients. Typically, pond productivity and biomass is driven by nutrient-sufficient benthos, whereas the water column is ultra-oligotrophic and nutrient limiting for plankton (Bonilla et al. 2005; Rautio et al. 2011). Cyanobacteria-dominated benthic mats are characterized by their multilayered structures, with UV-protective substances in the top layers and a high concentration of light harvesting pigments below (Vincent et al. 1993; Bonilla et al. 2009). Plankton is usually taxonomically diverse and rich in carotenoids (Vincent et al. 1993; Bonilla et al. 2009).

Snow and ice algae not only occur in the Arctic and Antarctic, but they are also quite common in high alpine environments throughout the world. Therefore, investigations of their stress resistance have mostly been based on materials from non-polar regions. Remias (2012) reviewed the current knowledge of their morphology and physiology. Briefly, snow algae live in liquid water between snow crystals. These microalgae usually have complex life cycles with different stages that enable them to adapt quickly to changing conditions. Flagellate stages actively migrate vertically through the snow body, and resistant cysts with accumulated secondary carotenoids are produced for their survival under unfavorable conditions (Müller et al. 1998; Remias 2012). Leya et al. (2009) investigated various snow algae from the Arctic

and concluded that members of Chlorophyceae accumulate secondary carotenoids in response to nitrogen starvation and light stress, whereas members of Trebouxiophyceae (*Raphidonema* sp.) do not; they instead have large xanthophyll cycle capacity and high amounts of the antioxidant α-tocopherol. This observation supports the hypothesis that *Raphidonema nivale* is not a "true" snow alga but rather an occasional visitor from the soil environment (Stibal & Elster 2005).

In contrast to seasonal snowfields, glaciers are usually inhabited by immotile algae. Bare ice surfaces primarily host members of the class Zygnematophyceae, e.g., *Ancylonema nordenskioeldii* (Remias et al. 2011) and *Mesotaenium berggrenii* (Remias et al. 2009, 2012). Arctic *A. nordenskioeldii*, which was investigated by Remias et al. (2011), does not produce any specialized cells but is instead stress-resistant in the vegetative state. This microalga has low temperature requirements for photosynthesis and accumulates dark vacuolar pigments with combined UV and PAR absorption (Fig. 4 b). *A. nordenskioeldii* is very well-adapted to its extreme habitat; even sexual reproduction was observed under natural conditions (Remias et al. 2011).

Shallow puddles, wetlands, wet soil, streams and so on are another type of polar hydro-terrestrial environment. In addition to winter freezing, these environments can be subject to desiccation and other stresses during the summer. They are often dominated by Cyanobacteria, which are generally more stress-resistant than eukaryotic microalgae and therefore rather perennial, whereas eukaryotic microalgal mats have rather annual characters due to their high winter mortality (Davey 1991b; Tang et al. 1997; Elster 2002). Interestingly, most species do not form specialized cells, but their vegetative cells are able to survive stress conditions, as they possess thick cell walls and accumulate storage materials (Sheath et al. 1996).

Seaburg et al. (1981) investigated the temperature range for the growth of 128 cultures of Antarctic microalgae including strains isolated from hydro-terrestrial habitats. All isolates could grow at temperatures between 7.5 and 18°C, and most of them were able to grow at or under 5°C. Several strains of the genus *Klebsormidium* isolated from glacial melt streams are cold-adapted; they did not grow at temperatures above 20°C. These results indicate that Antarctic microalgae are psychrotrophs rather than psychrophiles, which is probably due to the instability of the environmental conditions. Nevertheless, the success of microalgae under natural conditions in the Antarctic is determined by the lowest temperature that allows growth rather than by the growth rate at their temperature optimum (Seaburg et al. 1981).

In addition, microalgae isolated from various polar hydro-terrestrial habitats were, in general, resistant to both freezing and desiccation (Šabacká & Elster 2006; Elster et al. 2008). Desiccation at 20°C was much more injurious than desiccation at 0 or 4°C. The microalgae also survived freezing down to -40°C, although with reduced viability (Šabacká & Elster 2006; Elster et al. 2008). Nevertheless, the eukaryotic microalgae were much less resistant to both stresses compared to Cyanobacteria, which predetermines the annual character of their mats (Šabacká & Elster 2006).

Numerous studies have investigated stress resistance of trebouxiophycean green algae of the genus *Prasiola* in both the Arctic and Antarctica. *Prasiola* can be found in various hydro-terrestrial habitats including marine supralittoral, streamlets, wet soil and the surface of ice. *Prasiola* is considered to be rather nitrophilous because it typically grows on moist soils fertilized by penguin guano or similarly, in the Arctic, in the vicinity of seagull colonies (Holzinger et al. 2006). A recent molecular phylogenetic study revealed a cryptic diversity within Antarctic *Prasiola* (Moniz et al. 2012). Antarctic specimens formed three well-supported clades corresponding to three different species. Remarkably, there were some differences in autecology, and the authors pointed out the need for careful species determination when interpreting the results of ecophysiological studies (Moniz et al. 2012). Unfortunately, such taxonomic information was not available for past studies of *Prasiola* stress tolerance.

Although the photosynthetic optimum for *Prasiola* occurs at high temperatures (Davey 1989; Kosugi et al. 2010), photosynthesis continued down to -7°C (Davey 1989) or even -15°C (Becker 1982), even though the thallus appeared frozen. It appears that *Prasiola* synthesizes free proline as a cryoprotectant because this amino acid was shown to accumulate at the onset of winter (Jackson & Seppelt 1995). *Prasiola* is also tolerant of hypersaline conditions (Jacob et al. 1992) and survived experimental desiccation as well (Davey 1989; Kosugi et al. 2010), but its recovery after rehydration depended on the remaining water content and the desiccation time; more than 90% water loss was lethal (Jacob et al. 1992). The effects of both freezing and desiccation stress were also enhanced by high irradiance (Davey 1989).

Antarctic *Prasiola* also tolerates high levels of UV radiation. Even 2 $W\,m^{-2}$ did not significantly alter its photosynthetic performance (Lud et al. 2001), and no UV was detected at the base of 15 mm thick mats (Post & Larkum 1993). Moreover, the upper layers bleached (Post & Larkum 1993; Lud et al. 2001), which indicates that mat-forming growth can protect the lower layers in a way similar to that of phytobenthic mats (Bonilla et al. 2005; Tanabe et

al. 2010). Furthermore, a UV absorbing compound was detected (Post & Larkum 1993) that was later classified as an MAA typical of Trebouxiophyceae (Karsten et al. 2005). Similarly, the Arctic supralittoral green alga *Prasiola crispa*, which lives under almost terrestrial conditions, is also well-adapted to UV, as demonstrated by chlorophyll fluorescence and ultrastructural investigations (Holzinger et al. 2006).

Although the hydro-terrestrial environment of both the Arctic and Antarctic may be viewed as rather inhospitable, it in fact hosts a diversity of microbial life. Microalgae belong to the main phototrophs of these habitats and often produce high amounts of biomass. Some representatives, e.g., *Prasiola*, have been quite intensively studied, and their resistance mechanisms are relatively well-understood. Nevertheless, there are still many aspects of life in this extreme environment that await further investigation.

1.5 Polar *Zygnema*

For this Ph.D. thesis, conjugating green microalgae with vegetative *Zygnema* sp. morphology were selected. These green microalgae belong to the most common eukaryotic components of microalgal mats in both the Arctic (Sheath et al. 1996; Kim et al. 2008; Holzinger et al. 2009) and Antarctic (Hawes 1989, 1990; Davey 1991b; Skácelová et al. 2013). They typically occur in hydro-terrestrial environments, such as slow-flowing meltwater streamlets, shallow puddles and ephemeral pools, where they produce high amounts of mucilaginous biomass (Fig. 5 d). Such shallow, temporary, unstable habitats represent a transition between the freshwater and terrestrial environments, where microalgae are exposed to multiple stress conditions, above all, high solar irradiance including UV, freezing and desiccation. Although *Zygnema* seems to be successful in polar regions, the mechanisms of its stress resistance have not been completely elucidated.

Zygnema sp. (class Zygnematophyceae) is a filamentous microalga with two stellate chloroplasts per cell (Fig. 5 a). Zygnematophyceae belong to the group Streptophyta, and their close relationship to land plants (Embryophyta) has been recently addressed (Wodniok et al. 2011; Timme et al. 2012; Zhong et al. 2013, 2014). Therefore, elucidating their stress resistance mechanisms is also of interest for understanding the process of evolution and the transition of plants to land. Zygnematophyceae do not produce any flagellate stages, and they sexually reproduce by the process of conjugation, which leads to the formation of zygospores (Fig. 5 e, f).

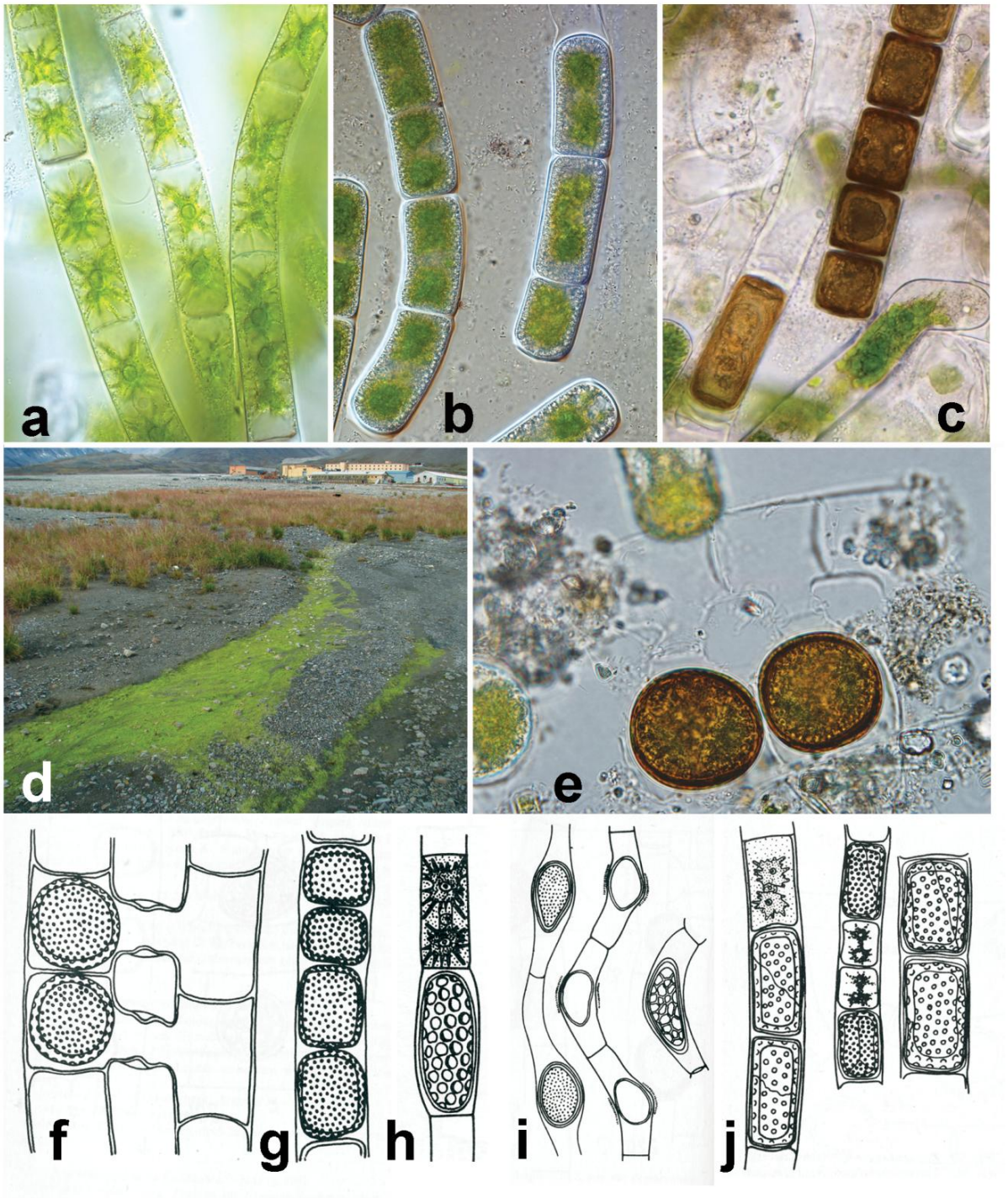

Figure 5. Morphological features of *Zygnema*. **a-c** *Zygnema* sp. CCALA 880 from the Antarctic; **a** Vegetative cells; **b** pre-akinetes (stationary-phase-like cells); **c** akinetes with distinctly coloured cell walls; **d** *Zygnema* sp. hydro-terrestrial mat near Pyramiden, Svalbard; **e** scalariform conjugation and zygospores of *Zygnema* sp. from Svalbard; **f** scalariform conjugation of *Zygnema giganteum*; **g** aplanospores of *Z. giganteum*, after Randhawa; **h** aplanospore of *Z. collinsianum*, after Transeau; **i** parthenospores of *Z. terrestre*, after Randhawa; **j** akinetes of *Z. cylindricum*, after Krieger and Transeau; **f-j** from Kadlubowska (1984).

Zygospores are highly specialized, stress-resistant cells with algaenan (sporopollenin-like material) in their cell walls (de Vries et al. 1983; Kadlubowska 1984; Poulíčková et al. 2007). The morphology of mature zygospores and sporangial shape are essential for species determination (Transeau 1915). According to traditional taxonomy, the genus *Zygnema* is divided into four sections (Kadlubowska 1984), but first studies based on molecular phylogeny showed that the traditional taxonomic concept does not correspond to natural groups (Stancheva et al. 2012). The genus *Zygnema* turned out to be monophyletic and the investigated strains formed two major subclades; Fig. 6 (Stancheva et al. 2012).

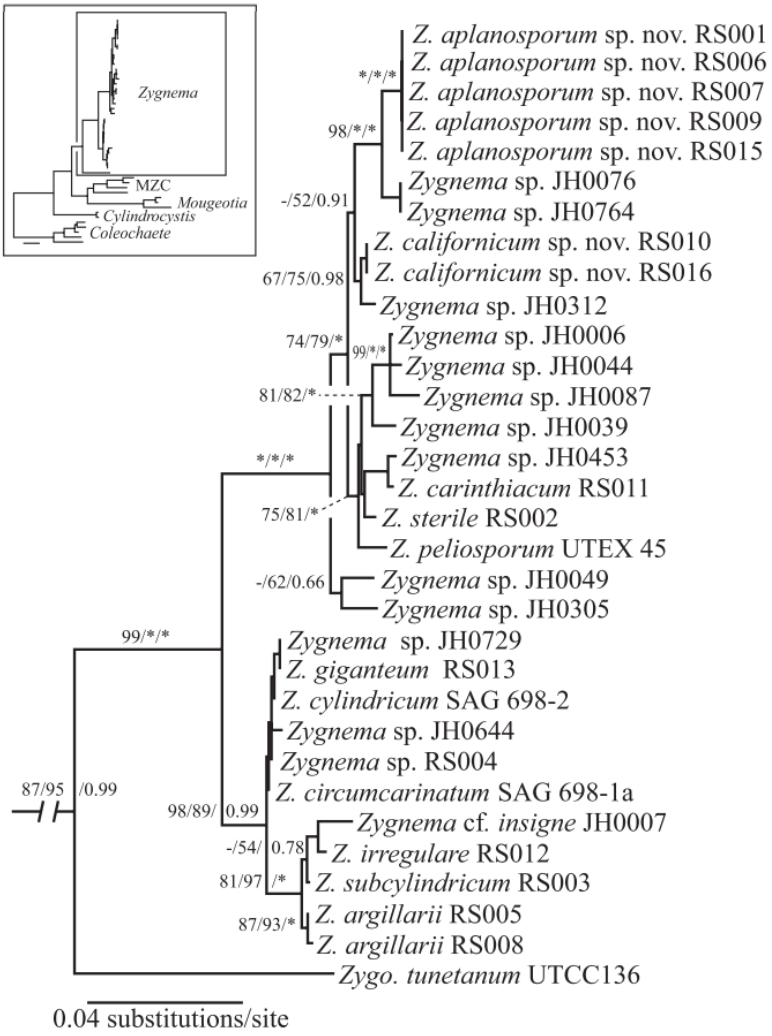

Figure 6. Maximum-likelihood phylogenetic tree of *Zygnema* and their sister lineage of *Zygogonium tunetanum* based on *rbc*L data. Numbers above the branches are bootstrap values from parsimony analysis and maximum likelihood analysis, and posterior probabilities from a Bayesian analysis, respectively. An asterisk indicates bootstrap support of 100 or a posterior probability of 1.0. A dash indicates bootstrap support of <50 or a posterior probability <0.5. From Stancheva et al. (2012).

Nevertheless, the conjugation process in *Zygnema* has never been observed in Svalbard or Antarctica and, therefore, species determination is not possible. It has even been suggested that the lack of reproductive stages may be a consequence of a trade-off between sexual reproduction and growth in extreme environments (Holzinger et al. 2009). Reports of a conjugation process and zygospore formation in *Zygnema* cf. *leiospermum* are limited to Central Ellesmere Island (79° N) (Elster & Svoboda 1996; Elster et al. 1997), indicating that sexual reproduction actually occurs in the High Arctic, although it is probably very rare in this region compared to lower latitudes.

Several studies have been published that investigated the stress resistance of *Zygnema* spp., not only in polar regions. In general, filamentous Zygnematophyceae are very sensitive in the vegetative state (McLean & Pessoney 1971; Agrawal & Singh 2002), and the formation of resistant cells is important for their survival. In addition to zygospores, other specialized cell types have been described in *Zygnema*, namely parthenospores, aplanospores and akinetes (Kadlubowska 1984; Poulíčková et al. 2007; Stancheva et al. 2012), Fig. 5 g-j. Aplanospores are formed inside vegetative cells when protoplasts are enclosed by a new cell wall, independent of the original wall (Kadlubowska 1984). Parthenospores result from incomplete conjugation; they form directly from gametes (Kadlubowska 1984).

Akinetes are formed directly from vegetative cells via thickening of the cell wall and accumulation of storage materials (Figs 5 b, 7), which is exactly the mechanism described for other algal akinetes in Chapter 1.4.2. Akinetes have granulated cell contents, reduced chloroplast lobes and brownish-yellow coloration (McLean & Pessoney 1971; Fuller 2013). Such cells formed as a result of prolonged cultivation of a strain isolated from Texas (Fig. 7). These cells were released from filaments and were found to be desiccation-tolerant (McLean & Pessoney 1971). Nevertheless, in some species of *Zygnema*, the cell walls of mature akinetes can have a similar structure, ornamentation and coloration to that of zygospores; Fig. 5 c, j (Kadlubowska 1984). Therefore, some authors distinguish these two types of cells, using the term “akinete” only for mature, specialized cells with distinctive cell-wall characteristics; Fig. 5 c, j (Genkel & Pronina 1979; Fuller 2013). In this case, the old, stationary-phase-like vegetative cells (Fig. 5 b) are referred to as “resting cells” (Genkel & Pronina 1979) or “pre-akinetes” (Fuller 2013).

Genkel & Pronina (1979) studied *Zygnema stellinum* in Belarus. The authors concluded that “resting cells” are involved in survival under short-term stress conditions in the summer, whereas zygospores, parthenospores or “akinetes” are required for survival in the winter. Fuller (2013) studied *Z. irregulare* from California. Pre-akinetes and akinetes formed after

several weeks of slow desiccation on agar plates, but the author suggested that in addition to desiccation, nutrient limitation may also be involved in their formation. The two types of cells differed in one important aspect: After rehydration, stationary-phase-like pre-akinetes gradually regained their vegetative appearance, whereas mature akinetes with zygospore-like cell walls had to burst open at a suture prior to germination. Fuller (2013) also noted the importance of secondary pectin layer of the cell walls of pre-akinetes – pectin acts as sponge and provides extracellular protection against desiccation – and hypothesized that cell walls of akinetes contain sporopollenin-like substances impermeable for water.

Unfortunately, the ambiguity of the term "akinete" in *Zygnema* can be confusing. In this thesis, the term "akinete" is used for stationary-phase-like vegetative cells, as is the case for other algae (Coleman 1983). If such cells still form a filament that has not yet disintegrated, they are referred to as "pre-akinetes" (Fig. 5 b). When akinetes with zygospore-like walls are discussed, they are described again to avoid confusion and are referred to as "true" akinetes.

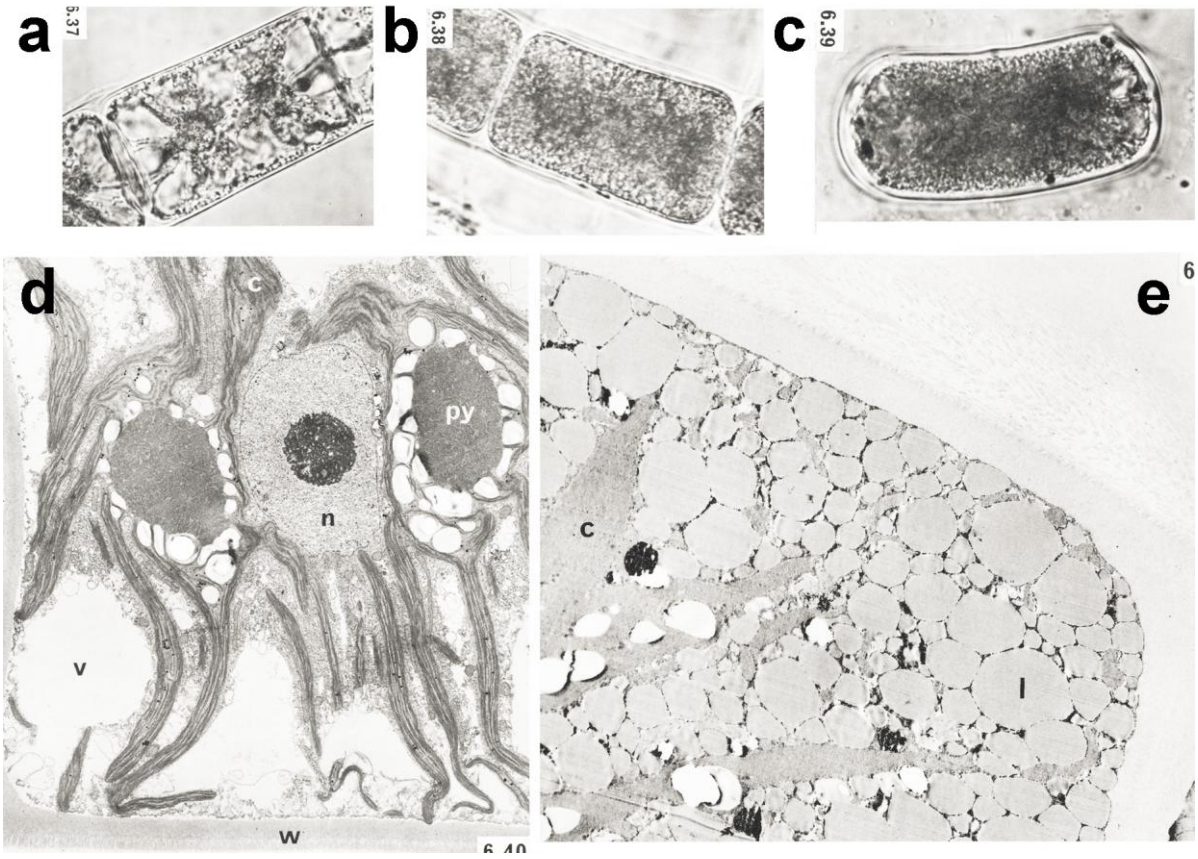

Figure 7. Morphology and ultrastructure of *Zygnema* sp. during akinete formation, after McLean & Pessoney (1971). **a** vegetative cell; **b** filament from an older culture; **c** mature akinete separated from the filament; **d** ultrastructure of a young vegetative cell with large vacuoles (*v*) and distinct chloroplast lobes (*c*); **e** ultrastructure of an akinete with typical accumulation of lipid bodies (*l*). From Pickett-Heaps (1975).

A few studies have also investigated Arctic or Antarctic *Zygnema*. The Antarctic *Zygnema* sp. was able to survive freezing down to -15 °C but only when a very slow cooling rate was applied, and thus, intracellular ice nucleation was avoided (Hawes 1990). The author did not observe the formation of any specialized cells, but all cells possessed dense cytoplasm resembling pre-akinetes. Cyclic freezing and thawing (between -4 and +5) had no effect on photosynthesis or viability, but prolonged exposure to -20°C led to high mortality; only two out of 7000 morphologically identical cells were viable. The author also detected sucrose as the main carbohydrate, but at relatively low concentrations to depress the freezing point (Hawes 1990).

The maximum growth rate of Antarctic *Zygnema* sp. was achieved at 20°C, but at low-light intensities the optimum growth temperatures were lower (Davey 1991a). Interestingly, *Zygnema* sp. was able to grow even in the very low PAR illumination level of 1 μmol m^{-2} s^{-1} (Davey 1991a), and other Arctic and Antarctic *Zygnema* sp. strains also turned out to be rather low-light adapted (Kaplan et al. 2013). Antarctic *Zygnema* sp. was also able to survive desiccation, but it was rather intolerant; only a few cells survived more than one day at the lowest humidity levels (Davey 1991a).

Arctic *Zygnema* sp. is also UV resistant, as experimental UV exposure had no negative effect on its photosynthesis or ultrastructure (Holzinger et al. 2009). However, the mechanism of resistance remains unknown. Nevertheless, the production of phenolic substances has also been revealed in other Zygnematophyceae, e.g., *Mesotaenium berggrenii* (Remias et al. 2012), *Zygogonium ericetorum* (Aigner et al. 2013), *Zygnemopsis decussata* (Figueroa et al. 2009) and *Spirogyra* (Nishizawa et al. 1985), suggesting that these compounds might also be produced by *Zygnema* and may be involved in its UV tolerance.

Recently, quite low (negative) values of cellular osmotic potentials were detected in *Zygnema* spp. from polar habitats, revealing certain levels of protection against water loss (Kaplan et al. 2013). The Arctic strains showed better osmotic stress tolerance than the Antarctic strains, but the authors of this study examined cultures, and the performance of *Zygnema* under natural conditions remained unknown. In addition, Vilumbrales et al. (2013) studied the effect of salt stress on various fluorescence parameters in Antarctic *Zygnema* sp. and concluded that *Zygnema* sp. is less tolerant to salt stress than *Klebsormidium* sp. isolated from similar localities (Vilumbrales et al. 2013).

In summary, there is only limited knowledge of the stress resistance mechanisms of *Zygnema* spp. in the Arctic and Antarctic. On one hand, *Zygnema* is very common in these regions, which indicates that this alga is well-adapted to life under extreme conditions. On the

other hand, both freezing and desiccation were found to be very stressful, with only a few surviving cells (Hawes 1990; Davey 1991b). Almost nothing is known about the formation of specialized cells in polar *Zygnema* spp. and their role in stress resistance. Moreover, the production of UV-screening substances in *Zygnema* spp. has never been studied in detail. Finally, it is unknown whether Arctic and Antarctic mats are formed by one or multiple species, and analyses based on molecular data are urgently needed.

2 Research objectives of this thesis

In the present thesis, I performed both field and laboratory observations and experiments to answer the following questions:

- **Do *Zygnema* spp. under natural conditions in Svalbard form any specialized cells?**

  Natural populations of *Zygnema* were investigated during two field expeditions that covered the entire summer season and one winter sampling. The samples were observed under a light microscope to detect changes in morphology and the formation of specialized cells. These observations are summarized in paper II, which focuses on the formation of pre-akinetes at the end of the summer.

- **What is the role of these cells in stress resistance?**

  Stress resistance of pre-akinetes was investigated under both field (paper II) and laboratory (paper III) conditions. In paper II, we studied osmotic stress resistance and recovery using sorbitol solutions and compared the reactions among populations in different states of natural dehydration. In paper III, we investigated the effect of direct desiccation. In both studies, measurements of chlorophyll fluorescence were used to assess photosynthetic activity and stress during the experiments.

- **What conditions induce the formation and resistance of such cells?**

  Based on the field investigations (paper II), we hypothesized that starvation is the main factor leading to the formation of pre-akinetes in *Zygnema* and that pre-akinetes can be hardened by slow desiccation. In paper III, we tested this hypothesis under experimental conditions: selected strains were pre-cultivated in various types of media and then desiccated using various drying rates.

- **What are the mechanisms of UV resistance of *Zygnema* spp. from polar regions?**

  In paper I, we investigated the production of UV-screening compounds in selected strains of Arctic and Antarctic *Zygnema* after experimental UV exposure. The pigment content and photosynthetic performance were studied by means of HPLC and

chlorophyll fluorescence measurements. Moreover, the effect of UV on cellular ultrastructure was studied.

- **Is there a hidden diversity in polar *Zygnema* spp. mats?**

  As species could not be determined using morphological traits, molecular methods were applied to reveal phylogenetic relationships (based on *rbc*L sequences) of the strains and their positions among other *Zygnema* spp. (papers I and III). For these investigations, samples of Arctic *Zygnema* were isolated into monoclonal cultures and Antarctic strains were obtained from culture collections.

3 Original papers

3.1 Manuscript I

Changes in phenolic compounds and cellular ultrastructure of Arctic and Antarctic strains of *Zygnema* (Zygnematophyceae, Streptophyta) after exposure to experimentally enhanced UV to PAR ratio

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Abstract

Ultraviolet (UV) radiation has become an important stress factor in polar regions due to anthropogenically induced ozone depletion. Although extensive research has been conducted on adaptations of polar organisms to this stress factor, few studies have focused on semi-terrestrial algae so far, in spite of their apparent vulnerability.

This study investigates the effect of UV on two semi-terrestrial Arctic strains (B, G) and one Antarctic strain (E) of the green alga *Zygnema*, isolated from Arctic and Antarctic habitats. Isolates of *Zygnema* were exposed to experimentally enhanced UV A and B (predominant UV A) to photosynthetic active radiation (PAR) ratio. The pigment content, photosynthetic performance and ultrastructure were studied by means of high-performance liquid chromatography (HPLC), chlorophyll *a* fluorescence and transmission electron microscopy (TEM). In addition, phylogenetic relationships of the investigated strains were characterized using *rbc*L sequences, which determined that the Antarctic isolate (E) and one of the Arctic isolates (B) were closely related, while G is a distinct lineage.

The production of protective phenolic compounds was confirmed in all of the tested strains by HPLC analysis for both controls and UV-exposed samples. Moreover, in strain E, the content of phenolics increased significantly (p=0.001) after UV treatment. Simultaneously, the maximum quantum yield of photosystem II photochemistry significantly decreased in UV-exposed strains E and G ($p<0.001$), showing a clear stress response. The phenolics were most probably stored at the cell periphery in vacuoles and cytoplasmic bodies that appear as electron dense particles when observed by TEM after high-pressure freeze fixation. While two strains reacted moderately on UV exposure in their ultrastructure, in strain G, damage was found in chloroplasts and mitochondria. Plastidal pigments and xanthophyll cycle pigments were investigated by HPLC analysis; UV A- and B-exposed samples had a higher deepoxidation state as controls, particularly evident in strain B.

The results indicate that phenolics are involved in UV protection of *Zygnema* and also revealed different responses to UV stress across the three strains, suggesting that other protection mechanisms may be involved in these organisms.

Introduction

Polar regions are characterized by extreme climatic conditions. Organisms living there have to possess adaptations that enable them to survive in such a harsh environment. Numerous abiotic stress factors have been connected with polar climate, including low temperature, drought, nutrient limitation and periodic freeze-thaw cycles during the summer [74]. Among those extreme abiotic factors, solar ultraviolet (UV) radiation seems to be not usually considered a major stress factor in polar regions [23, 24]. Solar elevation decreases towards higher latitudes, and therefore, irradiation is lower in polar regions than in temperate and tropical zones. Moreover, solar rays travel a longer path through the atmosphere in getting to the poles, resulting in a greater proportion of shortwave radiation being absorbed and scattered [23]. Over the last few decades and in light of anthropogenically induced ozone depletion, there has been greater interest in the biological effects of UV radiation on polar organisms [34, 49, 75]. It has been hypothesised that certain polar organisms may be unable to adapt to an increasing UV environment. Moreover, predictions for future scenarios allow speculations about an increase of UV radiation reaching the Earth's surface particularly in polar regions. These effects are expected to be further enhanced due to climate change [10].

Many damaging effects caused by UV irradiation have been described, of which the dominant targets are DNA and the photosynthetic apparatus, and secondary effects are caused by the production of reactive oxygen species (ROS) [e.g. 14, 19, 62-67, 76]. Photoautotrophic organisms may be especially threatened by increases in UV radiation because solar radiation is essential for their growth and survival. In polar regions, eukaryotic algae are significant primary producers with the ability to inhabit and even dominate practically all habitats [17]. Recently, extensive research has been performed on the UV resistance of polar algae, especially all marine macroalgae [e.g. 27, 30, 37, 38, 52, 53, 61], and the findings have been reviewed by Hanelt et al. [24], Holzinger and Lütz [29] and Karsten et al. [39]. In general, the resistance to UV stress in macroalgae corresponds to their depth zonation [7].

Few studies have examined the adaptation of polar freshwater or semi-terrestrial algae to UV radiation. This is rather surprising because, in habitats such as shallow pools, these algae are subject to relatively high levels of irradiation, low levels of dissolved organic carbon and low temperatures that slow down repair mechanisms [26]. Apart from that, algae from shallow aquatic localities or semi-terrestrial sites can be additionally subject to desiccation [18, 32, 38]. Additionally, in a study of the Arctic soil alga *Tetracystis*, extraplastidal carotenoids were found, which shield against irradiation [58]. In another study, Holzinger et

al. [31] investigated field collected samples of the filamentous Zygnematophyceae, *Zygnema* sp. from Svalbard (High Arctic). They concluded that this alga was well adapted to ambient conditions of high irradiation and insensitive to experimentally enhanced UV. Similarly, marine intertidal species of the Trebouxiophyceae, *Prasiola crispa* [28], and the Ulvophyceae, *Ulva* sp. [8] and *Urospora penicilliformis* [61], which need to survive temporary under almost terrestrial conditions, were also markedly resistant to UV.

Eukaryotic algae possess a range of mechanisms to reduce the impact of UV radiation. In addition to repair mechanisms, protection including UV-screening mechanisms plays an important role. The ability to produce UV-absorbing compounds has two main advantages: First, algae are not limited to the habitats where beneficial physical conditions are available, e.g. dissolved organic carbon or Fe^{3+} ions, which are able to effectively screen UV [4, 70]. Second, they are not impaired by nonspecific attenuation of both UV and visible light [14]. Various substances produced by algae with the ability to screen UV radiation are known, for example, mycosporine-like amino acids (MAAs) [13], secondary carotenoids [42], phenolics [60, 66] or sporopollenin [78].

The goal of the present study was to describe the effects caused by an enhanced UV to photosynthetic active radiation (PAR) ratio on three selected strains of *Zygnema* isolated from polar regions. Algae in the genus *Zygnema* are ideal for such investigations because they grow typically in shallow pools, streamlets or on the surface of wet soils. Moreover, they occur worldwide, including both the Arctic and Antarctica. Even in polar regions, *Zygnema* is quite common and easy to recognize due to its mat-forming growth [25, 31, 40]. As mentioned, no harmful effect of experimental UV exposure on *Zygnema* was observed in previous studies [20, 31], yet, the nature of UV protection in *Zygnema* remains unknown. We expect that the alga obtains UV resistance by the accumulation of phenolic compounds, which can be analyzed by high-performance liquid chromatography (HPLC). The UV-screening ability of phenolic substances found in some members of the class Zygnematophyceae has already been discussed [18, 60]. Moreover, high amounts of water-soluble pigments, probably also phenolics, have been reported to occur in *Zygnema cruciatum* [22], but their role in UV screening has not been tested. In this study, we examined if the production of phenolics in *Zygnema* is enhanced by experimental UV exposure. In addition, we determined pigment contents of chlorophylls and xanthophyll cycle pigment and measured photosystem (PS) II efficiency, and we also observed the ultrastructure of the cells to prove the effectiveness of UV tolerance. Finally, we established the phylogenetic relationships of the three strains of *Zygnema* according the *rbc*L sequences.

Material and methods

Origin of the strains

Three strains of *Zygnema*, originating from the polar regions, were chosen for our experiments. Two of the strains, strain B (CCALA 976) and strain G (CCALA 977), were isolated in 2010 on Svalbard (High Arctic) from shallow seepage pools in the Petunia Bay (78°40´N, 16°30´E) and deposited in the Culture Collection of Autotrophic Organisms in Třeboň, Czech Republic (CCALA, www.butbn.cas.cz/ccala/index.php). Strain E (CCCryo 278-06) was obtained from the CCCryo culture collection in Postdam-Golm (cccryo.fraunhofer.de) isolated in 2006 from a meltwater pool north of Artigas Base freshwater lake (also known as Lago Uruguay, Lake Profound or Artigas Base freshwater lake), Fildes Peninsula, Maxwell Bay, King George Island, South Shetland Islands, Antarctica. Prior to the experiment, all strains were cultivated in liquid Bold's basal medium (BBM) [9] at 15 °C with continuous light regime (~38 μmol m^{-2} s^{-1}).

DNA sequencing and phylogenetic analysis

DNA was isolated from the three polar strains of *Zygnema* (B, E and G) using the PowerPlant DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) basically as described in [36]. Polymerase chain reaction (PCR) amplification of the *rbc*L gene was performed using primers M28F, M1161R or M1390R [43, 47] or with newly designed primers 443F 5'-TCCAAGGTCCTCCTCATGGTATCC-3' and 1263R 5'-ACGGTTTGCACCTGCTCCAGGT-3'. PCR conditions were as listed in [35]. Products of cycle sequencing were analyzed on an ABI 3100 DNA Sequencer™ (Applied Biosystems, Foster City, CA, USA), with individual reads compiled into contigs in Sequencher 4.5 (Gene Codes Inc., Ann Arbor, MI, USA) and edited manually to resolve any ambiguity. *Rbc*L sequences from the study strains of *Zygnema* were compared to the NCBI database through BLAST searches [3], and the resulting top three matches of each new *Zygnema* sequence were used to produce an alignment. Published *rbc*L sequences from two members of Desmidiales, *Cylindrocystis* and *Mesotaenium*, were used as outgroup. The *rbc*L alignment includes 1,384 nucleotide positions, with a total of 146 parsimony informative sites and 89 parsimony sites for the in-group taxa only. Maximum likelihood (ML) analysis and the ML bootstrap analysis (200 replicates) were performed in PAUP* [72]. The Bayesian analysis was done in Mr. Bayes [33]. The model of sequence evolution used in the ML and Bayesian analyses was selected by jModelTest v0.1 [54] under the Akaike Information Criterion as GTR+I+gamma.

Two independent Bayesian analyses each were run for 1.1 x 10^6 generations with one cold plus three heated chains, with a subsample frequency of 200. Convergence within and between runs was determined using Tracer v1.4.1 [56]. Trees from the initial 10^5 generations were discarded as burn-in before determining the majority-rule consensus tree.

Experimental cultivation

During the experiments, the algae were exposed on agar-solidified BBM [9] plates, three parallel Petri dishes for control and three for UV-exposed samples for each strain. Regularly, a thin layer of liquid medium was added to prevent additional desiccation stress. The dishes were placed into a climate chamber (Percival PGC_6L, USA), with temperature set to 15 °C and a continuous illumination of 38.1 ± 3.9 μmol m^{-2} s^{-1} (PAR) on average. Subsequently, UV was induced by using a combination of a UV A (Sylvania BL 350, Havells Sylvania, UK; with UV A in the range of 315-400 nm with a peak at 352 nm; for spectrum see the manufacturers web site: www.spezilamp.de/sylvania-mini-lynx-longlife-p-8556.html) and a UV B (LX-363 Drago-lux 12.0, Germany; www.dragonterraristik.de/Technik/Licht-Waerme-UV/DRAGO-LUX-DELUXE-COMPACT-120-36-W-24::1793.html) fluorescence tube. Acrylic glass filters (Plexiglas® XT) with a cut-off wavelength of approximately 280 nm were placed over the UV exposure and an additional filter with a cut-off at approximately 400 nm over the control samples, respectively. The amount of incident light energy slightly varied within the whole experimental area, that is why the dishes were shifted in a circular manner twice a day to provide overall identical conditions for all replicates. The aim of the exposure was to study the effects caused by enhanced UV/PAR ratio, but not to simulate the spectral proportions of outdoor solar irradiation. Thus, the mean intensities of UV irradiation were 3.22 ± 1.69 W m^{-2} UV A and 0.018 ± 0.015 W m^{-2} UV B measured with a PMA2100 (Solar Light, USA). This gives a ratio of UV B:UV A of approximately 1:160, resulting in a predominant UV A treatment. The UV irradiation was provided for 8 h day^{-1}. The UV penetrating the cut-off filter was negligible (0.28 ± 0.25 W m^{-2} UV A and 0.0003 ± 0.0002 W m^{-2} UV B).

Light and transmission electron microscopy

Potential cellular changes induced by UV stress were observed on the morphological and ultrastructural level. A Zeiss Axiovert 200 M Light Microscope (Carl Zeiss AG, Oberkochen, Germany) equipped with a Zeiss Axiocam MRc5 camera was used for capturing micrographs.

Control and 4-day UV-exposed samples were high-pressure frozen and freeze-substituted according to the methods of Aichinger and Lütz-Meindl [2] with modifications. Prior to freezing, samples were transferred to 150 mM sucrose for 1 min. Freeze substitution was carried out in 2% OsO_4, 0.05% uranyl acetate in acetone at -80°C for 60 h. Temperature was raised to -30°C at a dT of 10°C h^{-1} within 5 h and substitution continued for 4 h at -30°C. Subsequently, temperature was raised to 20°C at a dT of 2.5°C h^{-1} for 20 h, and samples were kept at 20°C for 10 h. Samples were then transferred to propylene oxide and embedded in low-viscosity embedding resin (Agar Scientific, England). For transmission electron microscopy (TEM), ultrathin sections (~60 nm) were prepared with a Leica Ultracut, counterstained with uranyl acetate and Reynold´s lead citrate, and investigated at a Zeiss LIBRA 120 transmission electron microscope at 80 kV. Images were captured with a ProScan 2k SSCCD camera.

Photosystem II efficiency

The physiological state of the photosynthetic apparatus was determined by measuring the maximum quantum yield of PS II photochemistry in a dark-acclimated state (F_V/F_M). The samples were taken immediately after the end of the experiment (i.e. after 7 days of exposure to UV/PAR) in 5 replicates within each of the 3 Petri dishes (n=15) per strain, dark-adapted for 30 min and then F_V/F_M was measured with a Handy PEA (Hansatech Instruments, UK).

High-Performance Liquid Chromatography

Control samples and samples exposed for 7 days to the previously mentioned UV regime were investigated by HPLC. At the end of the experiment, the biomass from individual Petri dishes was filtered onto glass fibre filters (Whatman GF/C), freeze-dried for 48 h and the dry weight measured. When sufficient biomass could be obtained (strains E and G), two filters were made from each Petri dish (n=6). Only one filter per Petri dish was made for strain B (n=3).

Chlorophylls and carotenoids were quantified by HPLC according to Remias et al. [57]. Briefly, freeze-dried filters were ground with a Micro-Dismembrator (Sartorius) in pre-cooled Teflon jars with a quartz ball and extracted in *N*,*N*-dimethylformamid. After centrifugation and prior to injection, 50% methanol was added to the extract (1:2). The HPLC system (Agilent ChemStation 1100) was run with a binary solvent gradient and using a LiChroSpher column (RP-C18, 150 x 4 mm). The detection wavelength was 440 nm. Peak assignment was carried out with individual retention times and absorption spectra in comparison to available pigment standards (DHI C14 Centralen, Denmark).

The samples were also screened for polar, soluble phenolics by alternative extraction of the ground cells with methyl *tert*-butyl ether, followed by phase separation with 20% methanol. The latter phase was prepared for HPLC injection by centrifugation (15,000 g, 10 min) and filtration through 0.45 μm regenerated cellulose syringe filters. Analysis was performed with the same HPLC system as for pigments, however, using a Phenomenex Synergi Hydro column (RP-C18, 150 x 2 mm) with oven set at 30°C, pump 0.3 ml/min, solvent A: water+0.5% formic acid, solvent B: methanol+0.5% formic acid. The binary linear gradient was as follows: start 0% B, 40 min. 100% B and then 8 min. post-run with 100% A. The injection amount was 20 to 40 μl; typical peaks had retention times between 5 and 25 min. A detection wavelength of 280 nm was used for semiquantitative peak integration to gain the relative phenol content per sample. The amount of phenolics was calculated as the total peak areas normalized to the algal dry weight.

Data analyses

Data were analyzed using the general linear model (GLM) analysis of variance (ANOVA) with nested design because of the hierarchical structure of the design. The effects of factors 'strain' (B, E and G) and 'UV treatment' (UV and control) were tested. For each factor combination, three Petri dishes were used. Five replicate measurements of F_V/F_M were done in each dish and one to two filters were prepared from each dish for the HPLC analysis. Therefore, the effect of individual Petri dishes was nested in 'strain'. 'Petri dish' was considered as a random effect factor and both 'strain' and 'UV treatment' were considered as factors with fixed effects. When significance of an effect was proved by GLM ANOVA, additional Tukey's test was performed for post hoc pairwise comparisons. All statistical analyses were performed in Statistica for Windows, Version 10, Statsoft.

Results

Molecular characterization

The *rbc*L sequences of the three new *Zygnema* strains were deposited in GenBank under accession numbers JX075101 (strain B), JX075102 (strain E) and JX075103 (strain G). The sequences obtained were 721 (strain G), 1,302 (strain B) and 1,358 (strain E) nucleotides in length. Phylogenetic analysis of the new *rbc*L data with related published *rbc*L sequences (Fig. 1) demonstrated that strains B and E are closely related (they differ by 7 nucleotides, representing 0.5% difference overall) and together are related to a strain of *Zygnema* cf.

insigne isolated by John Hall (JH0007) [71]. The strain *Zygnema* sp. G is in a separate clade from strains B and E, and instead is most closely related to *Zygnema peliosporum* (UTEX LB45). The distinction of strains B and E from G is supported strongly by both ML bootstrap and Bayesian analysis (Fig. 1).

Light microscopy

All three investigated strains had typical *Zygnema* appearance with two star-shaped chloroplasts per cell, each containing a pyrenoid in the centre of the chloroplast (Fig. 2 a, c). All samples, including PAR-only, had a remarkably dense cytoplasm with chloroplasts practically filling up the whole intracellular space. In strain B, the cells were considerably browner after UV treatment (Fig 2 b), which could be a result of an increased number of vacuoles at the cell periphery. On the other hand, there was no visible difference between control and UV-treated cells in strain E, both cultures appeared similar; UV-exposed samples remained unaltered (Fig 2 c, d). On the contrary, in strain G, obvious damages could be observed after UV exposure (Fig. 2 f). Most of the cells lost their cytoplasmic streaming completely and chloroplasts disintegrated into smaller, often globular pieces.

Transmission electron microscopy

When viewed by TEM, *Zygnema* sp. B showed star-shaped chloroplasts in the centre of the cells (Fig. 3 a). The chloroplasts had a central pyrenoid with starch grains and several lobes protruding towards the cell periphery (Fig. 3 a). In the cell periphery, medium electron dense compartments and electron-dense particles were observed (Fig. 3 a, c, d) and small vacuoles were found (Fig. 3 a, d). Mostly in close vicinity of the chloroplast, Golgi bodies with at least 10 cisternae were visible (Fig. 3 b). At the Golgi bodies, a clear distinction between *cis*- and *trans*-side was possible, the latter giving rise to the *trans* Golgi network (TGN; Fig. 3 b). The cell centre contained a nucleus (Fig. 3 c) with a marked nucleolus. The vacuoles in the cell periphery were polymorphic (Fig. 3 c, d); electron-dense particles had a diameter of approximately 400-600 nm (Fig. 3 d). Close to the nucleus, microtubules were occasionally observed; mitochondria showed an internal structure with cristae (Fig. 3 e). When *Zygnema* sp. B cells were exposed to UV, only little modifications of the ultrastructure were observed (Fig. 4). The chloroplast lobes covered medium electron-dense compartments, and electron-dense particles were still evident (Fig. 4 a, b). However, the amount of vacuolization increased (Fig. 4 b). In general, neither Golgi bodies nor mitochondria were

altered (Fig. 4 c). The chloroplast still contained starch grains (Fig. 4 c); only occasionally electron-dense spots were observed within the chloroplast lobes (Fig. 4 d).

Zygnema sp. E control cells (Fig. 5) had particularly long and narrow chloroplast lobes, covered by large medium electron-dense compartments. Electron-dense particles and electron-translucent vacuoles were also observed in the cell periphery (Fig. 5 a, b). The nucleus showed a distinct nucleolus, which is shown in part in Fig. 5c. The pyrenoid had a characteristic shape, with thylakoid membranes penetrating the central electron-dense area (Fig. 5 d). Golgi bodies (Fig. 5 e) were similar to those described for *Zygnema* sp. B. Upon exposure of *Zygnema* sp. E to UV radiation, again, no drastic changes of the ultrastructure were observed (Fig. 6). Medium electron-dense compartments, vacuoles and electron-dense particles remained in the cell periphery. Golgi bodies and mitochondria remained intact (Fig. 6 a). Only occasionally, chloroplasts showed accumulations of plastoglobules or electron-dense areas (Fig. 6 b, c). The nucleus remained visibly intact with one nucleolus (Fig. 6 b). In some cases, particularly large vacuoles were observed (Fig. 6 d).

While *Zygnema* sp. G control cells (Fig. 7 a-c) had a similar appearance as the previously mentioned control cells, the exposure to UV lead to more drastic consequences in this strain (Fig. 7 d-f). Control cells contained narrow chloroplast lobes between medium electron-dense compartments, electron-dense particles and vacuoles (Fig. 7 a). Nucleus, mitochondria and Golgi bodies had the expected appearance in TEM control samples (Fig. 7 b, c). *Zygnema* sp. G exposed to UV showed swellings of the chloroplasts with plastoglobules and partially electron-dense areas (Fig. 7 d, f); mitochondria were particularly electron-dense and showed a rearrangement of cristae (Fig. 7 e).

Photosytem II efficiency

The mean values of the maximum quantum yield of PS II (F_V/F_M) were substantially lower after UV exposure than in the control (Fig. 8). However, the response to UV differed among the tested strains, and this decrease was significant only in strain E and strain G. The results of the statistical analyses testing the difference of measured values of F_V/F_M are presented in Table 1; the pairwise comparisons confirmed the significant difference between control and exposure in strain E ($p<0.001$) and strain G ($p<0.001$), but not in strain B ($p=0.086$). For comparison of the values in individual strains, see Fig. 8.

Interestingly, F_V/F_M differed significantly among individual controls as well, showing a considerable level of stress response even without experimental UV treatment – most pronounced in strain G. Apparently, this strain was already impaired under control conditions.

Low values (around 0.2) measured after UV exposure indicated that the photosystems were practically destructed.

Plastidal pigments

The content of plastid-bound pigments was measured by HPLC. The pigments were categorized into three groups: First, chlorophyll *a* and *b*, including some degenerative phaeophytin derivates, which occurred in several samples; second, xanthophyll cycle pigments, i.e. violaxanthin (V), antheraxanthin (A) and zeaxanthin (Z); and third, the remaining primary carotenoids, including beta-caroten, lutein and neoxanthin. Finally, the deepoxidation state of xanthophyll cycle (A+Z)/(V+A+Z) was estimated.

Neither of the investigated group of pigments differed significantly between controls and UV-exposed samples (Table 1). However, the total pigment content per dry weight was significantly lower in strain B than in the other two strains (Table 2); $p<0.005$. Therefore, the relative pigment compositions for each individual strain are presented (Fig. 9).

When viewed from the deepoxidation state of the cultures, significant differences were revealed between UV-treated samples and controls (Table 1), showing that the UV samples were in higher deepoxidation state than the controls. The individual strains differed as well, as strain G was significantly more deepoxidized than the other two (Tables 1 and 2). Finally, virtually no secondary carotenoids were detected by using the same HPLC protocol as for the pigment analysis.

Phenolic compounds

By HPLC, all three *Zygnema* strains showed the presence of several compounds with the ability to screen UV radiation. The putative phenolic nature of these peaks was derived from their characteristic spectral absorption maxima around 280 nm typical for aromatic compounds and also from their water-soluble, hydrophilic nature.

The mean values of the content of phenolic compounds were always higher in UV-exposed samples than in the controls (Fig. 10 a), but the effect of UV was different among individual strains. The detailed results of statistical tests can be seen in Table 1. Markedly, the pairwise comparisons clearly showed the difference between control and exposure to be significant within only one of the strains tested – strain E ($p=0.001$). In general, strain B possessed fewer phenolics per dry weight than the other two strains. Furthermore, the same phenolics were present also in control samples, suggesting that certain amounts of protective

compounds are present independently of environmental UV stimuli. Figure 10 a illustrates the summarized peak areas of the phenolics for each species and their increase after UV exposure.

Figure 10 b demonstrates a representative HPLC chromatogram with the phenolic peaks of *Zygnema* strain E after exposure. The first and the last main peaks (retention times, 6.57 and 18.823 min) have HPLC-online absorption maxima of approximately 278 nm, thus absorbing mainly UV B radiation. Two further main peaks (8.356 and 16.141 min) show an additional absorption in the UV A region (shoulder at 352 nm and a minor maximum at 367 nm, respectively). The second peak (retention time 8.356) can be even seen in the visible (VIS) light region with a minor maximum at 473 nm. This fact corresponds most likely with the yellowish colour of the aqueous extracts. All the minor peaks had similar absorption spectra. The control samples contained all these compounds as well, however, in lower concentrations per dry weight. The other two species had very similar chromatograms (data not shown).

Discussion

We investigated the effect of UV on the cell structure and ultrastructure, the physiological performance and the production of intracellular soluble compounds in three polar strains of *Zygnema*. All strains were isolated from polar habitats and, therefore, a comparable level of stress resistance could be speculated about. After UV irradiation, we found a significant increase in accumulation of phenolic compounds in strain E and a significant decrease in PS II efficiency in strains E and G, when compared to control cells. Both effects are stress responses, however, occurring to a different extent in the investigated strains.

The UV scenario created in our experiments did not reflect the natural solar spectrum, but included a shift in the ratio of UV to PAR, a method that is widely used for provoking UV effects in algae [e.g. 28, 31, 38, 52, 61, 63, 79]. Despite the experimental limitations, we found significant effects of UV treatment on the phenolic compounds in strain E. To our knowledge, this is the first study to use an HPLC protocol rather than spectrophotometric assays to quantify changes of phenolic compounds in streptophyte green algae after exposure to UV; recently, a red algal species, *Hypnea*, was investigated concerning phenolic compounds by HPLC [66].

Phylogenetic relationships

One possible explanation for the differences observed among the investigated strains is their phylogenetic relationship. The species concept of the genus *Zygnema*, and in nearly all algae, is dominantly based on morphological characters [35], but it has been shown that traditional classification may not be consistent with phylogeny [71]. Further, the morphological distinction among *Zygnema* species rests on information from zygospores. Sexual reproduction was not observed in our strains and it was noted that other *Zygnema* from polar regions do not reproduce sexually [31, 40].

Therefore, the three strains under investigation were characterized by phylogenetic analysis of their *rbc*L sequences. The genus *Zygnema* contains over 137 species. The systematics of only a small number of *Zygnema* species was recently clarified using analysis of *rbc*L and cox3 data, but there are many species for which molecular data are not available [71]. Thus, we were unable to assign the strains used in this study to species. That said, the published sequences generated by this study should ultimately allow taxonomic assignment of the strains in the future and the information provided by these polar strains contributes to the understanding of the diversity and biology of the genus *Zygnema*. Interestingly, the two Arctic strains, *Zygnema* sp. B and G, are not most closely related. Instead, *Zygnema* sp. B is very closely related to the Antarctic strain (E) and to *Zygnema* cf. *insigne*.

Structural and ultrastructural aspects

Morphologically, samples of the three strains under control conditions looked similar. They all had a markedly condensed protoplasmic content and contained quite a high amount of small coloured bodies. Such a cytosolic appearance is not typical for young vegetative cells of *Zygnema* and resembles so-called pre-akinetes [46]. Many algae produce such modified vegetative cells by accumulating lipids and other metabolic products and by thickening the cell walls. Akinetes are formed at the onset of unfavourable conditions, such as nutrient starvation, and are considered resistant resting stages [15]. Markedly, natural populations of *Zygnema* sp. containing a high amount of lipid bodies have already been reported from the Arctic and Antarctica [25, 31]. Also, the Zygnematophycean freshwater ice alga *Mesotaenium berggrenii* that survives harsh environmental conditions at glacier surfaces without making cysts contains high amounts of purpurogallin-derived, phenolic compounds [60]. It seems that such a cytology is an adaptation to extreme climatic conditions, where cells could be suddenly exposed to stress scenarios.

To date, a limited number of studies have addressed UV effects on the ultrastructure of freshwater algae from polar habitats [28, 31, 61], whereas red and brown algae are better studied (for a summary, see Karsten et al. [39]). The desmid *Micrasterias denticulata* was remarkably resistant to UV B; alterations in the ultrastructure were seen only as an effect of irradiation with wavelengths lower than 284 nm [44, 48].

The ultrastructural changes found in the present study as a consequence of UV exposure were moderate in strains B and E, but pronounced in strain G, where serious damage could be observed, especially in chloroplasts and mitochondria. Damage to these organelles is usually the first sign of UV-induced damage in sensitive species [24, 29]. While an earlier study on Arctic *Zygnema* was made with field-collected samples followed by an exposure to an increased UV to PAR ratio only for 24 h [31], the present study employed cultivated samples exposed for 4 days to UV (8 h day^{-1}) irradiation. Moreover, all earlier studies used conventional chemical fixation [28, 31, 61], but with the laboratory-grown samples, we were able to use sophisticated high-pressure freezing, followed by freeze substitution, a method that is generally accepted to allow better preservation of the ultrastructure.

We speculate that the electron-dense particles with a diameter of 400-600 nm frequently found in the cell cortex contain phenolics. These structures have a similar appearance like physodes, phlorotannin/phenolics containing structures frequently found in brown algae [26, 68, 69]. In the present study, most of these electron-dense particles appeared round, likely due to better preservation by high-pressure freezing. In an earlier study, similar electron-dense structures with irregular outer shape, likely caused by shrinkage processes during dehydration, were detected in Arctic *Zygnema* after chemical fixation [31]. We, therefore, assume that these substances are not lipids, as one could conclude by their electron density. Moreover, electron-dense structures have been described by McLean and Pessoney [46] as ‘inclusions’ in beginning akinetes of older *Zygnema* sp. cultures. These structures were evident in untreated as well as in UV-treated cells in our study, which goes along with the finding that phenolics were also detected in both by HPLC analysis, even after prolonged UV exposure. Moreover, the irregularly shaped medium electron-dense compartments in the cell cortex could contain phenolic compounds as well due to the hydrophilic nature of phenolics. However, we could not observe a quantitative enhancement of these medium electron-dense compartments in UV-exposed cells. The nature of these compartments remains obscure; they might either be some sort of lytic vacuoles, which could only be proven by investigating their contents. Due to the texture of these medium electron-dense compartments, one could even

speculate that they are thylakoid-free parts of the chloroplast; however, we did not observe any connection of these compartments with the chloroplast.

There was little evidence for the accumulation of lipids based on TEM, which agrees with the observations of Bakker and Lokhorst [5], who also did not show accumulation of lipids in cultured *Zygnema* cells. This is in contrast to field-grown cells, which contain massive amounts of medium electron-dense bodies ('grey lipid bodies'), likely induced by nutrient starvation or harsh environmental conditions of the Arctic [31].

Physiological effects of UV irradiation

The presence of putative phenolic compounds was proven in all three *Zygnema* strains by using HPLC in control samples, as well as in 7-day UV-exposed samples; the prolonged exposure time in comparison to the TEM investigations had methodical reasons, but we speculate that this prolonged time caused only a quantitative increase in the amount of phenolics. This is in contrast to earlier reports, where only spectrophotometric assays (e.g. Folin-Ciocalteu) were used for a more imprecise determination [e.g. 18, 20, 41]. The presence of such phenolics, which are not common in freshwater microalgae, supports the hypothesis that their production might be crucial in UV protection of *Zygnema*. In all three strains, the mean value of total phenolic content rose after UV exposure. Nevertheless, only in strain E was this increase significant. The lack of significance in *Zygnema* sp. B and G was mainly due to large variation in phenolic content among individual replicates. As a matter of fact, in strain B, not enough biomass was available for more replications.

The screening effects of phenolics are enhanced by the fact that they are stored in electron-dense particles and vacuoles at the cell periphery, therefore protecting presumably chloroplasts and other organelles in the centre of the cells. This arrangement of organelles was confirmed in our *Zygnema* strains by TEM. High accumulation of secondary pigments in cytoplasmic compartments to shade the chloroplast was observed in other algae, too [29, 59]. Moreover, some of the phenolic compounds revealed in *Zygnema* also have an absorption in the VIS region, causing a yellowish to brownish colour of the vacuoles and of the aqueous extracts, respectively. Consequently, such compounds may also serve as protectants against excessive VIS irradiation, which otherwise could cause photoinhibition or intracellular ROS production. Stancheva et al. [71] found that zygotes of *Zygnema* exhibit a yellow, brownish or bluish secondary coloration, probably caused by such phenolic pigments. Strain E, which had the highest levels of phenolics after UV exposure, reached the highest values of F_V/F_M, suggesting a protective role of the phenolics for photosynthetic apparatus. Accordingly, the

best photosynthetic rate (rETR) and F_V/F_M values were found in cells of the chlorophyte *Zygnemopsis decussata* with high content of phenolics [18].

Remarkably, the strains investigated in this study also produced phenolic compounds without UV exposure; the values were highest in strain G, which could point towards influence by other stress. This indicates an accumulation in advance of any harmful irradiation events, probably similarly as in *Zygogonium ericetorum*, which exhibits pinkish vacuoles [32]. Phenolics are widely distributed in brown algae as phlorotannins [1, 16, 50]. In non-Streptophycean green algae, phenolics are not widespread, only few examples like the marine green alga *Dasycladus vermicularis* are known to contain phenolics [51]. In contrast, these compounds have been found in members of the Zygnematophyceae [12, 60], which might be explained by their close relationship to land plants [6, 77]. In addition, a high amount of uncharacterized pigments was detected when attempting to isolate RNA from *Z. cruciatum* [22]. It could be speculated that these pigments were also phenolics, but there are many other compounds in the aqueous phase. Passive UV absorption is a nature of phenolic substances, given by their structure containing aromatic groups, but their original function in regards of plant physiology may be different [14]. Thus, the accumulation of phenolics is triggered not only by UV irradiation [50] but by different environmental factors (salinity, PAR and temperature) as well [51]. In fact, the applied temperature of 15°C during the experiments could contribute to higher repair rates under these relatively low UV intensities.

In this study, a significant ($p<0.001$) decrease of PS II efficiency was observed after UV exposure in strains E and G, but not in strain B. This parameter is widely used as an indicator of quantum efficiency of PS II photochemistry and gives a good measure of photoinhibitory damage caused by environmental stresses [11, 45]. On the contrary, in the study of Holzinger et al. [31], no photoinhibition was observed at 926 μmol photons m^{-2} s^{-1} in field-collected samples of *Zygnema* from Svalbard. Also, Germ et al. [20] showed no decrease in F_V/F_M under UV B exposure. In other species, however, it has already been shown that UV exposure can result in a decline in F_V/F_M. Interestingly, some authors assign this to the effect of UV B [52, 79]. In contrast, other authors conclude that it is UV A that directly affects PS II and causes inhibitory stress [18, 76], which could also be the case in this study, as we used a predominant UV A radiation treatment. UV exposure generated similar declines in *Tetracystis* sp. and *Chlamydomonas nivalis*, but the initial F_V/F_M values were reached again after an overnight recovery [58].

All three investigated strains contained a considerable amount of antheraxanthin and zeaxanthin. The highest deepoxidation state of the xanthophyll cycle was found in strain G,

which goes along with the highest levels of phenolics in control cells of this strain, which could as well be a consequence of other stress factors, already suppressing this strain prior to UV exposure. This argumentation would further be supported by the finding that strain G had the lowest values of F_V/F_M in control cells. In the other strains, the deepoxidation was nearly 30% in strain B. This strain shows some signs of light stress, as the xanthophyll cycle pool size and the deepoxidation state increased; however, this was the only strain where UV treatment did not affect F_V/F_M values significantly. The plastidal pigments were investigated, as quantitative changes could point towards intracellular stress events, e.g. chlorophyll degeneration or increase of xanthophyll cycle pigments. Moreover, with the same HPLC protocol, the presence or absence of any secondary carotenoids (e.g. astaxanthin) was tested and found to be negative. Xiong et al. [79] reported slight degradation of chlorophyll in UV B-sensitive green algal species. Such degradation products occurred in samples of *Zygnema* strain B and strain G in the present study.

Other compounds may be involved in UV protection of *Zygnema* as well. No secondary carotenoids were detected, and MAAs have not been found in other Zygnematophyceae so far [60]. However, other ways of protection than producing intracellular compounds may play a role, and it has been suggested that extracellular structures help to screen UV (e.g. sporopollenin in the cell walls) [78]. *Zygnema* is capable of producing this resistant polymer, but its presence so far has been demonstrated only in the cell walls of zygospores [55]. Such putative substances may not have been extracted from the cell walls by our methods. Extracellular mucilage may also protect cells from UV [29]. Remarkably, in our study, the highest amount of mucilage production was observed in strain B. The presence of mucilage can also explain its significant low amount of overall pigments per dry weight because a large proportion of dry weight was in fact due to extracellular matrix. Finally, another possibility for explaining the UV tolerance may be the formation of macroscopic mats in the habitat, which is also typical for *Zygnema* [31]. Mat-forming growth forms provide self-shading and protect cells against excessive irradiation [9, 73].

Conclusions

Our study showed the accumulation of various phenolic-like compounds by polar, semi-terrestrial-derived *Zygnema*. The use of an HPLC protocol supported a reliable quantification than comparatively unspecific, colorimetric spectrophotometer assays. The phenolics are obviously involved in UV protection as significantly shown by the response of strain E. Even

in *Zygnema* sp. strain G, which was severely stressed by the exposure conditions, a comparably high content of phenolics per dry weight was measured. The different strains, however, started under different physiological conditions. The lower optimal quantum yield in control cells of strain G in comparison to strain B and E could have influenced the UV resistance of this strain. It can be taken as a hint for another stress interacting with the UV in this strain, which is supported also by the very high deepoxidation state. However, it has to be stated that phenolic pigments generally play an important role in stress response of Zygnematophyceae, above all in species of extreme habitats like glacier surfaces [60], high mountain lakes [18] or semi-terrestrial habitats of polar regions as described in this study. In such locations, synthesis of phenolics has a substantial advantage – they are carbohydrates [21], which make their production 'cheaper' in comparison to, e.g. MAAs, containing nitrogen [13].

Our study, gives evidence for the importance of phenloics in UV protection in *Zygnema*, but lacks a clear-cut correlation between the degree of damage and putative phenolic contents. Strain G shows damage in the ultrastructure and PS II quantum efficiency, but has as high phenolic content as strain E which did not show damage. On the other hand, strain B has the lowest total phenolics content and shows no signs of damage. This could have different reasons: First, it is possible that the applied UV intensities were too low to provoke significant UV damages in the investigated *Zygnema* strains. Second, the different responses to UV stress in the investigated *Zygnema* strains might as well suggest that various other strategies, such as mucilage production, compounds localized in the cell wall or mat-forming growth, might be involved in overall UV protection. This variation also illustrates the value in using more than a single isolate per genus or major taxon in physiological studies. There are great differences in ecophysiological performance among individual strains, and therefore, generalizations based on the investigation of a single strain might be misleading.

Further work is needed to compare the resistance of polar strains with that of algae from temperate or high-altitude regions where UV stress is naturally stronger. Moreover, to our knowledge, the structure of such phenolics occurring in *Zygnema* has not been determined. The isolation and characterisation of these compounds is currently under work. Finally, a detailed knowledge of special adaptation mechanisms in polar organisms may also be of potential use and commercial interest.

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Tables

Table 1 Summary of the results of statistical analyses (GLM, *p* values of nested ANOVA)

Effect	*df*	F_V/F_M	Total phenolics	Chlorophylls *a*+*b*	V+A+Z	Other primary carotenoids	Deepoxidation state
UV treatment	1	<0.0001	0.0012	0.9884	0.0813	0.2829	0.0473
Strain	2	0.0001	0.0037	0.0816	0.0058	0.0129	0.0022
Petri dish (strain)	6	0.0319	0.5595	0.15	0.3896	0.2545	0.3961
Strain × treatment	2	<0.0001	0.0404	0.9163	0.7539	0.3247	0.0776

'UV treatment' and 'strain' were considered as fixed effects, 'Petri dish' was considered as a random effect. *Petri dish (strain)* Petri dish nested in strain, F_V/F_M PS II efficiency, *V*+*A*+*Z* xanthophyll cycle pigments, *Deepoxidation state* the ratio (A+Z)/(V+A+Z)

Table 2 Total pigment content of the investigated strains of *Zygnema* (in micrograms per gram dry weight) and deepoxidation state (A+Z)/(V+A+Z)

Strain	B		E		G	
Treatment	Control	UV	Control	UV	Control	UV
Total pigment–mean	0.8076	0.8750	2.8083	2.7018	2.5265	3.0529
Total pigment–SD	0.3834	0.1060	0.7915	1.1058	1.3739	0.6734
Depoxidation state–mean	0.2947	0.5926	0.4320	0.4615	0.7814	0.7707
Depoxidation state–SD	0.1139	0.1058	0.0710	0.0700	0.0253	0.2015

Mean values are given as well as standard deviations.

Figures

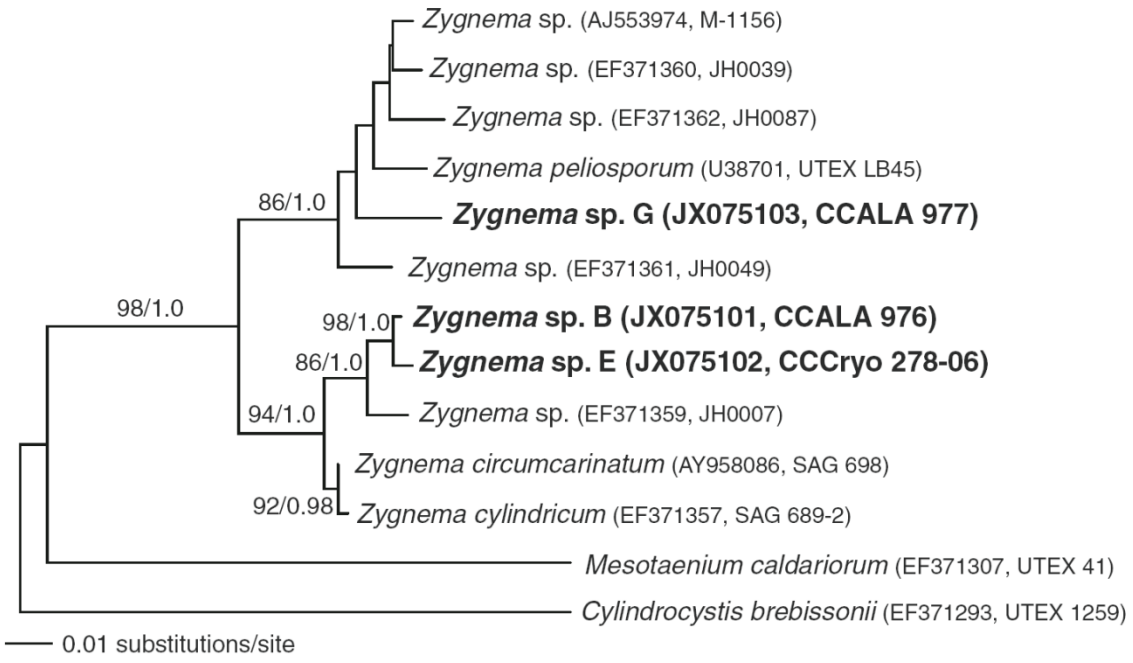

Figure 1 ML tree (score -lnL=4,104.333) from phylogenetic analysis of *rbc*L sequence data from different strains of *Zygnema* plus two related saccoderm desmid genera. Taxon labels include GenBank accession numbers and strain designations. Labels listed in boldface font were obtained for this study. The GTR+gamma model parameter values were set during the search: RA-C=3.0900652; RA-G=7.7050449; RA-T=7.7120372; RC-G=2.7439213; RC-T=28.160416; RG-T=1; gamma shape=0.813032; pinvar=0.441081. ML bootstrap support values (out of 200 replicates) are indicated, followed by Bayesian posterior probabilities. Scale bar corresponds to the number of expected substitutions/site

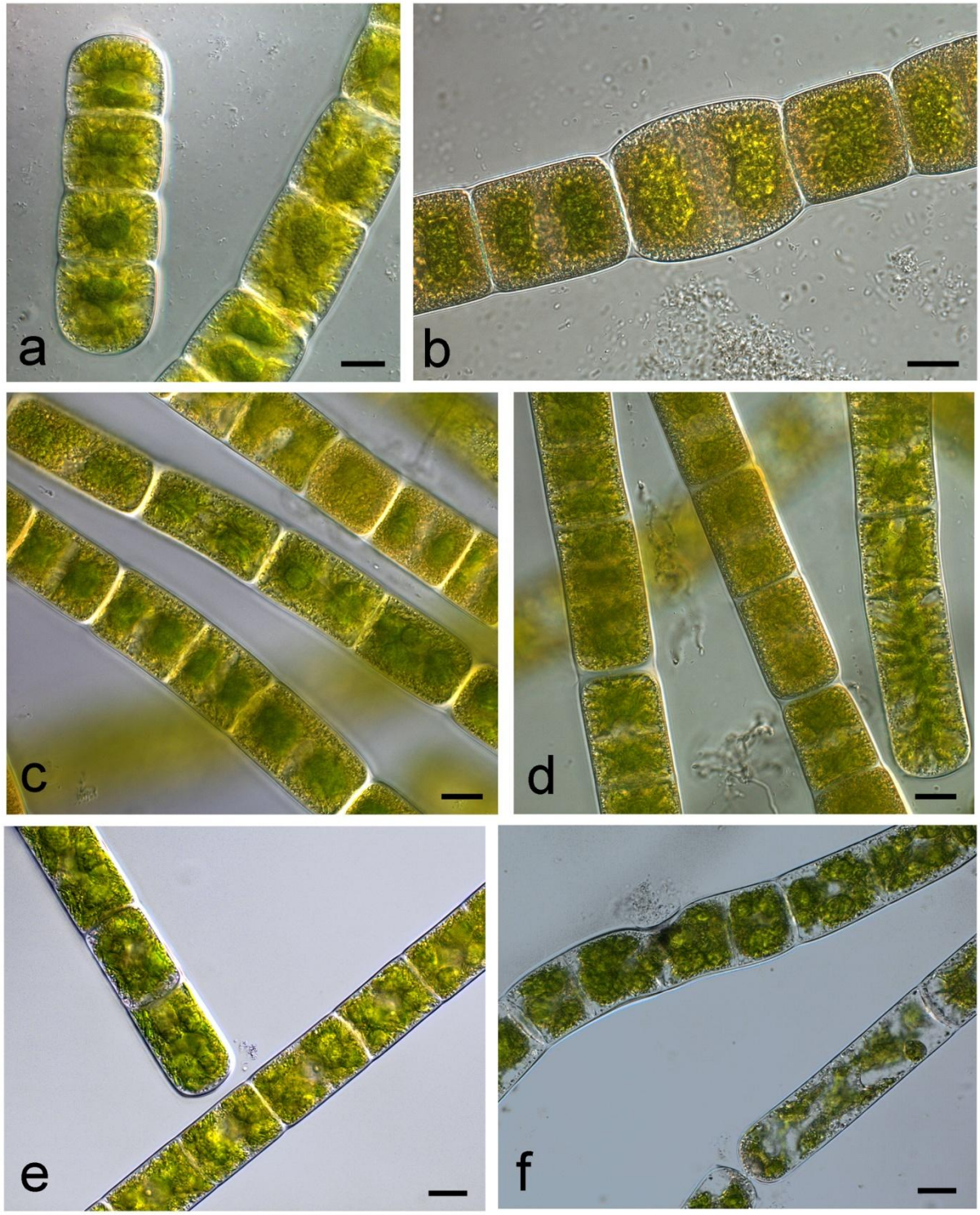

Figure 2 Light microscope images of the investigated *Zygnema* strains. **a** strain B–control, **b** strain B–after UV exposure, **c** strain E–control, **d** strain E–UV exposure, **e** strain G–control, **f** strain G–UV exposure. Scale bars, 10 μm

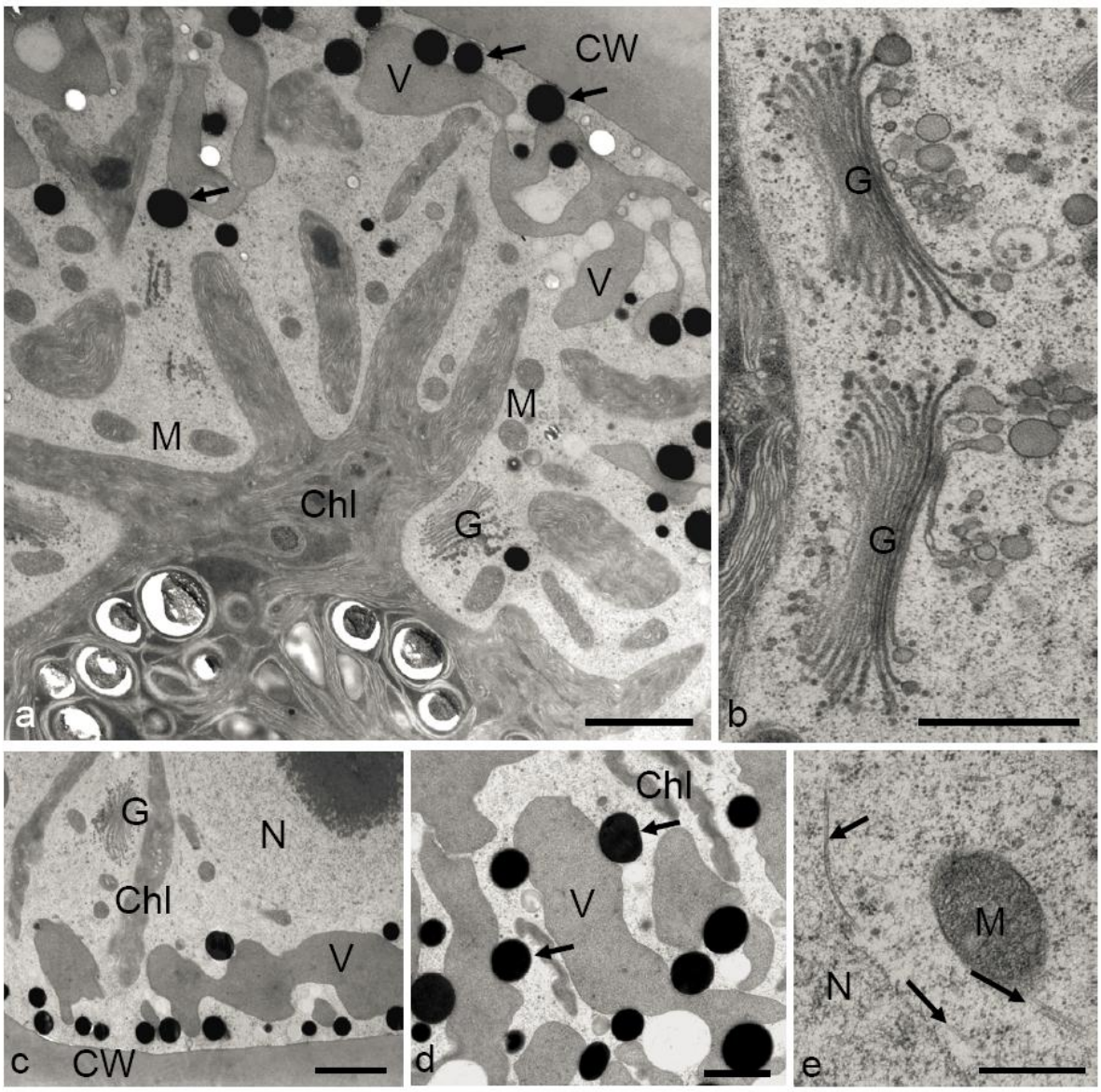

Figure 3 Details of the ultrastructure of control cells of *Zygnema* sp. B. **a** Star-shaped chloroplast in the centre of the cell, medium electron-dense compartments (*asterisks*) and electron-dense particles (*arrows*) at the cell periphery; **b** Golgi bodies in close contact with chloroplast, a TGN is clearly visible (*arrows*); **c** cell periphery with medium electron-dense compartments (*asterisk*), nucleus in the centre of the cell; **d** multiple shapes of medium electron-dense compartments (*asterisks*) and electron-dense particles (*arrows*); **e** microtubules (*arrows*) close to the nucleus and mitochondrion. *Chl* chloroplast, *CW* cell wall, *G* Golgi bodies, *M* mitochondrion, *N* nucleus, *Py* pyrenoid. Scale bars: **a**, **c** 2 μm; **b**, **d** 1 μm; **e** 500 nm

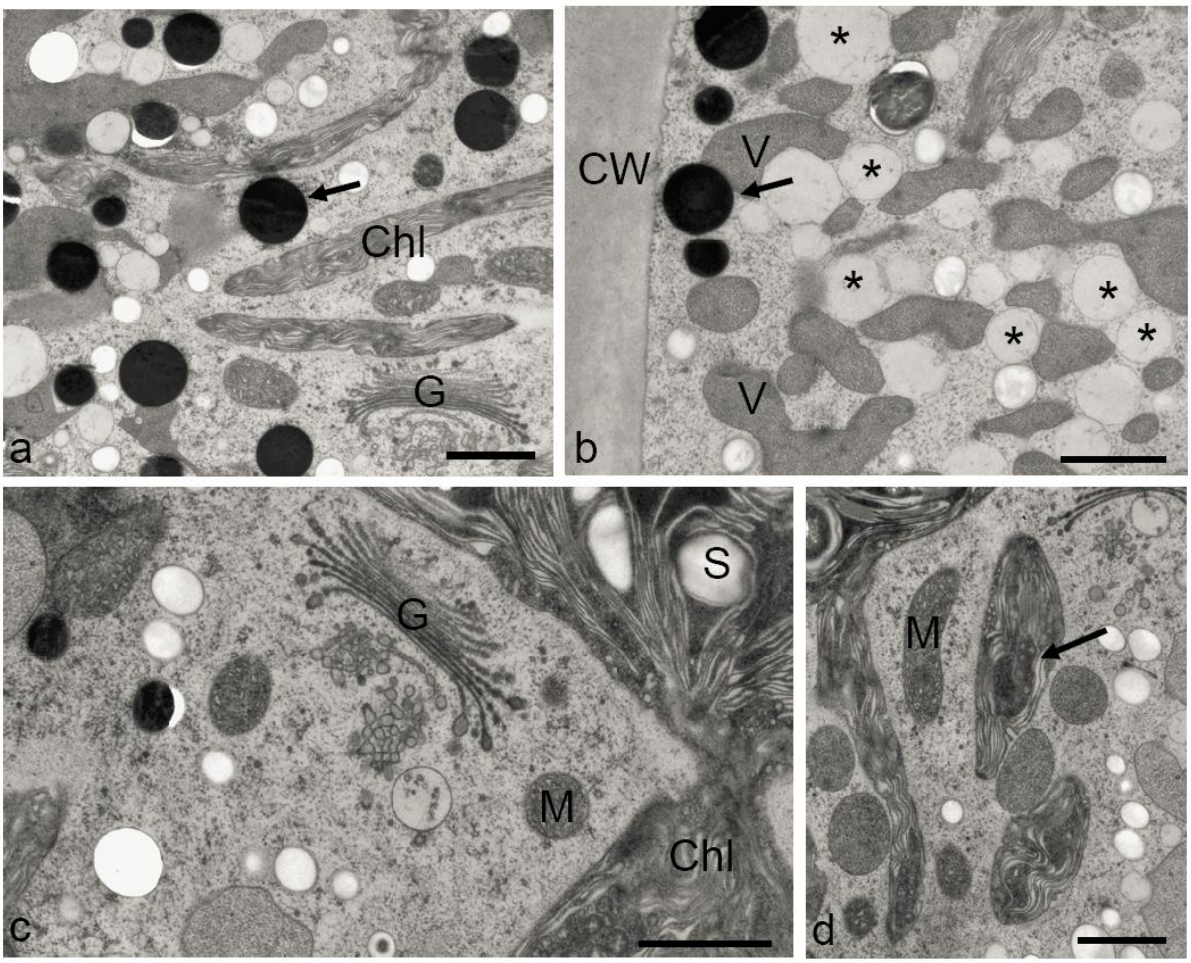

Figure 4 Transmission electron micrographs of *Zygnema* sp. B after UV exposure. **a** Chloroplast lobes, Golgi bodies, medium electron-dense compartments (*asterisk*) and electron-dense particles (*arrow*) appear unchanged; **b** while medium electron-dense compartments (*asterisks*) and electron-dense particles (*arrows*) appear unchanged, additional occurrence of electron-translucent vacuoles was found; **c** Golgi body with normal appearance, chloroplast with starch grain; **d** unchanged mitochondria, chloroplast lobe with electron-dense inclusion (*arrow*). *Chl* chloroplast, *CW* cell wall, *G* Golgi bodies, *M* mitochondria, *S* starch grain, *V* vacuole. Scale bars: **a-d** 1 μm

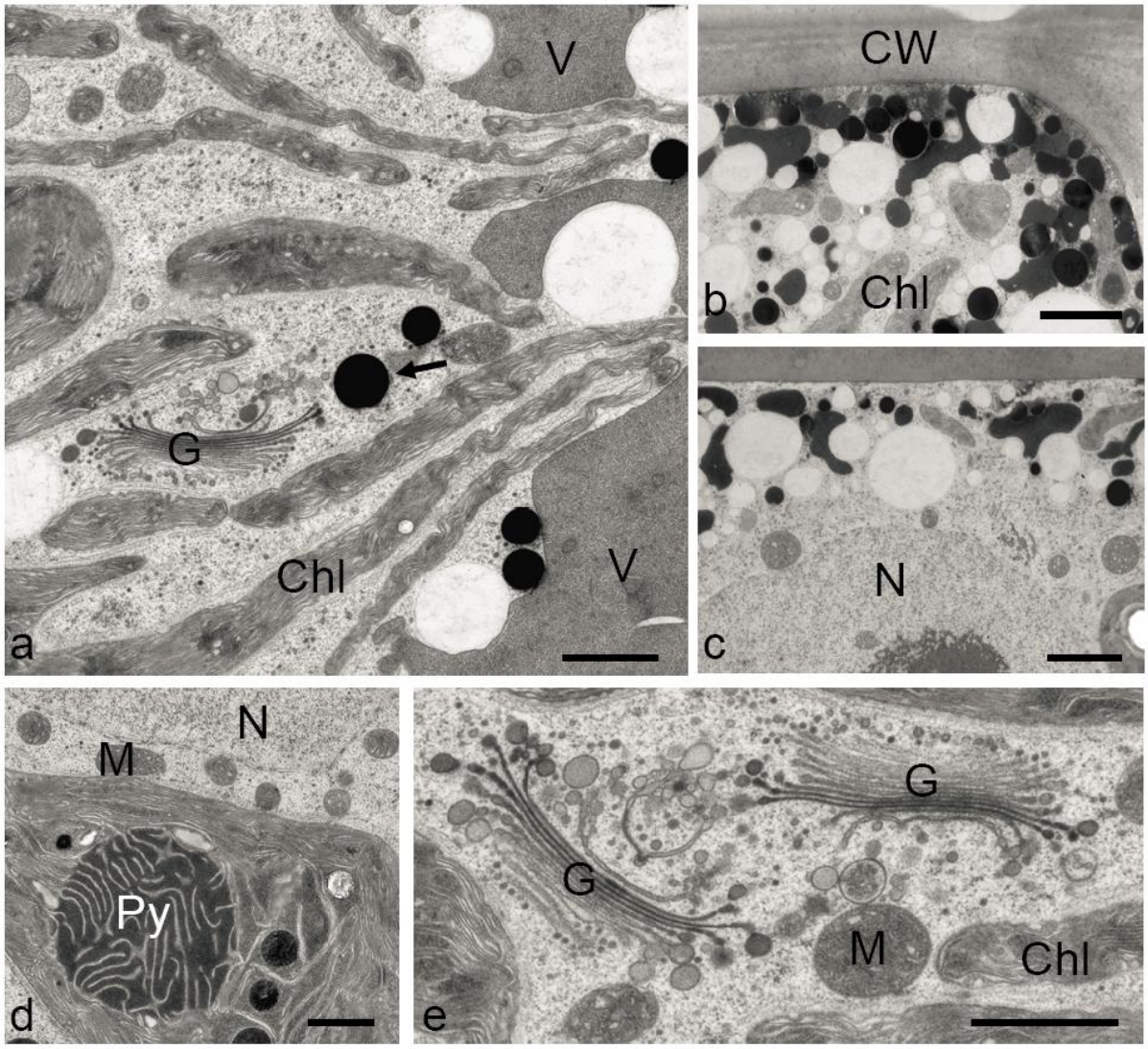

Figure 5 Details of the ultrastructure of control cells of *Zygnema* sp. E. **a** Long and narrow chloroplast lobes covered by large medium electron-dense compartments (*asterisks*) and electron-dense particles (*arrow*); **b** cortical area with electron-dense particles and vacuoles; **c** central area of the cell with nucleus and part of the nucleolus (*arrow*), vacuoles in the cell cortex; **d** chloroplast detail with pyrenoid, mitochondria and nucleus; **e** detail with Golgi bodies, mitochondria and chloroplast lobes. *Chl* chloroplast, *CW* cell wall, *G* Golgi body, *M* mitochondrion, *N* nucleus, *Py* pyrenoid, *V* vacuole. Scale bars: **a**, **d**, **e** 1 μm; **b**, **c** 2 μm

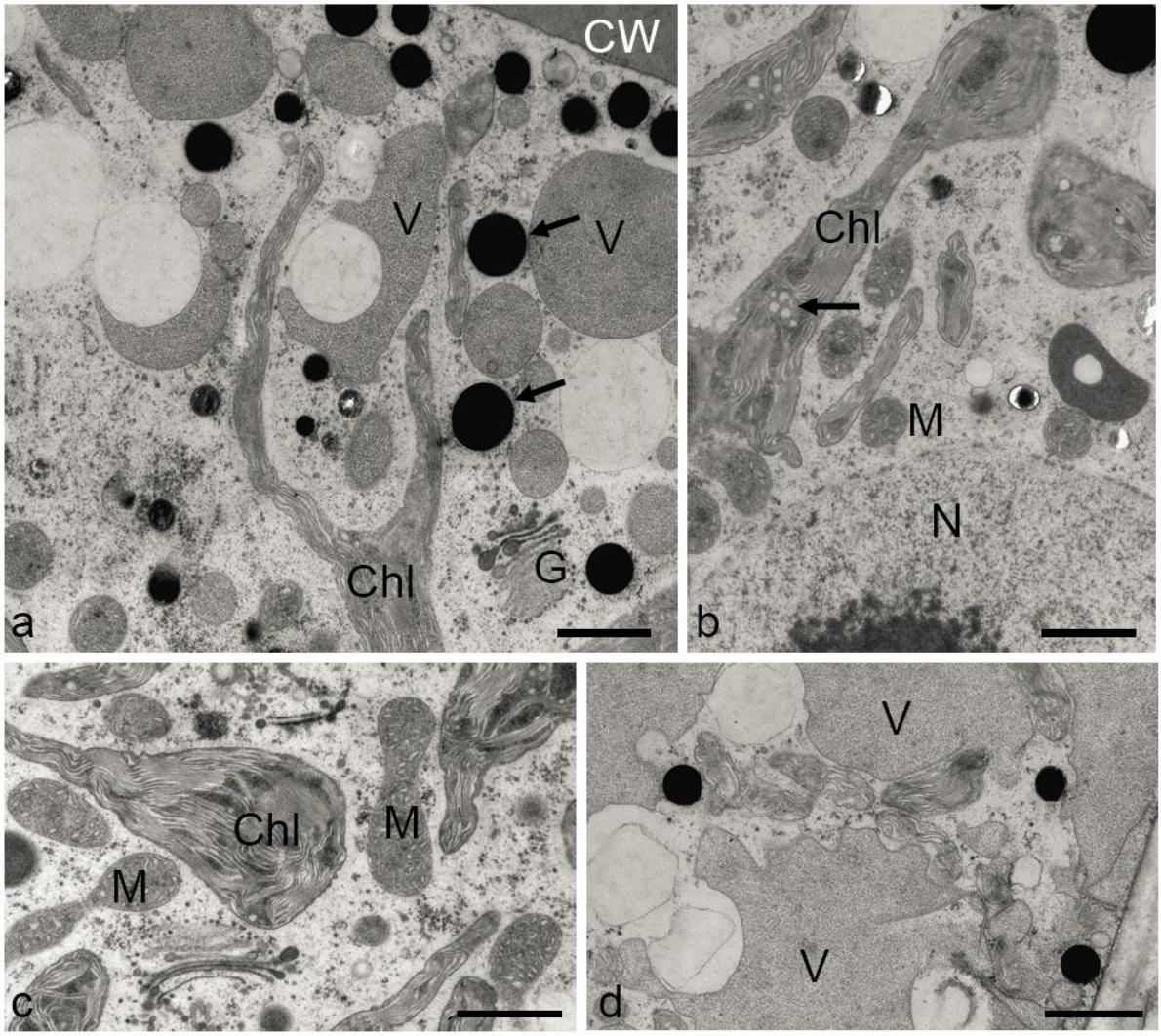

Figure 6 Transmission electron micrographs of *Zygnema* sp. E after UV exposure. **a** Detail of the cell cortex with chloroplast lobes, substantial amount of vacuolization, medium electron-dense compartments (*asterisks*) and electron-dense particles (*arrows*); **b** central area, nucleus with nucleolus, mitochondria intact, the chloroplasts contain electron-dense areas and plastoglobules (*arrow*); **c** mitochondria with normal appearance, chloroplast partly swollen; **d** extensive medium-electron dense compartments (*asterisks*) in the cell cortex, vacuoles and chloroplast lobes are found in the same area. *Chl* chloroplast, *CW* cell wall, *G* Golgi body, *L* lipid body, *M* mitochondrion, *V* vacuole. Scale bars: **a**-**d** 1 μm

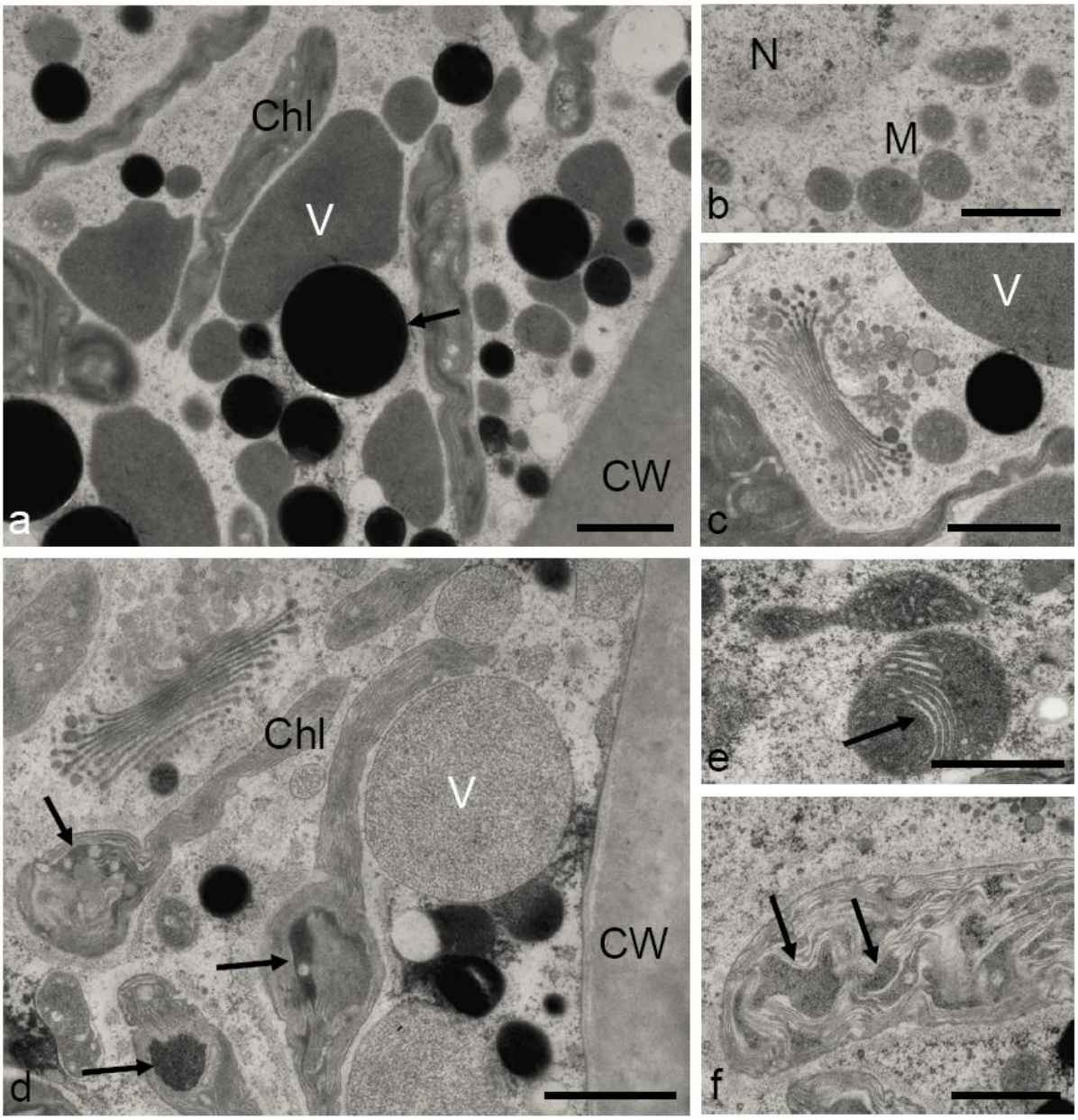

Figure 7 Details of the ultrastructure of *Zygnema* sp. G control cells (**a**-**c**) and cells after UV exposure (**d**-**f**). **a** Chloroplast lobes between medium electron-dense compartments (*asterisks*), vacuoles and electron-dense particles (*arrow*) in the vicinity of the cell wall; **b** central area with nucleus and mitochondria; **c** Golgi body next to a chloroplast lobe and medium electron-dense compartment (*asterisk*); **d** cortical area with altered chloroplast with swellings (*arrows*), partially filled with electron-dense content or plastoglobules, medium electron-dense compartment (*asterisk*); **e** altered mitochondria with rearranged parallel-oriented cristae (*arrow*); **f** chloroplast with electron-dense contents (*arrows*). *Chl* chloroplast, *CW* cell wall, *G* Golgi body, *M* mitochondrion, *N* nucleus. Scale bars: **a**-**f** 1 μm

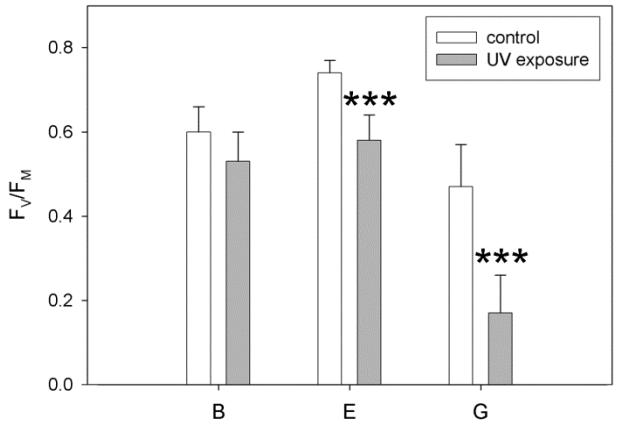

Figure 8 Maximum quantum yield of PS II in dark-adapted state for both control and UV-exposed samples; mean values+SD. Significant differences ($p<0.001$) between control and UV-exposed samples were found in strains E and G and are marked with *three asterisks* (*n*=15)

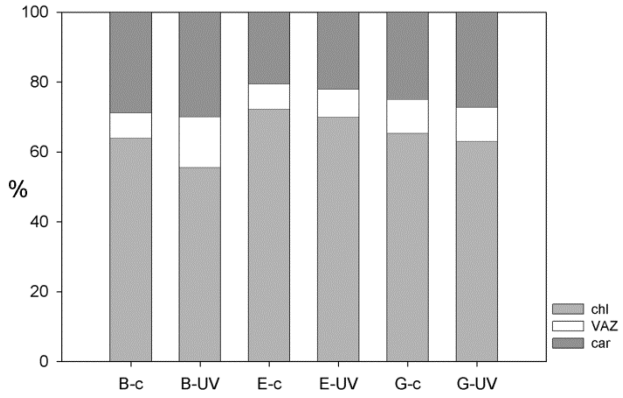

Figure 9 Proportion of individual pigment groups to the total chloroplast pigment content for each strain and treatment (mean values). *Chl* chlorophylls *a* and *b*, *VAZ* violaxanthin, antheraxanthin and zeaxanthin (xanthophyll cycle pigments), *car* other primary carotenoids

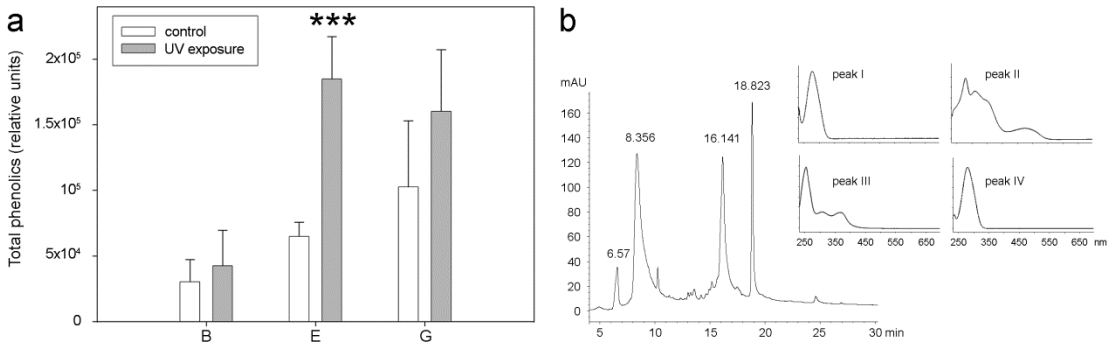

Figure 10 a Total content of phenolic compounds expressed as peak area per 1 mg dry weight. Strain E control and 7-day UV-exposed samples differ significantly (p=0.001) in their amount of phenolics, as indicated with two *asterisks*; the differences in the other strains are not significant (*n*=6 for E and G, *n*=3 for B). Mean values+SD. **b** Representative HPLC chromatogram at 280 nm of a UV-exposed culture (E) revealing several different phenolic peaks. The control samples contained these compounds as well, only in lower concentrations per dry weight. Online absorption spectra of the four largest peaks are also given

3.2 Manuscript II

Osmotic stress and recovery in field populations of *Zygnema* sp. (Zygnematophyceae, Streptophyta) on Svalbard (High Arctic) subjected to natural desiccation

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Abstract

Zygnema is a genus of filamentous green algae belonging to the class of Zygnematophyceae (Streptophyta). In the Arctic, it typically forms extensive mats in habitats that regularly dry out during summer, and therefore, mechanisms of stress resistance are expected. We investigated its natural populations with respect to production of specialized desiccation-resistant cells and osmotic acclimation. Six populations in various stages of natural desiccation were selected, from wet biomass floating in water to dried paper-like crusts. After rewetting, plasmolysis and osmotic stress effects were studied using hypertonic sorbitol solutions, and the physiological state was estimated using chlorophyll *a* fluorescence parameters. All populations of *Zygnema* sp. formed stationary-phase cells filled with storage products. In green algal research, such cells are traditionally called akinetes. However, the populations differed in their reaction to osmotic stress. Whereas the wet-collected samples were strongly impaired, osmotic stress resistance of the naturally dried samples was comparable to that of true aeroterrestrial algae. We showed that Arctic populations of *Zygnema* acclimate well to natural desiccation via hardening that is mediated by slow desiccation. As no other types of specialized cells were observed, we assume that the naturally hardened akinetes also play a key role in winter survival.

Introduction

Eukaryotic microalgae are predominantly known as aquatic organisms. In spite of that, numerous microalgal representatives of various taxonomic groups have adapted to life in aeroterrestrial habitats, such as soil crusts (Lewis, 2007), tree bark (Lüttge & Büdel, 2010), rocks (Knowles & Castenholz, 2008) or even man-made surfaces (Häubner *et al*., 2006). Particularly in polar regions, many other microalgae live in hydroterrestrial environments where they are regularly exposed to drying atmospheres: in shallow pools, snow-fed streams, or wetlands (Elster, 2002). Therefore, similar physiological adaptations in algae from aero- and hydroterrestrial habitats are expected.

Habitats with unpredictable fluctuations in water availability are characterized by a whole range of stressful environmental conditions, such as temperature extremes, high irradiance (both photosynthetic active radiation and UV), or lack of nutrients (Gray *et al*., 2007; Karsten *et al*., 2007; Rindi, 2007); however, water deficiency is most critical. Dehydration, that is, lowering of the intracellular water potential, accompanies not only desiccation, but freezing and osmotic (salt) stress as well (Bisson & Kirst, 1995). Therefore, all these stresses are interrelated and similar in their physiological effects, and the acclimation to one such stress is assumed to induce resistance to the others as well (Morison & Sheath, 1985; Pearson & Davison, 1994; Welsh, 2000). Nevertheless, differences between the effects of individual stresses were revealed by experimental studies. For instance, freezing and desiccation were found to have a different effect on photosynthesis and viability of the same algae, with desiccation being more deleterious (Davey, 1989; Šabacká & Elster, 2006; Souffreau *et al*., 2010).

In aero- or hydroterrestrial environments, green microalgae belong to the most abundant algal groups (Rindi *et al*., 2009). Green microalgae of the subgroup Streptophyta are of particular interest because Streptophyta also comprises all land vascular plants (Embryophytes; e.g., Becker & Marin, 2009). Numerous investigations have recently focused on the stress resistance of various aeroterrestrial Streptophyta. Several members of this group are remarkably desiccation tolerant (e.g., Elster *et al*., 2008; Holzinger *et al*., 2010; Graham *et al*., 2012; Karsten & Holzinger, 2012; Aigner *et al*., 2013) suggesting that poikilohydry evolved early in the history of Streptophyta (Graham *et al*., 2012). There are also several studies of osmotic stress using hypertonic salt solutions or osmotically active sugars (Affenzeller *et al*., 2009; Karsten & Rindi, 2010; Kaplan *et al*., 2012, 2013). The cellular osmotic potential is a function of the concentration of (in)organic osmolytes that help to

maintain cellular homeostasis under desiccation stress (Gustavs *et al.*, 2010; Karsten *et al*., 2010). Therefore, it is believed to be directly correlated with water-holding capacity and, thus, with desiccation tolerance (Kaplan *et al.*, 2012, 2013).

Many green microalgae survive unfavorable conditions by forming specialized highly resistant dormant cells (e.g. zygospores, cysts). In addition, senescent or growth-limited vegetative cells that enter the stationary growth phase are often observed in microalgae. Such asexually developed resistant cells with markedly thickened cell walls and accumulated storage products are usually called akinetes in green algae (Coleman, 1983) and xanthophytes (Nagao *et al.*, 1999). They can be considered alternative dormant stages without distinct morphological differentiation. The akinetes of *Klebsormidium rivulare* are characterized by increase in dry weight, accumulation of storage products and low-molecular-weight solutes, lower pigment content, thickened cell walls, and preferential carbohydrate and lipid production over protein-synthesizing metabolism (Morison & Sheath, 1985).

In this study, we focused on the alga *Zygnema* sp. (Zygnematophyceae, Streptophyta) in its natural habitats on Svalbard (High Arctic). Polar regions are characterized by many interrelated stress factors (Convey, 2000; Elster, 2002), and microalgae isolated from such an extreme environment represent good model organisms for studying adaptive strategies (Elster & Benson, 2004). *Zygnema* is one of the most common streptophyte algae in the Arctic and Antarctica, usually forming extensive mats in shallow pools or on wet soil, living at the transition between aquatic and aeroterrestrial environments (Hawes 1990; Kang *et al*., 2007; Kim *et al*., 2008, 2011; Holzinger *et al*., 2009). In the studied region, liquid water is available mainly during the spring melt, and many freshwater habitats dry out completely in the summer. Furthermore, *Zygnema* is subjected not only to desiccation but also to other stresses, such as freezing (Hawes, 1990) or UV irradiation (Holzinger *et al.*, 2009; Pichrtová *et al.*, 2013).

Vegetative filaments of *Zygnema* lack constitutive desiccation tolerance (McLean & Pessoney, 1971), and the effect of field acclimation (hardening) or dormant stages production is therefore assumed. The formation of various resistant cell types has been frequently reported in *Zygnema*, namely zygospores, parthenospores, and akinetes (Kadlubowska, 1984; Poulíčková *et al*., 2007; Stancheva *et al.*, 2012); their role in stress resistance is not yet fully understood. *Zygnema* commonly forms green algal akinetes as described earlier—developed from vegetative cells, with thick cell walls and accumulated storage products. Akinetes were described as individual cells that form from stationary-phase cells in starved cultures (McLean & Pessoney, 1971). *Zygnema* akinete production can occur in naturally desiccated

sites, and akinetes were found that could survive experimental desiccation in a *Zygnema* sp. from Texas (McLean & Pessoney, 1971) and *Zygnema stellinum* from Belarus (Genkel & Pronina, 1979). Nevertheless, it must be noted here that, in *Zygnema*, the term 'akinete' is ambiguous because it also refers to a special type of cell in the life cycle of *Zygnema*. Such akinetes are rectangular cells with cell walls colored and structured in the same way as in zygospores, but developing asexually within vegetative cells. They represent a highly specialized, morphologically distinct, species-specific cell type (Kadlubowska, 1984; Stancheva *et al*., 2012).

Only recently, the osmotic potential and plasmolysis under laboratory conditions were studied in *Zygnema* sp. strains originating from the Arctic and Antarctica (Kaplan *et al.*, 2013). The authors applied the method of incipient plasmolysis detection (Oparka, 1994) to estimate the osmotic potential of *Zygnema* cells. They investigated the structural and ultrastructural changes connected with osmotic stress as well as physiological performance. The Arctic and Antarctic strains of *Zygnema* sp. had in general less negative osmotic potentials in comparison with previously studied *Klebsormidium* (Kaplan *et al.*, 2012), and the authors concluded that *Zygnema* was less desiccation tolerant than *Klebsormidium.* Nevertheless, as these experiments were carried out using liquid cultures, the performance of naturally desiccated *Zygnema* remains unclear.

Therefore, we followed the work of Kaplan *et al*. (2013) but focused on natural populations in field conditions. We hypothesized that the formation of hardened, desiccation-resistant cells takes place during the natural desiccation process. We selected populations in various stages of natural desiccation and compared their cell morphology and response to controlled osmotic stress. We estimated the osmotic potential of plasmolysis as a good indication of the field acclimation level to desiccation and other stresses and assessed photosynthetic activity by chlorophyll fluorescence.

Materials and methods

Algal material

The *Zygnema* samples were collected in the area surrounding Billefjorden in central Svalbard in August of 2011, and the experiments were conducted at field station Petuniahytta (Elster & Rachlewicz, 2012). Six natural populations of *Zygnema* were sampled. The populations represented three natural desiccation states: (1) 'wet' biomass floating in liquid water of seepage pools (populations 1 and 2) that had not experienced dehydration since the

spring, (2) 'moist' biomass on soil surface that probably had experienced recent dehydration or rehydration, but which was apparently fully hydrated at the time of collection due to the capillary water between filaments (population 3), and (3) 'dry' biomass on dry soil surface forming paper-like crusts, visibly dried out (populations 4-6). Prior to all measurements and experiments, biomass from all sampling sites was placed into stream water and kept overnight (8 h) in shade conditions at photosynthetic photon flux density (PPFD) of 15-30 μmol m^{-2} s^{-1}.

Experimental design

All experiments were conducted with three replicate samples with similar amounts of biomass. The replicates were always taken from the area of up to 0.3 m^2 within the same population.

The cellular osmotic stress was induced in sorbitol solutions (D-Sorbitol, FCC grade, SAFC). The osmotic potential of the solutions was assessed using thermocouple psychrometry (C-52 Sample Chamber linked to Wescor HR-33T microvoltmeter, Wescor) against NaCl standard solutions (Sun, 2002).

Two separate experiments were conducted. First, samples of *Zygnema* sp. biomass were placed into four sorbitol solutions of concentrations 300, 450, 600, and 750 mM (Ψ_π = –0.64, –1.08, –1.52, and –1.96 MPa, respectively). After 24 h of incubation, the presence/absence of plasmolysis was determined by observing a retraction of protoplast from the cell wall in a light microscope. The solute concentration at which 50% of the cells plasmolyse is frequently used as a measure of incipient plasmolysis and serves as an estimation of the mean osmotic pressure of the cells (Oparka, 1994; Iwata *et al*., 2001; Kaplan *et al*., 2012, 2013). Thus, we assessed the osmotic potential (Ψ_π) of the cells at turgor loss ($\Psi_{t=0}$), that is, when the cellular osmotic potential is equal to the osmotic potential of the external solution.

The aim of the second experiment was to study the effect of a stronger osmotic stress on physiological performance. The samples were incubated in a hypertonic solution of 2 M sorbitol (Ψ_π = –5.63 MPa) for 4 h. Then, the samples were rinsed in water, blotted, and transferred into stream water for 24 h where they were allowed to recover their structural and physiological characteristics.

Algal material was kept in polystyrene 6-well plates and incubated outdoors to ensure conditions comparable to those in the field (5–8 °C, diffusive irradiance). In both experiments, parallel controls were kept in stream water in the same place as experimentally treated samples.

The particular sorbitol concentrations and incubation times used in our experiments were based on similar studies (Kaplan *et al*., 2012, 2013) and chosen according to the results of pilot experiments. It should, however, be noted that published osmotic potentials of the sorbitol solutions measured by Kaplan *et al*. (2012) were slightly lower than in our study (e.g. –5.87 vs. –5.63 MPa for 2 M sorbitol).

Chlorophyll a fluorescence

Chlorophyll *a* fluorescence of the algal biomass was measured by an imaging modulated fluorimeter FluorCam (PSI, Czech Republic). We optimized a quenching analysis protocol to study slow chlorophyll fluorescence induction kinetics. Samples were dark-acclimated for 30 min prior to the measurements. The minimum fluorescence signal from open PSII reaction centers (F_0) was recorded, followed by the maximum chlorophyll fluorescence (F_M) during the application of a saturation pulse. Then, the Kautsky effect was induced by actinic light (PPFD of 100 μmol m^{-2} s^{-1}). Steady-state fluorescence (F_S) was reached after 4 min when the second saturation pulse was applied to measure maximum chlorophyll fluorescence in the light (F'_M). Two relative measures were computed: maximum quantum yield of PSII ($F_V/F_M = (F_M - F_0)/ F_M$) and steady-state quantum yield of PSII in the light ($\Phi_{PSII} = (F'_M - F_S)/ F'_M$). F_V/F_M characterizes the overall physiological state of PSII because its decline can be interpreted as photoinhibitory damage connected with environmental stress. Φ_{PSII} measures the proportion of light absorbed by chlorophyll associated with PSII that is used in photochemistry and consequently serves as a good estimation of photosynthetic activity (Maxwell & Johnson, 2000). Its linear relationship with CO_2 assimilation and O_2 evolution has been frequently observed (Baker, 2008 and references therein).

The fluorescence parameters were measured (1) before experimental treatments, (2) during the incubation in the sorbitol solutions (after 24 h incubation in 300–750 mM and after 4 h incubation in 2 M sorbitol) and (3) also during subsequent recovery (from 2 M sorbitol) in stream water (after 4 and 24 h).

Light and epifluorescence microscopy

The morphology of the cells and the occurrence of plasmolysis were observed in an Olympus BX53 microscope equipped with a 100 W ultrahigh-pressure mercury lamp. Both bright field and epifluorescence observations were performed. Chloroplasts were visualized by means of chlorophyll autofluorescence, and the cell viability was estimated before the experiments and after a 24-h recovery in water with the application of the SYTOX Green dye

(SYTOX® Green nucleic acid stain, Molecular Probes). This dye penetrates into cells via damaged membranes and binds to DNA. Thus, cells with damaged membranes emit a bright green fluorescence. The membrane integrity as a proxy for cell viability is widely used in algae and other microorganisms (de los Ríos *et al*., 2004; Knowles & Castenholz, 2008; Tashyreva *et al*., 2013). For the visualization of SYTOX Green dye fluorescence, a U-FBWA cube with 460–495/510–550 nm was used. The samples were treated with 3 μM SYTOX solution for 10 min prior to counting. The concentration and incubation time were assessed by trial tests on cultured material following a method described by Tashyreva *et al*., (2013). The number of viable and dead cells was counted in three randomly chosen fields of view in each of three independent samples of biomass (> 500 cells).

Data evaluation and presentation

The initial values of fluorescence parameters were tested by general linear model (GLM) two-way factorial analysis of variance (ANOVA) to determine whether there were any significant differences between populations and also between samples of the same population used for the separate experiments. The two factors tested – 'population' and 'experiment' were regarded as fixed effects. The differences in fluorescence parameters between the initial state and after 24 h incubation in individual sorbitol concentrations were tested by separate paired *t*-tests. Finally, GLM repeated-measures ANOVA was used to compare changes in parameters in time (in 2 M sorbitol and after 4 and 24 h of recovery). Factor levels were compared with Tukey's HSD *post hoc* tests. We also performed principal component analysis (PCA) to visualize correlations between the measured parameters and studied populations. Populations that had not plasmolysed even in 750 mM sorbitol were arbitrarily given the value 1,000 as the point of plasmolysis for PCA calculation purposes. No data transformations were performed, and the parameters ('species' data) were standardized and centered to make them comparable. ANOVA was performed in Statistica 10 for Windows and PCA in Canoco for Windows version 4.5 (ter Braak & Šmilauer, 2002). SigmaPlot 9.01, Adobe Photoshop 7.0 and Microsoft Office Power Point 2007 were used for the graphical elaboration of the results.

Results

Morphology and physiological performance in field-collected Zygnema

All investigated populations, including those in the natural desiccated state, were viable after collection in the field. Only a small proportion of dead cells were observed in some populations by means of light microscopy or epifluorescence (Fig. 1). In the light microscope, cells of all populations appeared similar (Fig. 2a–f). Their dense (brownish) cytoplasmic content was rich in storage material, so their typical star-shaped chloroplasts were not clearly discernible. Thick mucilaginous sheaths were observed around filaments in moist and dry samples (Fig. 2c–f). The filaments tended to break into short fragments in populations 5 and 6. Typical vegetative cells of *Zygnema* sp. with clearly visible stellate chloroplasts were observed only rarely.

The initial values of chlorophyll fluorescence parameters after rewetting differed among populations (Fig. 3). The highest initial value of F_V/F_M (Fig. 3a) was measured in population 2 (wet) and the lowest in population 6 (dry). Φ_{PSII} showed a more distinct pattern: Both wet populations showed much higher values than those measured in moist and dry populations (Fig. 3b).

Occurrence of plasmolysis

Plasmolysis first occurred in both wet populations in 450 mM sorbitol (Ψ_π = –1.08 MPa; Table 1); in 600 mM sorbitol (Ψ_π = –1.52 MPa), at least 50% of the cells were visibly plasmolyzed. According to the incipient plasmolysis method, this value corresponds to the cellular osmotic potential at turgor loss ($\Psi_{t=0}$). On the other hand, the moist and dry populations only started to plasmolyse in 750 mM sorbitol (Ψ_π = –1.96 MPa) or did not plasmolyse yet, indicating a more negative $\Psi_{t=0}$. Interestingly, even a minor osmotic stress induced filament disintegration in sample 5 (dry) since single-celled fragments formed already after 24 h incubation in 300 mM sorbitol.

Actual values of Φ_{PSII} decreased with increasing sorbitol concentration in wet and moist populations (Fig. 4). In contrast, in populations 4 and 6 (dry), the mean values of Φ_{PSII} after 24-h cultivation in various sorbitol concentrations were even higher than the initial values (> 100%, Fig. 4). The Φ_{PSII} increased in controls of dry samples (4–6) incubated for 24 h in water as well (Fig. 4).

Osmotic stress and recovery

Plasmolysis occurred in all populations after a 4-h incubation in 2 M sorbitol (Ψ_π = –5.63 MPa; Fig. 2g–l). The plasma membrane retracted only partly from the cell wall in the dry samples, whereas in the wet samples, clear convex plasmolysis was observed. Furthermore, a high proportion of the cells in the wet samples were irreversibly damaged after the osmotic stress (Fig. 2g and h). We observed in the light microscope that some cells lost their integrity in 2 M sorbitol, and instead of plasmolyzing, their cell content was homogenously spread out. These cells did not recover in water, and further observation proved that they were dead.

Fluorescent parameters, Φ_{PSII} in particular, decreased after 4 h of incubation in 2 M sorbitol (Fig. 5, Table 2). The decline in F_V/F_M of the wet samples continued after their transfer to water, whereas dry populations almost recovered initial values of F_V/F_M after 4 h in water; population 5 recovered fully. Even after 24 h in water, neither the wet nor moist populations reached the initial values of F_V/F_M. Recovery of Φ_{PSII} followed a similar pattern: 4 h in water was enough for the full recovery in dry samples, but after 24 h, the moist population (No. 3) also fully recovered its Φ_{PSII} values (Fig. 5, Table 2). Moreover, microscopic observations performed after a 24-h recovery period showed that filaments from samples 3 (moist) and 5 (dry) disintegrated into single cells.

Overall similarity of the samples

Principal component analysis was performed to visualize the similarity of populations (each represented by two to three individual samples; Fig. 6). The first axis that explained 70 % of the total variation was mainly formed by parameters related to recovery from stress. The second axis (19 %) was related to parameters describing the initial state of the field-collected samples. Thus, wet populations with poor recovery were clearly separated from the others along the first axis. It was notable that the moist samples (No. 3) clustered together with some of the dry samples (Fig. 6).

Discussion

Morphology and physiological performance in field-collected Zygnema

Our results clearly support our hypothesis that naturally desiccated populations of *Zygnema* become hardened, possessing an osmotic mechanism of desiccation resistance that prevents plasmolysis. The natural populations were mostly formed by old, stationary-phase cells that are usually called akinetes in green algae (McLean & Pessoney, 1971; Coleman,

1983). In fact, they were the only special cell type recorded in our samples. They were mostly not disintegrated from filaments, in which case we call them pre-akinetes in accordance with McLean & Pessoney (1971). It must be emphasized that these resistant stationary-phase cells are markedly different from the true *Zygnema* akinetes with zygospore-like cell walls, which we have never observed in the Arctic.

Notably, even the wet populations formed pre-akinetes (*sensu* McLean & Pessoney, 1971). Thus, we assume that osmotic stress connected with slow desiccation is not the key factor controlling pre-akinete formation in *Zygnema*. This is in contrast to *Klebsormidium rivulare,* which produced akinetes only after prolonged desiccation but had a markedly different morphology when collected in water (Morison & Sheath, 1985). Nevertheless, akinetes of various algae were also induced by salt stress (Meindl *et al*., 1989) and nutrient starving (Darling *et al*., 1987; Nagao *et al.*, 1999). We hypothesize that the extensive mats of biomass deplete mineral nutrients during the summer, and the algae experience conditions similar to laboratory-induced starvation, which leads to the formation of stationary-phase cells also in water environments.

In spite of being morphologically similar, the populations sampled in various stages of desiccation differed in their physiological characteristics. The water status was reflected mainly by $\Phi_{PSII,}$ which was lower in the dry samples than in the wet samples. This difference was clear, although we rehydrated the dry-collected samples 8 h prior to the experiments in order to reactivate their physiological processes before measurements. A much longer time of rehydration would be needed for complete recovery and particularly dehardening, leading to the loss of their stress tolerance. For example, the dehardening took several days in *K. rivulare* akinetes (Morison & Sheath, 1985).

Osmotic stress and osmotic potential

Populations in various stages of desiccation differed in their reaction to the experimental osmotic stress treatments. The wet-collected populations (1 and 2) act similarly to laboratory-grown, nonhardened cultures of *Zygnema* without any stress-induced osmotic adjustment: chlorophyll fluorescence was unaffected after 24 h in 400 mM sorbitol, but 800 mM sorbitol led to a significant reduction of F_V/F_M in cultivated material (Kaplan *et al*., 2013). Moreover, the incipient plasmolysis occurred at –1.67 MPa (Kaplan *et al.*, 2013), very similar value to our results. However, this value is still much more negative than that in related freshwater algae (e.g. *Micrasterias* clearly plasmolyzed in 339 mM sorbitol; Affenzeller *et al.*, 2009).

Notably, 2 M sorbitol represented a rather strong osmotic stress and led to high mortality and lack of the recovery of PSII quantum yield (Fig. 5, Table 2).

The dry-collected samples differed markedly from the wet ones in their reaction to osmotic stress (Fig. 6). In general, they were much more resistant to osmotic stress. For comparison, a true aeroterrestrial alga *Klebsormidium crenulatum* plasmolyzed at 800 mM sorbitol (–2.09 MPa; Kaplan *et al*., 2012). *Klebsormidium nitens* plasmolyzed in 600 mM sorbitol (–1.67 MPa), but it showed an ability to acclimate to the actual osmotic conditions because after 24-h cultivation, the cells were not plasmolyzed anymore even in 800 mM sorbitol. *Klebsormidium nitens* also fully recovered oxygen evolution after 3 h of recovery from a 2 M sorbitol treatment (Kaplan *et al*., 2012). Thus, the field-desiccated (naturally hardened) *Zygnema* cells can be compared with the true aeroterrestrial algae on the basis of cellular osmotic potential and their ability to recover fluorescence parameters.

The moist-collected population (3) clustered together with the dry samples (Fig. 6), which may be a result of its expected exposition to several desiccation-rehydration cycles earlier in the season. It was moist at the moment of collection, but the desiccation history of the sites remained unknown. Nevertheless, the moist population showed an intermediate response in the recovery rate as compared to the dry and the wet populations (Fig. 5, Table 2).

Sources of variation among samples

Interestingly, significant differences among populations in the same degree of natural desiccation were also observed. The dry populations (4–6) reacted inconsistently to mild osmotic stress (Figs 4 and 6). Disintegration into single cells was observed only in population 5. Moreover, individual samples of the population 5 showed high variation mainly connected with a wide range of initial F_V/F_M values (Fig. 6). This variability is not very surprising because the category ‘dry’ was established arbitrarily based on the macroscopic appearance in the field at the moment of collection and may therefore comprise samples that were not dried to the same degree for the same period. Such a difference could not be discerned in the field but was revealed in the performance of individual populations during experiments.

Another possible explanation for population differences might be a hidden genetic diversity. The genus *Zygnema* comprises more than 130 morphologically defined species (Kadlubowska, 1984), but the first molecular analyses showed that traditional systematics of the genus do not correspond with molecular phylogeny (Stancheva *et al.*, 2012). We hypothesize that the difference in physiological performance between populations 1 and 2 in the natural state (Fig. 3) might reflect species-specific characteristics. This is supported by

morphology, because population 2 differed from the others, with wider filaments and different chloroplast morphologies (Fig. 2). Moreover, two phylogenetically distant genotypes were revealed within the same seepage pools in Svalbard recently (Pichrtová *et al.*, 2013). Namely, the published *Zygnema* G (Pichrtová *et al.*, 2013) was isolated from the site 1 in this study and *Zygnema* B from the site 2. However, this assignment cannot be proven as no molecular methods were applied in the present study. Nevertheless, our results show that the performance of the samples under osmotic stress conditions clearly depends on their natural hydration status *in situ* regardless of possible genotype-related variability.

Role of pre-akinetes in Zygnema survival

Genkel and Pronina (1979) studied *Z. stellinum* in Belarus and concluded that resting cells—identical to the pre-akinetes we observed—play a role in survival during short periods of unfavorable conditions, whereas production of much more specialized cells, such as zygospores and parthenospores, is essential for winter survival. However, as such specialized cells have never been observed in the Arctic or Antarctic *Zygnema* (Hawes, 1990; Kim *et al*., 2008, 2011; Holzinger *et al*., 2009), we expect that pre-akinetes (McLean & Pessoney, 1971) play a key role during winter survival. This hypothesis is also supported by our observation: Frozen biomass collected in April 2012 consisted of viable pre-akinetes (M. Pichrtová & J Elster, unpublished data).

However, our results show that pre-akinete morphology itself is not closely correlated with better stress resistance. Similarly, Kaplan *et al*. (2013) experimentally proved that in cultures, plasmolysis occurs at similar osmolarities in both young and senescent (pre-akinete) cells. As field-desiccated akinetes showed much better osmotic stress resistance compared with akinetes from the wet-collected samples, this indicates that field acclimation to the natural dehydration must take place and that desiccation (probably slow) is necessary for the development of desiccation tolerance.

Synthesis and accumulation of organic osmolytes (notably sugars and sugar alcohols) is one of the main mechanisms involved in the osmotic acclimation of algae (Bisson & Kirst, 1995), although sugar alcohols are missing in streptophyte algae such as *Klebsormidium* (Karsten & Rindi, 2010). These compounds osmotically equilibrate the cells with their medium (Karsten *et al*., 2007; Oren, 2007) and as compatible solutes stabilize proteins and membranes by substituting water. They are involved not only in desiccation tolerance, but also in cold acclimation and subsequent freezing resistance (Nagao *et al*., 2008). The only analysis of osmolytes in *Zygnema* revealed sucrose to be the dominant one – it represented

95 % of extractable sugars in Antarctic samples (Hawes, 1990). Nevertheless, the author noted that the total content of soluble sugars in field samples was too low to decrease considerably the freezing point of the cells. To our knowledge, production of organic osmolytes has never been studied in Zygnematophyceae in detail, and the biochemical nature of cellular protection mechanisms in *Zygnema* remains unresolved.

Besides the formation of resistant cells, other protection mechanisms play a role as well. The extracellular matrix or mucilaginous sheaths reduce water loss (Karsten *et al*., 2007; Knowles & Castenholz, 2008). We also observed massive mucilage surrounding filaments, especially in the moist and dry populations. Another adaptive mechanism is the mat-forming growth. Cells from the upper layers provide protection for the cells in lower layers not only from desiccation (Holzinger & Karsten, 2013) but also from excessive irradiation (Aigner *et al*., 2013; Pichrtová *et al*., 2013).

In conclusion, our results indicate mechanisms of *Zygnema* survival in the changing environment of temporary melt water pools in the Arctic. During the summer, the cells gradually change to pre-akinetes, possibly as a result of nutrient starving. Such cells are, however, not resistant to drying without further acclimation. It is only (naturally slow) desiccation that induces hardening against severe desiccation and presumably freezing stress as well.

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Tables

Table 1. Occurrence of plasmolysis in four sorbitol concentrations and proportion of viable cells. +, first occurrence of plasmolysis; ++, at least 50% of cells were plasmolyzed. Viable cells were counted before and after the treatment with 2 M sorbitol, and then the relative viability was calculated.

Population	1	2	3	4	5	6
Plasmolysis occurrence in sorbitol solutions						
300 mM	-	-	-	-	-	-
450 mM	+	+	-	-	-	-
600 mM	++	++	-	-	-	-
750 mM	++	++	+	-	-	+
Viable cells after strong osmotic stress (2 M sorbitol) relative to the initial state						
%	40	25	100	100	100	100

Table 2. Means of chlorophyll fluorescence parameters measured after a 4-h incubation in 2 M sorbitol (Sorb) and subsequent recovery after 4 and 24 h in water and ANOVA results ($n = 3$). Populations sharing the same letter do not differ significantly in initial values of the fluorescence parameters (one-way ANOVA, Tukey's *post hoc* tests, $p > 0.05$). Differences between initial and following (Sorb, 4-h rec, and 24-h rec) means computed by GLM repeated-measures ANOVA are denoted by asterisks: * $0.05 > p > 0.01$, ** $0.01 > p > 0.001$, *** $p < 0.001$

	F_V/F_M				Φ_{PSII}			
Population	Initial	Sorb	4-h rec	24-h rec	Initial	Sorb	4-h rec	24-h rec
1	0.56a	0.32***	0.25***	0.37***	0.39ab	0.08***	0.12***	0.23***
2	0.63ab	0.31***	0.16***	0.36***	0.48a	0.04***	0.05***	0.20***
3	0.57ab	0.37***	0.43**	0.43**	0.25c	0.08***	0.16**	0.23
4	0.66b	0.52***	0.55*	0.58	0.34b	0.12***	0.31	0.33
5	0.58ab	0.45***	0.51	0.54	0.32bc	0.1***	0.28	0.30
6	0.57ab	0.34***	0.45*	0.48	0.33bc	0.16***	0.32	0.30
ANOVA *p*	0.03				< 0.0001			

Figures

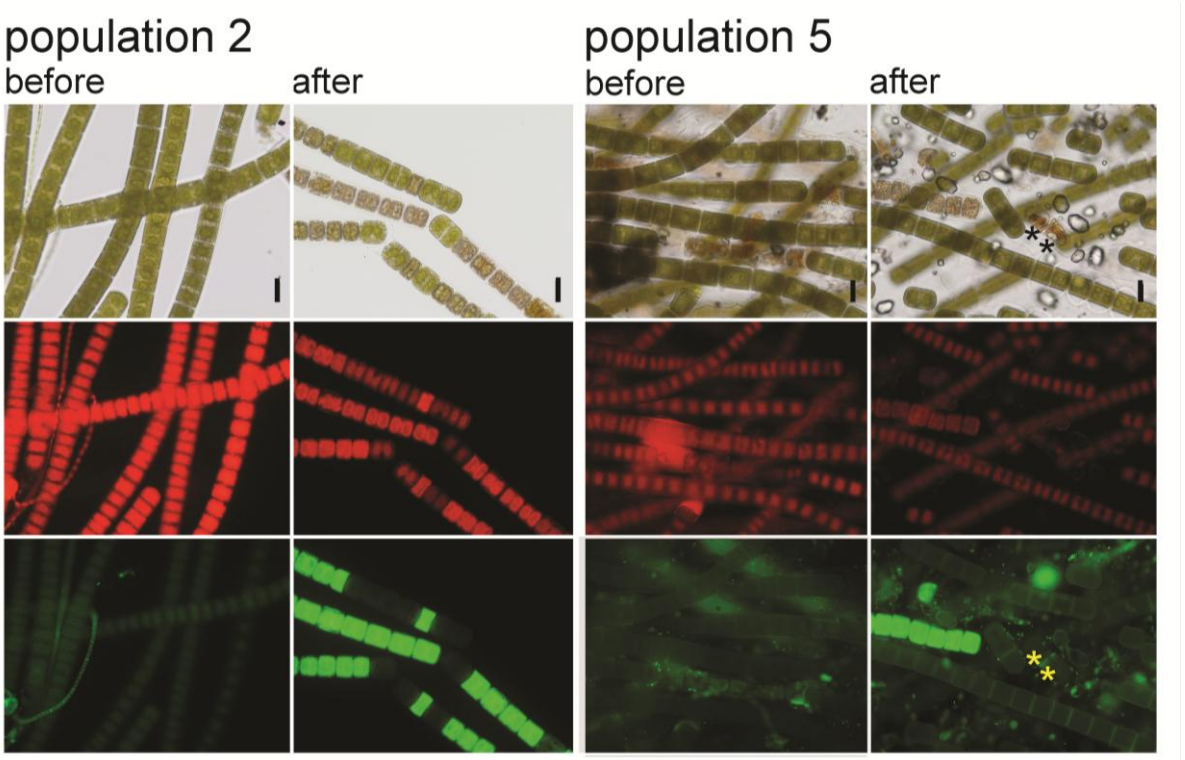

Fig. 1. Comparison between light microscope (top row), chlorophyll autofluorescence (middle row), and epifluorescence (bottom row) images of populations No. 2 (collected wet) and No. 5 (collected dry) in the initial state before incubation in 2 M sorbitol and after a 24-h recovery in water. The epifluorescence highlights the nucleic content of dead cells stained by SYTOX Green stain. Asterisks denote unlabelled dead cells without nucleic content. Intensity of chlorophyll autofluorescence may temporarily increase after cell death (population 2, after recovery), and therefore, chlorophyll autofluorescence does not provide reliable estimate of cellular physiological state. Scale bars: 30 μm.

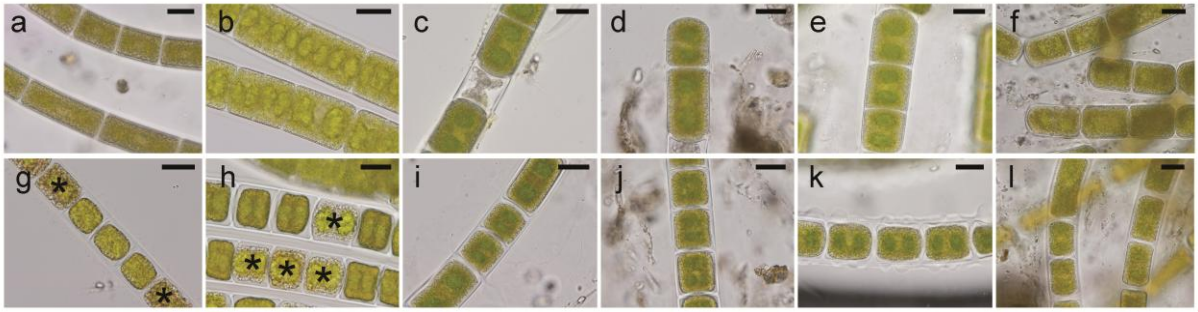

Fig. 2. Light microscope images showing *Zygnema* cells in their natural state in water (a–f) and plasmolysed cells in 2 M sorbitol (g–l). Population 1: a, g; 2: b, h; 3: c, i; 4: d, j; 5: e, k; 6: f, l. Cells irreparably damaged are marked by asterisks. Scale bars: 20 µm.

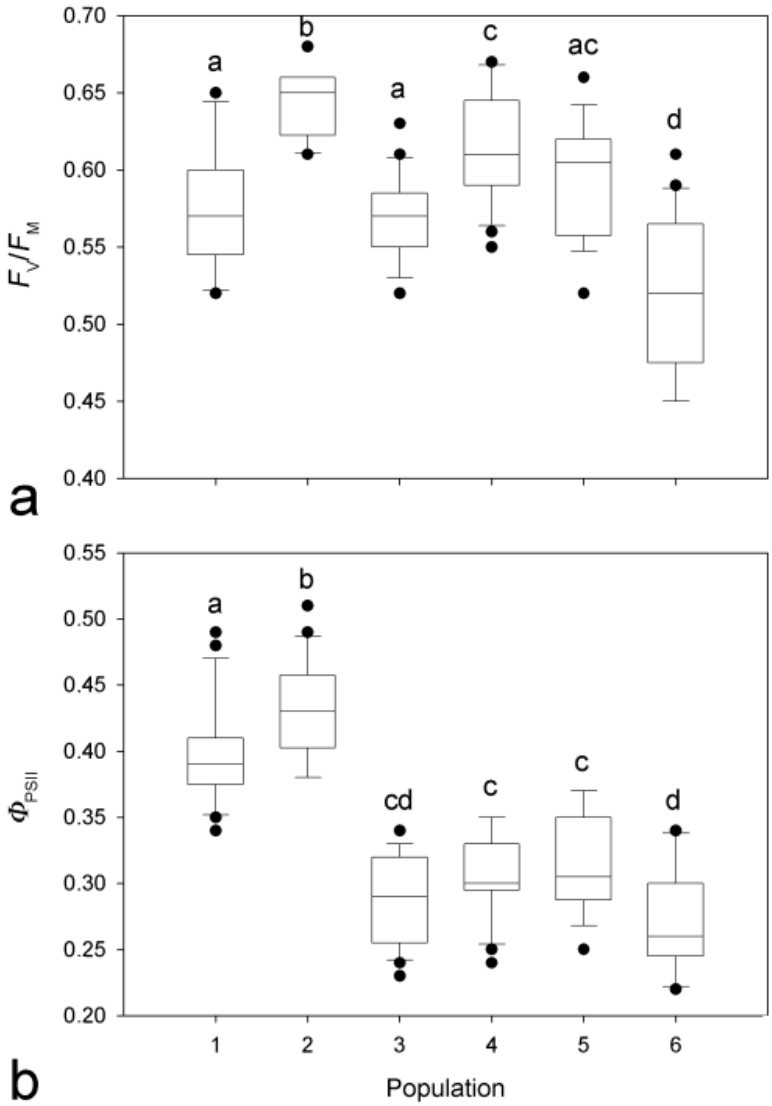

Fig. 3. Initial values of chlorophyll fluorescence parameters measured prior to the experiments on all samples from individual populations (n = 21). The samples were kept in water for 8 h after collection in the field; (a) maximum quantum yield of PSII (F_V/F_M), (b) steady-state quantum yield of PSII in the light (Φ_{PSII}). Populations 1 and 2: wet biomass; 3: moist biomass; 4, 5, 6: dry biomass when collected. Different letters represent different means ($p < 0.05$; GLM two-way ANOVA, Tukey's *post hoc* tests). The line within the box marks the median, the boundaries indicate the 25th and 75th percentiles, the error bars indicate the 10th and 90th percentiles and the individual points denote outliers – samples with values out of this range.

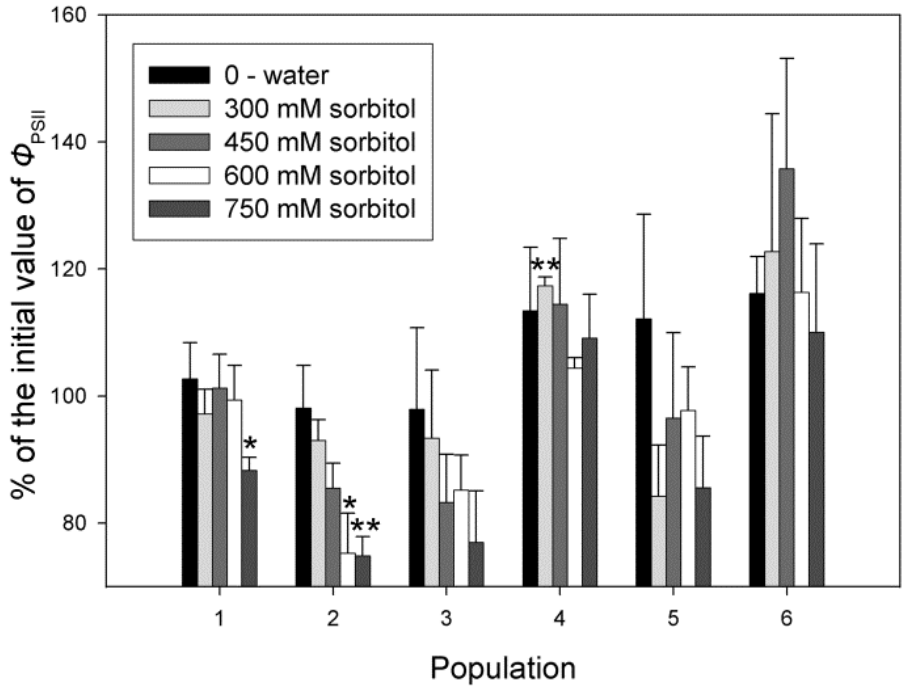

Fig. 4. Relative values (compared to the initial state) of steady-state quantum yield of PSII in the light (Φ_{PSII}) after 24-h incubation in water and four sorbitol concentrations (means + SD). The mean absolute measured values at the beginning of the experiment were as follows: population 1 (Φ_{PSII} = 0.40), population 2 (0.42), population 3 (0.30), population 4 (0.30), population 5 (0.31), and population 6 (0.26). Differences between the initial state and value measured after 24 h of experimental treatment are marked by asterisks ($0.001 < p < 0.01$ **, $0.01 < p < 0.05$ *; paired *t*-test, $n = 3$).

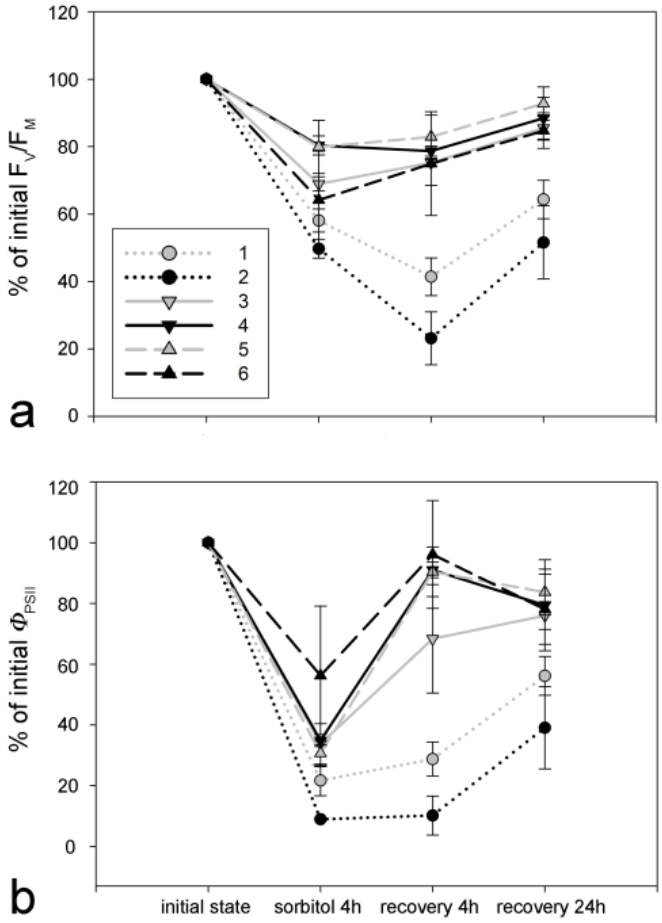

Fig. 5. Changes in chlorophyll fluorescence parameters during incubation of *Zygnema* samples in 2 M sorbitol and subsequent recovery. Values relative to the initial values before the experiment are shown (mean ± SD): (a) maximum quantum yield of PSII (F_V/F_M), (b) steady-state quantum yield of PSII in the light (Φ_{PSII}). The initial absolute values of F_V/F_M were as follows: 1 (0.56), 2 (0.63), 3 (0.57), 4 (0.66), 5 (0.58), and 6 (0.57) and of Φ_{PSII}: 1 (0.39), 2 (0.48), 3 (0.25), 4 (0.34), 5 (0.32), and 6 (0.33).

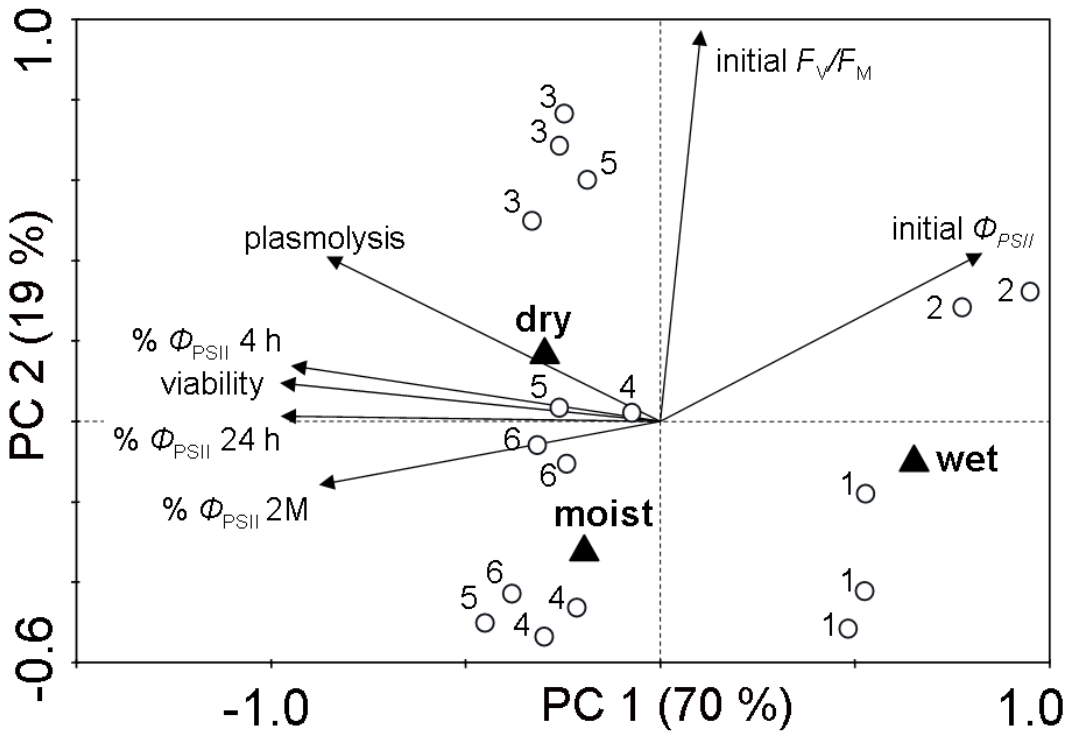

Fig. 6. PCA ordination plot of samples from populations 1–6 stressed by the incubation in 2 M sorbitol solution based on various measured parameters. PC1 and PC2, principal component axes with percentage of explained variability; viability, relative viability after the incubation in 2 M sorbitol compared with the initial viability; plasmolysis, sorbitol concentrations where plasmolysis occurs, populations 4 and 5 were arbitrarily given the value 1000; initial F_V/F_M, initial values of F_V/F_M; initial Φ_{PSII}, initial values of Φ_{PSII}, % Φ_{PSII} 2M: relative values of Φ_{PSII} measured after 4 hours in 2 M sorbitol; % Φ_{PSII} 4h, relative values of Φ_{PSII} measured after 4 hours of recovery in water, % ΦPSII 24h, relative values of Φ_{PSII} measured after 24 h of recovery in water. Centroids of the three groups with a different water status were projected *post hoc* onto the correlations.

3.3 Manuscript III

Nitrogen limitation and slow drying induce desiccation tolerance in conjugating green algae (Zygnematophyceae) from polar habitats

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Abstract

Filamentous Zygnematophyceae are typical components of algal mats in the polar hydroterrestrial environment. Under field conditions, they form mature cells (pre-akinetes), which are resistant to osmotic stress following natural desiccation. Here, pre-akinete formation was investigated in four strains of algae with vegetative *Zygnema* sp. morphology isolated from the Arctic and Antarctic. Phylogenetic analysis using *rbc*L sequences revealed that one strain, from the Arctic, belonged to the genus *Zygnemopsis*, whereas the other three were closely related strains of *Zygnema*. Algae were grown for 9 weeks in liquid or on solidified medium, with or without nitrate, before desiccation at three different drying rates. Light and transmission electron microscopy (TEM) and chlorophyll a fluorescence were used to determine the effect of drying rate on viability and recovery of physiological activity. Pre-akinete formation was induced by nitrogen starvation, as pre-akinetes made up a greater proportion of the cells in nitrate-free cultures. Vegetative cells were observed only after cultivation in full medium but pre-akinetes survived at least some of the desiccation regimes. Both recovery and viability were clearly dependent on drying rate: slower desiccation led to higher levels of survival. Nevertheless, cells survived rapid drying (at 10% rh) after pre-cultivation on agar or after acclimation by very slow desiccation. This demonstrates that mature cells (pre-akinetes) develop during starvation but must be acclimated by mild desiccation stress to survive rapid drying. Pre-akinetes play a key role in stress resistance to climate extremes in polar regions, where sexual reproduction leading to the formation of dormant zygospores is largely suppressed.

Introduction

Green algae, despite being widely considered aquatic organisms, occur often in hydroterrestrial or aeroterrestrial environments [1,2], and their exceptional ability to adapt to life on land is well-known. The transition to land has appeared independently many times within this group and aeroterrestrial algae are found in various green algal lineages [3,4]. The most important land colonization event in the Earth's history appeared within streptophytes, in the ancestor of all land plants (Embryophyta), which occurred during the Ordovician Period [5]. There is an ongoing discussion concerning which of the algal streptophyte classes is the actual sister group to Embryophytes; currently, either Zygnematophyceae or a clade consisting of Zygnematophyceae and Coleochaetophyceae are considered the closest algal relatives of land plants [6–9].

Zygnematophyceae, the most species-rich lineage of streptophyte algae, are filamentous or unicellular algae that do not form any flagellate stages and reproduce sexually by conjugation, producing a dormant zygospore [10]. Many Zygnematophyceae are found in conditions that expose them to desiccation stress, such as those found in aeroterrestrial habitats [11], airborne particles [12], and surface of glaciers [13,14]. In extreme habitats, such as the Arctic and Antarctic, no conjugation process has been observed in nature [15,16]. Filamentous Zygnematophyceae are also typically found in ephemeral pools or streams that are subject to occasional or regular desiccation. Such algal mats are a noticeable feature of polar tundra habitats in particular, where they are among the most important primary producers [17]. The genus *Zygnema* has been repeatedly reported in such habitats in the Arctic and in Antarctica [15–19].

Mechanisms of desiccation tolerance in green algae have been reviewed recently [2,20,21]. Certain algae are considered desiccation-tolerant, but their actual survival capability is dependent upon the conditions under which desiccation occurs. Various factors significantly influence recovery after dehydration: for example, the duration of desiccation [22], light availability during desiccation [23], and relative air humidity, which corresponds with the drying rate [22,24].

Green microalgae can rarely fully withstand desiccation in the vegetative state; this ability has been found in some desert species, mostly in those belonging to the Chlorophyte lineage [23]. More often they form specialized cells, including zygospores, aplanospores or akinetes, which are capable of surviving periods of desiccation. Akinetes are mature (senescent), stationary phase cells rather than morphologically specialized dormant stages.

However, they have been found to be much more stress-resistant than fresh, actively growing vegetative cells. They usually possess thick cell walls and are filled with storage materials [25–27]. The formation of such stress-resistant stationary phase cells by *Zygnema* sp. has been observed under both experimental [25,28] and field conditions [15,19]. In accordance with the published literature, we term these cells "akinetes" after they have disintegrated into single cells, and "pre-akinetes" if they are still part of a filament; the use of the term "akinete" in *Zygnema*, which in some species refers to specialized stages with a colored and sculptured mesospore, has been recently addressed [19].

Under some experimental conditions, the formation of resistant stages (akinetes) may be induced by desiccation in, for example, *Klebsormidium rivulare* [27]. It is also induced in ageing cultures of *Zygnema* sp. [25] and is most likely the result of nitrogen-starvation treatment, as nutrients are depleted by intensive growth under batch culture conditions. During nitrogen starvation, protein synthesis is suppressed and metabolism shifts to the production of carbohydrates and lipids, which are substances of great biotechnological interest [27,29,30]. The occurrence of storage product-filled cells in Arctic and Antarctic *Zygnema* sp. has been reported in field samples [15,17,19].

When the formation of pre-akinetes was observed under natural conditions in the Arctic, their resistance to osmotic stress was found to be significantly related to their natural hydration status [19]. The authors concluded that the experience of mild dehydration stress was crucial for akinete hardening, and hypothesized that other environmental factors (possibly nutrient starvation) must be involved in their initial formation [19].

In this study, we test this hypothesis; four strains, isolated from putative *Zygnema* sp. mats growing in polar regions that readily produced pre-akinete stages, were used for the experiments. First, we studied the effects of mild dehydration stress and nitrogen starvation on the formation of pre-akinetes. Next, desiccation stress resistance of pre-akinetes was investigated under various rates of drying. Photosynthetic activity during the experiments was assessed regularly by measuring chlorophyll fluorescence. As no conjugating stages were observed, which make it impossible to determine species according to their morphological traits, the strains were also characterized by their *rbc*L sequences. Finally, we used electron microscopy to characterize the ultrastructure of pre-akinetes before and after desiccation.

Materials and Methods

Cultivation of algal material and light microscopy observations

Four strains (B, C, E and L) with a vegetative *Zygnema* morphology were used for the experiments. Capital letters were assigned to the strains in accordance with previously published studies on polar *Zygnema* spp. [28,31]. *Zygnema* sp. B (CCALA 976) was isolated in 2010 from a shallow seepage pool on Svalbard in the high Arctic. Strain L was isolated in 2011 from the same locality in Svalbard (Arctic) as *Zygnema* sp. B and was deposited in the same culture collection (accession number XXX). The filaments of *Zygnemopsis* sp. L are narrower than those of *Zygnema* sp. B. *Zygnema* sp. C was obtained from the Culture Collection of Autotrophic Organisms in Třeboň, Czech Republic (CCALA, www.butbn.cas.cz/ccala/index.php; strain No. 880). It was isolated by Josef Elster and Jana Šnokhousová in 2008 from a sample originally collected from a seepage reaching Monolith Lake on James Ross Island, Antarctica. *Zygnema* sp. E (CCCryo 278-06) was isolated in 2006 from a meltwater pool north of Artigas Base freshwater lake (also known as Lago Uruguay or Lake Profound), Fildes Peninsula, Maxwell Bay, King George Island, South Shetland Islands, Antarctica.

Cultures of each of the four strains were transferred in the exponential growth phase into either standard Bold's Basal medium (BBM) [32] or into BBM lacking nitrate (termed henceforth 'BBM' and 'BBM-N', respectively). The cultures were grown in 6-well microplates in either liquid ('L') medium or on medium solidified with 1.5% agar ('A'). Thus, four combinations of pre-cultivation conditions for each strain were achieved; these are henceforth referred to as 'A BBM', 'A BBM-N', 'L BBM' and 'L BBM-N'. The plates were kept under optimal growth conditions of 20°C and continuous light (intensity: 35 μmol m^{-2} s^{-1}) for 9 weeks.

Light microscopy of the algal strains was undertaken using a Zeiss Axiovert 200 M light microscope (Carl Zeiss AG, Oberkochen, Germany) equipped with a Zeiss Axiocam MRc5 camera. The cultures were investigated after 9 weeks of pre-cultivation to determine the percentages of pre-akinetes. Further observations were made using light microscopy following the desiccation experiment and 48 hours of subsequent rehydration, when the proportion of surviving cells was estimated.

DNA isolation and phylogenetic analysis

DNA was isolated using the Invisorb Spin Plant Mini kit (Invitek, Germany), according to the manufacturer's instructions. The chloroplast-encoded gene *rbc*L was amplified using the polymerase chain reaction (PCR) and primers RH1 and 1385R [33]. Each reaction contained 13.9 µl sterile Milli-Q water, 2 µl $MgCl_2$ (25 mM), 2 µl AmpliTaq Gold 360 Buffer (Applied Biosystems, Carlsbad, CA, USA), 0.4 µl dNTP mix (10 mM), 0.25 µl each primer (25 pmol/µl), 0.2 µl AmpliTaq Gold 360 DNA Polymerase, and 1 µl DNA (10 ng/µl). The PCR programme consisted of an initial denaturation for 10 minutes at 95°C, followed by 35 amplification cycles, each with a 1 minute denaturation step at 94°C, 1 minute annealing at 48°C. and 2.5 minutes extension at 72°C, and a final extension stage at 72°C for 10 minutes. Finally, the PCR products were purified using the GenElute PCR Clean-Up kit (Sigma-Aldrich, USA), according to the manufacturer's protocol, and sequenced by Macrogen Inc. (Seoul, South Korea). The GenBank accession numbers for the sequences are xxx (*Zygnema* sp. B), xxx (*Zygnema* sp. C), xxx (*Zygnema* sp. E) and xxx (*Zygnemopsis* sp. L).

Published sequences from GenBank database were selected on the basis of recent *Zygnema*/Zygnematophyceae phylogenies [34,35]. The resulting alignment comprised 26 sequences, each of which was up to 1292 nucleotides long.

Three different phylogenetic analyses were performed: maximum likelihood (ML), weighted parsimony (wMP, character weighting), and Bayesian inference (BI). For ML and BI, the sequence evolution model was determined as GTR+I+gamma by MrModelTest 2.3 [36] using the Akaike Information Criterion. The BI phylogenetic trees were constructed using Three different phylogenetic analyses were performed: maximum likelihood (ML), MrBayes 3.2.1 [37]. Two parallel Markov chain Monte Carlo runs were carried out for 3×10^6 generations, each with one cold and three heated chains. Convergence of the two cold chains was checked by the average standard deviation of split frequencies: the value was 0.001865. Trees and parameters were sampled every 100 generations, and trees from the initial 100 generations were discarded using the sumt burnin function. The BI tree was rooted using the midpoint root function in FigTree 1.3.1 [38]. Bootstrap analyses (ML, wMP) were calculated using Garli 2.0 [39] and PAUP* Portable version 4.0b10 [40]. ML analyses consisted of rapid heuristic searches (100 pseudo-replicates) using automatic termination (genthreshfortopoterm command set to 100,000). The wMP bootstrapping was performed using heuristic searches with 100 random sequence addition replicates, tree bisection reconnection swapping, and random addition of sequences (the number was limited to 10,000 for each replicate).

Desiccation stress treatments

Initially, samples pre-grown on agar were used in the desiccation experiments because they were expected to have developed a level of stress resistance. Desiccation stress resistance was compared between well-developed akinetes from cultures grown on A BBM-N and morphologically diverse cultures grown on A BBM. The experiments followed a standardized setup developed for monitoring physiological performance during controlled dehydration and rehydration (Karsten *et al.*, unpublished data). Small samples of each of the four different strains were placed on glass fibre filters (Whatman GF/C). Four of these filters were placed on a perforated metal grid inside a 200 ml polystyrene box sealed with a transparent lid prior to experimental drying. Different drying rates were achieved by desiccating samples inside the containers at different relative humidities (rh): rate 1, a rapid rate of drying, at approximately 10% rh was achieved using 100 g of freshly dried silica gel (Silica Gel Orange, Carl Roth, Karlsruhe, Germany), whereas rate 2, a slow drying rate at approximately 86% rh, was achieved using saturated KCl solution. For generating a very slow rate of drying (rate 3), each biomass sample was wetted with 10 µl L BBM medium. Additionally, another set of samples, desiccated in this 'very slow' mode for 12 hours, was put over silica gel for a further 24 hours.

After desiccation for 24 hours, the samples were rehydrated by the addition of 0.5 ml fresh L BBM medium and placed into new polystyrene boxes with 100 ml tap water to ensure an environment with very high relative humidity (98–100%). The filters were sprayed regularly with sterile distilled water to prevent additional desiccation stress and allowed to recover for 72 hours. During the experiments, the containers were kept at ambient room temperature under continuous illumination (light intensity: 50 µmol m^{-2} s^{-1}).

Samples from liquid cultures (L BBM and L BBM-N) were also desiccated under the three drying rates described above to determine whether pre-akinetes developing under such conditions (i.e., without exposure to mild desiccation stress) reacted to experimental desiccation in a similar manner to those grown on agar.

Measurement of the effective quantum yield

The effective quantum yield of photochemical energy conversion in PSII (Φ_{PSII}) [41] was measured using a PAM 2500 fluorometer (Heinz Walz GmbH, Effeltrich, Germany). Φ_{PSII} is a relative parameter computed as $(F_M'-F)/F_M'$, where F is steady state fluorescence in the light-adapted state and F_M' the maximum fluorescence in the light-adapted state measured after the application of a saturation pulse. Measurements of Φ_{PSII} were performed directly

through the closed transparent exposure chambers with a constant distance of 5 mm between the probe and the samples. The first measurement was taken immediately after placing the filters into the desiccation chambers; subsequent measurements were then made every 2 minutes for samples desiccated at 10% rh, every 5 minutes for samples desiccated at 86% rh or every 60 minutes for samples undergoing very slow desiccation. Measurement continued until the values of Φ_{PSII} reached zero or settled at an above-zero value. The effective quantum yield was also measured 1, 6, 12, 24, 48 and 72 hours following rehydration.

Transmission electron microscopy

For transmission electron microscopy (TEM), samples of all cultures were taken from cultures pre-grown on A BBM-N, either immediately or following desiccation at 86% rh for 2.5 hours. Samples were fixed according to the methodology described previously [15] with the following modifications. Briefly, samples were fixed for 1 hour in 2.5% glutaraldehyde, 20 mM caccodylate buffer (pH = 6.8), rinsed, and then post-fixed in the same buffer containing 1% OsO_4 at 4°C for 18 hours. Samples were then dehydrated in increasing concentrations of ethanol and transferred *via* propylene oxide to modified Spurr's resin [42]. Ultrathin sections (~60 nm) were prepared with a Reichert ultracut microtome, counterstained with uranyl acetate and Reynold's lead citrate, and viewed using a Zeiss Libra 120 transmission electron microscope. Digital images were captured with a ProScan 2k SSCCD camera controlled by OSIS iTEM software, and further processed using Adobe Photoshop (7.0) software.

Statistical analysis

All measurements of the effective quantum yield were made using four independent replicate samples per strain. The differences in Φ_{PSII} between individual strains after different pre-cultivation conditions (i.e., the initial values before desiccation) were tested separately for agar and liquid cultures by general linear model (GLM) two-way factorial analysis of variance (ANOVA). The factors tested ('strain' and 'nitrogen') were regarded as fixed effects. Factor levels were compared using Tukey's post-hoc tests. The ANOVA was performed in STATISTICA 10 for Windows.

SIGMAPLOT 9.01 and Adobe Photoshop (7.0) were used for the graphical elaboration of the results showing performance of the cultures during desiccation and subsequent recovery.

Results

Phylogenetic analyses

Phylogenetic analyses (Fig. 1) of *rbc*L sequences revealed that strains B, C and E were members of the genus *Zygnema* but strain L belonged to another genus, *Zygnemopsis*.

The Antarctic strains, C and E, shared an identical *rbc*L sequence and were closely related to *Zygnema* sp. B from the Arctic. All these three *Zygnema* strains formed a well-supported cluster (BI/ML/MP: 1.0/95/95) together with the strain *Zygnema irregulare* (RS012), isolated from California [35]. The *rbc*L sequences within this group differed from each other by, at most, seven base-pair substitutions.

The strain *Zygnemopsis* sp. L fell into a separate *Zygnemopsis* clade (1.0/99/100) and was most closely related to the strain *Zygnemopsis* sp. CCAP 699/1, isolated by Ott in 1965 from an unspecified freshwater habitat in the USA. Sequences of these two strains differed at four sites.

Investigations using light microscopy

Samples of algal cultures were observed after 9 weeks pre-cultivation in four combinations of culture conditions. The cultures grown on A BBM showed high morphological variation. The whole range of cell types, from normal vegetative cells to mature stationary phase cells (pre-akinetes), appeared in all *Zygnema* strains (Fig. 2A–D). It was difficult to perform an exact quantification of different cell types because of the gradual transitions in morphology. Nevertheless, in all cultures of *Zygnema* spp. (B, C and E) more than 50% of cells showed a pre-akinete morphology. In cultures of strains C and E, pre-akinetes with very thick cell walls appeared, but these made up for less than 1% of cells. In *Zygnemopsis* sp. strain L, around 30% of the cells were dead following the pre-cultivation period, and the remainder were mature cells (pre-akinetes).

By contrast, the cells from all the starved (A BBM-N) cultures had a more homogenous appearance: all viable cells were pre-akinetes (Fig. 2E–H). The cells from starved cultures were filled with storage material that appeared as large globular hyaline inclusions. The chloroplasts of these cells were small, lacked the typical stellar shape, and were rather yellowish in color, when compared with cells from A BBM cultures. Cells with very thick cell walls appeared occasionally in starved cultures; this was observed in strains C and E, in particular (Fig. 2F and G). Moreover, algal filaments were enveloped by mucilaginous sheaths, which was also apparent macroscopically in cultures grown on agar plates. Very

rarely, we also observed true *Zygnema* akinetes with distinctly colored cell walls, but these appeared only in strain C after A BBM pre-treatment (Fig. 2B).

A similar effect of nitrogen starvation on cell morphology was observed in cultures grown in liquid media (L BBM, L BBM-N; Fig. S1). Nitrogen-starved cultures consisted only of mature pre-akinetes whereas cultures grown in L BBM medium also contained vegetative cells with bright-green colored chloroplasts and no distinct storage inclusions.

Physiological performance during desiccation

The initial values of Φ_{PSII} differed between the A BBM and A BBM-N cultures for each strain and were always lower in the starved culture (GLM ANOVA, Tukey's post-hoc tests, n = 12, $p < 0.0002$; Fig. 3). Following the transfer into the desiccation chambers, Φ_{PSII} started to decrease and the rate of the decline differed according to the desiccation scenario. Over silica gel (rh around 10%), the samples began to desiccate almost immediately and Φ_{PSII} dropped to zero within 10–20 minutes, indicating the complete cessation of physiological activity (Fig. 3A–D). By contrast, it took up to 50 minutes for the Φ_{PSII} to reach its lowest value in samples desiccated at 86% rh (Fig. 3E–H). When the samples were moistened with 10 µl L BBM medium prior to desiccation at 86% rh, the Φ_{PSII} remained unchanged for several hours before the values began to drop, with the lowest values being reached as late as 8–9 hours after the beginning of the experiment (Fig. 3I–L). Markedly, in some strains, cultures pre-grown on A BBM lost their physiological activity much faster than those pre-cultivated on A BBM-N (Fig. 3A, E, F, G and K). *Zygnemopsis* sp. L cultivated on A BBM-N showed very small Φ_{PSII} values (around 0.1), even prior to any experimental manipulation, indicating the very low performance of this strain under starvation conditions. These initial values did not change, regardless of the desiccation regimes applied (Fig. 3D, H and L).

Further experiments with liquid cultures (L BBM and L BBM-N) revealed very similar responses. The initial values of Φ_{PSII} were also significantly lower in nitrogen-starved samples for each strain (GLM ANOVA, Tukey's post-hoc tests, n = 12, $p < 0.0002$) and the decline in physiological activity again correlated with the drying rate.

Physiological performance and viability after rehydration

The values of Φ_{PSII} started to recover following rewetting of cultures. Noticeably, the recovery rate differed with regard to individual strains, desiccation scenarios, and pre-treatments (Fig. 4, Table 1). In accordance with the recovery rate, the proportion of surviving cells (observed 48 hours after rehydration) also varied (Table 2): strains C and E, pre-

cultivated on A BBM-N, survived even very quick desiccation at 10% rh; however, only a small proportion of cells were viable 48 hours after rehydration (Table 2). The samples that had been desiccated at 86% rh showed much better survival capacity: only strain B, pre-treated on A BBM, entirely failed to survive this treatment. All cultures, however, survived the very slow desiccation regime (desiccation at 86% rh plus additional moistening with 10 µl L BBM). The recovery of photosynthetic activity was also fastest after this treatment (Fig. 4, Table 1).

When samples were exposed first to the very slow desiccation regime for 12 hours and then transferred to 10% rh for additional 24 hours, all *Zygnema* strains produced at least a small proportion of surviving cells (Table 2); however, no cells of *Zygnemopsis* sp. L survived following exposure to 10% rh, even under these modified conditions (Table 2).

An additional set of desiccation experiments using liquid cultures gave very similar results. Similar to the samples pre-cultivated on agar, all the samples grown in liquid culture survived the very slow desiccation regime. In addition, all the liquid cultures, except *Zygnema* sp. B (L BBM) and *Zygnemopsis* L (L BBM-N), survived desiccation at 86% rh. However, in contrast to the agar cultures, no liquid culture survived desiccation at 10% rh, indicating that pre-cultivation on a solidified medium improves the acquisition of desiccation tolerance.

Ultrastructural investigation of starved cultures

TEM was performed on cultures cultivated for 9 weeks on A BBM-N (Fig. 5) and on the same cultures following desiccation for 2.5 hours at 86% rh (Fig. 6). All four strains investigated (*Zygnema* sp. B, Fig. 5A; *Zygnema* sp. C, Fig. 5B; *Zygnema* sp. E, Fig. 5C and *Zygnemopsis* sp. L, Fig. 5D) had similar ultrastructural appearances. In all cases, large lipid bodies with a diameter of several µm were apparent in the cell periphery (Fig. 5A–D). Accumulation of starch grains indicated the chloroplasts were still active (Fig. 5A and D). Electron-dense granules between the lipid droplets were observed in all the investigated strains. The chloroplasts occasionally contained plastoglobules (Fig. 5C). The cell walls were homogenous, about 2–4 µm thick and, particularly in *Zygnema* sp. E, were covered with a fibrillose mucilage layer.

Following desiccation for 2.5 hours, the cytoplasm appeared denser (Fig. 6A and B) but individual organelles, such as the chloroplasts (Fig. 6A) and Golgi bodies (Fig. 6B), were still visible. The peripheral lipid bodies tended to accumulate and form large structures by fusion (Fig. 6A, B and D). The nucleus was still intact; however, occasionally less electron-dense areas were observed in the heterochromatin (Fig. 6C). The marked mucilage layer outside the

cell wall in *Zygnema* sp. E was still visible (Fig. 6F). The chloroplasts contained starch grains (Fig. 6F), as well as plastoglobules (not shown). Particularly dense accumulations of ribosomes surrounding the nucleus were observed (Fig. 6F). In *Zygnemopsis* sp. L, the cytoplasm contained numerous smaller vesicles as well as compartments with a medium electron-dense contrast (Fig. 6G).

Discussion

The aims of this study were to investigate the culture conditions that led to the formation of (pre)akinetes in *Zygnema* spp. and *Zygnemopsis* sp., and to study their tolerance of desiccation under controlled laboratory conditions. We confirmed the hypothesis that nitrogen starvation induces akinete formation, as a greater proportion of akinetes developed in both agar-solidified and liquid BBM-N cultures than in regular BBM cultures. Moreover, the ability to survive exposure to 10% rh was induced by either pre-cultivation on agar medium or by very slow desiccation at 86% rh, implying that akinetes can be acclimated to desiccation by exposure to mild desiccation stress (‘matric stress’; [43]).

Phylogenetic position

Both the Antarctic strains (C and E) shared the same *rbc*L sequence, which indicates that they are likely to be different isolates of the same species of *Zygnema*. This was an unexpected result, as they originated from different Antarctic islands: strain C was isolated from a seepage on James Ross Island whereas strain E was isolated from a meltwater pool on King George Island. It can be assumed that both sites, obviously shallow and temporary, provide similar ecological conditions. These two strains were also very similar in morphology and performance.

The Arctic strain *Zygnema* sp. B, although very closely related to strains C and E, apparently belongs to a different species, as assessed by differences in the *rbc*L sequence (seven base-pair changes relative to the Antarctic strains), geographical origin, and characteristic phenotypic features, including filament width, mucilage production and stress tolerance under experimental conditions. Species names could not be assigned to any of our strains as no zygospores were observed.

The identity of the *Zygnemopsis* sp. L strain was not known before the application of the molecular analysis. In its vegetative state, *Zygnemopsis* can be easily confused with *Zygnema* because of their similar chloroplast morphology. This study is the first report, to our

knowledge, of the occurrence of *Zygnemopsis* sp. in Arctic samples. Interestingly, the two genera are not closely related [34].

Starvation and (pre)akinete formation

It has long been known that some green algae survive unfavorable conditions by entering stationary phase and forming so-called akinetes [25–27]. Akinetes are usually characterized by thickened cell walls and a lower pigment content, and are filled with storage material [19,25,27]. In our cultures, they appeared darker and brownish in color, in comparison to vegetative cells from fresh cultures where the cells showed a high degree of vacuolization and a clearly stellate chloroplast [28,31]. Very thick cell walls were formed predominantly by the strains C and E.

A previous report indicated that fully developed, single-celled akinetes were present after 6 weeks of cultivation [25]; by contrast, even after 9 weeks of cultivation, our BBM cultures still contained a mixture of all types of cells from normal vegetative cells through to stationary phase cells. Nevertheless, when strong nitrogen starvation was applied by 9 weeks growth in nitrogen-free medium (both A BBM-N and L BBM-N), all cells appeared as pre-akinetes. The formation of akinetes is induced by nutrient starvation in xanthophytes *Heterococcus endolithicus* [44] and *Tribonema bombycinum* [45].

TEM confirmed the ultrastructural changes that accompanied nitrogen starvation and the formation of akinetes. Our observation is in accordance with an earlier study [25], which showed a massive accumulation of lipids in the cell cortex and a reduction of chloroplast lobes. In contrast to pre-akinetes, TEM revealed larger vacuoles in vegetative cells from young cultures of *Zygnema* sp. B and *Zygnema* sp. E [28,31].

The accumulation of lipids typically accompanies nitrogen limitation in algae [27,29,30] but lipids themselves are not involved in desiccation tolerance [27]. Lipid composition also changes with starvation; this has been described at the transcriptomic level in an unsequenced microalga [46]. Moreover, air drying has also been found to be a potential trigger for stimulation of triacylglycerol biosynthesis in *Chlorella kessleri* [47]. The production of lipids by algae is of great potential interest for biotechnological reasons; however, the lipid composition of Zygnematophyceae remains unknown.

We first studied directly the ultrastructure of akinetes in the desiccated state. Surprisingly, their cell walls appeared rather unimpaired, indicating a different strategy to that used by *Klebsormidium* spp., which possess very flexible cell walls that allow massive shrinkage during desiccation [22,48]. In general, a condensation of the cytoplasm was

observed in all *Zygnema* spp. strains following desiccation, as previously reported in other streptophyte green algae [22,48]. The slight changes in heterochromatin appearance and the accumulation of ribosomes, particularly around the nucleus, can be regarded as common stress reactions. Moreover, the mucilage layer outside the cell walls, observed particularly in *Zygnema* sp. E, may additionally contribute to desiccation tolerance. A similar mucilage layer has been observed in a natural *Zygnema* population from an Arctic habitat [15].

Desiccation tolerance and drying rate

In our study, vegetative or morphologically intermediate cells, even if pre-grown on agar, did not survive any of the desiccation treatments, indicating desiccation tolerance can be induced only in pre-akinetes. Thus, the recovery experiments reflect only the response of the pre-akinetes in both BBM and BBM-N cultures. Nevertheless, Figure 4 clearly demonstrates that pre-akinetes from starved cultures (BBM-N) of the three *Zygnema* strains were, in general, more resilient than those from BBM, as they survived more rapid desiccation. By contrast, in *Zygnemopsis* sp. L, nitrogen depletion resulted in severe damage and produced very low initial values of the effective quantum yield. As a consequence, desiccation tolerance seems to be strongly dependent on the state of the culture and this should be taken into account when attempting to understand and interpret desiccation experiments. Numerous experimental desiccation studies on various algal cultures have been published so far (for a recent review see [2]). Typically, log-phase cultures are used in such experiments, but we suggest that more mature or starved cultures should also be tested. Mature cells usually reflect natural conditions better, and the comparison of their responses will contribute to a better understanding of an alga's real capacity for desiccation tolerance.

The mature cells (pre-akinetes), which developed during the period of experimental pre-cultivation, tolerated, in most cases, conditions of moderate desiccation (86% rh and 'very slow' desiccation). Nevertheless, the cultures survived rapid desiccation (10% rh) after previous acclimation *via* desiccation at 86% rh or by being pre-grown on agar (strains C and E), showing that experiencing mild desiccation stress may induce desiccation tolerance. Moreover, recovery of photosynthetic activity and viability during rehydration were clearly dependent on speed of drying, i.e., the slower the desiccation, the better the level of survival. These data agree very well to field observations where slow desiccation enhanced osmotic stress tolerance [19]. Our results also suggest that the desiccation tolerances of *Zygnema* spp. and *Zygnemopsis* sp. are not constitutive, but detailed proteomic or metabolomic analyses will be needed to test this hypothesis.

The desiccation rate is in general an important factor affecting the survival and recovery after rehydration. For example, *Klebsormidium dissectum* showed much faster recovery of the optimum quantum yield when desiccated at 100% rh than at 55% or 5% rh [22]. The same effect was observed in a common lichen photobiont, *Trebouxia ericii* (now *Asterochloris ericii*; [24]); even the ultrastructural injuries were more pronounced after fast desiccation [49]. Similarly, fast drying (at 50% rh) was lethal for the moss *Fontinalis antipyretica*, which survived when desiccated at 95% rh [50].

Filamentous Zygnematophyceae typically form extensive mats and produce mucilage that provides a natural protection against quick dehydration and allows sufficient time for hardening [2,15,16]. Nevertheless, the ability to survive strong desiccation stress is still important for these algae; the akinetes liberated by filament fragmentation are ideal propagules for dispersion by means of asexual reproduction [25].

Besides mature cells (akinetes), algae also produce other types of resistant cells that enable them to survive stresses. Several cell types have been described in *Zygnema* [51], but none have ever been reported from polar regions. Nevertheless, in *Zygnema* sp. C cultures, true *Zygnema* akinetes with brown mesospores rarely occurred. Although the desiccation tolerance of true akinetes has been reported, [51], we did not prove this role in our experiments, as most of the brown cell-walled cells were obviously dead following desiccation experiments, whereas cultures that had produced pre-akinetes survived.

Light stress during desiccation

The survival capacity in a desiccated state is strongly influenced by other stress factors, the most important of which is light. Homoiochlorophyllous plants (most algae) retain chlorophyll when desiccated, which gives them the ability to recover their physiological activity quickly after rehydration [52]. There is, however, a strong danger of photodamage and photobleaching during desiccation because the chlorophyll molecules can still be excited when illuminated, but the energy produced cannot be transferred through photochemistry, leading to the production of reactive oxygen species [20]. Thus, algae desiccated under illuminated conditions are much less viable after rehydration than those desiccated in darkness [23].

Zygnema cells retained their chloroplasts following desiccation, although they were partly altered in mature akinetes. The chloroplasts lost the lobes characteristic of stellate chloroplasts and their photosynthetic activity diminished. A similar reduction in chloroplasts has been

observed in *Klebsormidium rivulare* akinetes [27]. We hypothesize that the reduction of chloroplast size is a general strategy that could reduce light stress during desiccation.

In our experiments, the cultures were continuously illuminated at a light intensity of 50 μmol $m^{-2}s^{-1}$. Such illumination is not stressful, as it is far below the prevailing irradiance in Svalbard during the summer, and well below the photoinhibition limits determined for Arctic and Antarctic *Zygnema* strains, which are around 500 μmol $m^{-2}s^{-1}$ [28]. Nevertheless, it is possible that the survival capacity of the strains tested would be even better when desiccated in darkness, but such a situation does not occur under natural conditions. However, in nature, self-shading, which reduces the light availability for cells from lower layers drastically, might be an important factor in mat-growing organisms like *Zygnema* sp. A similar phenomenon has been reported in the macroalga *Ulva* sp., where the subcanopy thalli are protected by top layers that usually bleach out [53].

Keeping our samples illuminated during desiccation and rehydration was necessary to allow regular measurements of the effective quantum yield, even at 2 minute intervals. This fluorescence parameter is measured under light conditions, to which the photosynthetic apparatus is adapted, and gives a good estimation of photosynthetic activity [54].

Desiccation tolerance in different Zygnematophyceae

All the strains used in this study survived desiccation under certain circumstances, but differences in stress resistance were found. The Antarctic *Zygnema* sp. C and *Zygnema* sp. E were best adapted to stressful conditions, as they were the only strains to survive direct exposure to 10% rh following pre-cultivation on A BBM-N. The Arctic strain *Zygnema* sp. B was less stress-resistant than the Antarctic strains; it survived moderate desiccation at 86% rh. Interestingly, an analysis of the osmotic stress tolerance of two of the *Zygnema* strains used in this study (B and E) found that *Zygnema* sp. B had a lower (more negative) osmotic potential (approximately 600 mM sorbitol, ψ -1.67 MPa) than *Zygnema* sp. E (approximately 300 mM sorbitol, ψ -0.8 MPa) [28]. This result suggests that *Zygnema* sp. B may be more resistant to osmotic stress; however, the study was performed with young cultures [28].

Very slow desiccation led to acclimation in all *Zygnema* strains, as reflected by their better rate of survival following a subsequent transfer to 10% rh. By contrast, *Zygnemopsis* sp. L did not survive in 10% rh even after such an acclimation period, which indicates that it is less desiccation-resistant than the *Zygnema* strains used in this study. Generalizing from this to the whole genera must be strictly avoided, because desiccation stress resistance was not

thoroughly investigated for any other strains; however, it would be interesting if future studies targeted strains from other clades within the genus *Zygnema*.

Stress tolerance is a widespread phenomenon in Zygnematophyceae and many species occur in extreme environments, such as the surface of glaciers [13,14]. Other members of the group, such as various desmids [55], *Zygnema stellinum* [25,56] or *Zygogonium ericetorum* [57–59], occur in ephemeral habitats that dry out regularly. Experimental air drying of *Zygogonium ericetorum* for 2.5 hours completely inhibited optimum quantum yield and, after rehydration, only 5–14% of the initial values were regained within 3 hours; however, subsequent cultivation for 7 days produced a return to 53% of the initial value [59]. Desiccation tolerance in Zygnematophyceae is of particular interest because of the close relationship between this class and all land plants [8,9], and thus the mechanisms that govern desiccation tolerance in this class and allow successful colonization of the terrestrial environment are expected to be shared by other plants as well.

Conclusions

We conclude that a 'hardening process' or acclimation, resulting in desiccation tolerance, can be induced in hydroterrestrial filamentous Zygnematophyceae under specific cultivation conditions. We confirmed the results of previous field observations and showed that nitrogen starvation leads to the formation of pre-akinetes, but these must be further acclimated, for instance by very slow desiccation or applying mild matric stress when growing on agar, to survive rapid desiccation at 10% rh.

Observations made using TEM indicate that a substantial amount of lipid is synthesized in the pre-akinete cells; however, the biochemical composition of pre-akinetes remains to be investigated in detail. This will provide deeper insights into the acclimation process.

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Tables

Table 1: Relative values of the effective quantum yield during recovery of algal samples pre-cultivated on agar (A BBM and A BBM-N cultures)

Strain		B		C		E		L	
Culture type		A BBM	BBM-N	BBM	BBM-N	BBM	BBM-N	BBM	BBM-N
	10% rh	ND	ND	ND	2%	ND	7%	ND	ND
t = 0	86% rh	ND	25%	0%	70%	32%	39%	14%	0%
	86% rh + BBM	5%	35%	30%	76%	83%	70%	53%	18%
	10% rh	ND	ND	ND	47%	ND	30%	ND	ND
t = 48 h	86% rh	ND	110%	12%	110%	64%	68%	16%	79%
	86% rh + BBM	17%	123%	70%	118%	100%	124%	92%	74%

The given percentages were computed as ratios between initial values of Φ_{PSII} and the values measured immediately after rehydration (t = 0) or after 48 hours of rehydration (t = 48 h). No data are shown for those regimes that were lethal for all of the cells (ND). Values shown are means of four, independent experimental replicates.

Table 2: Viability of cultures pre-cultivated on agar following 48 hours of rehydration in water (A BBM and A BBM-N cultures)

Strain	B		C		E		L	
Culture type	BBM	BBM-N	BBM	BBM-N	BBM	BBM-N	BBM	BBM-N
10% rh	-	-	-	++	-	+	-	-
86% rh	-	++	+	+++	+++	+++	+	+
86% rh + BBM	+	+++	++	+++	+++	+++	+++	+
86% rh + BBM => 10% rh	+	+	++	+++	+++	+++	-	-

– No living cells observed; + < 5% living cells; ++ 5–50% living cells; +++ 50–100% living cells

Figures

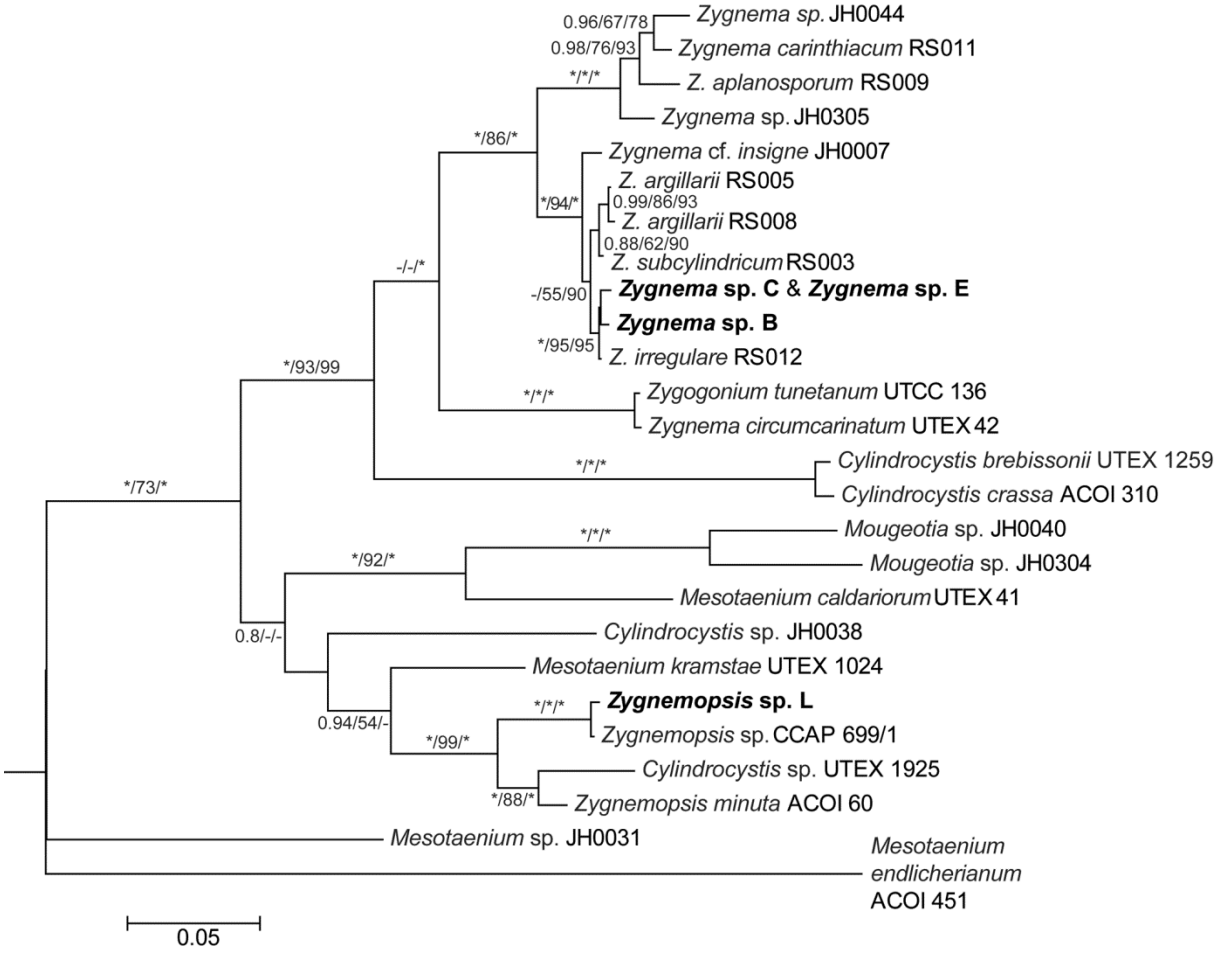

Figure 1: Phylogenetic tree of Zygnematophyceae showing the positions of the strains investigated in this study. A midpoint-rooted Bayesian tree of *rbc*L sequences is shown. Values at the branches indicate Bayesian posterior probabilities (BI PP), maximum likelihood (ML), and maximum parsimony (MP) bootstrap values (BS). Asterisks indicate BI PP = 1.00, and ML and MP BS = 100; dashes indicate BI PP < 0.8, and ML and MP BS < 50. Strains used in this study are in bold.

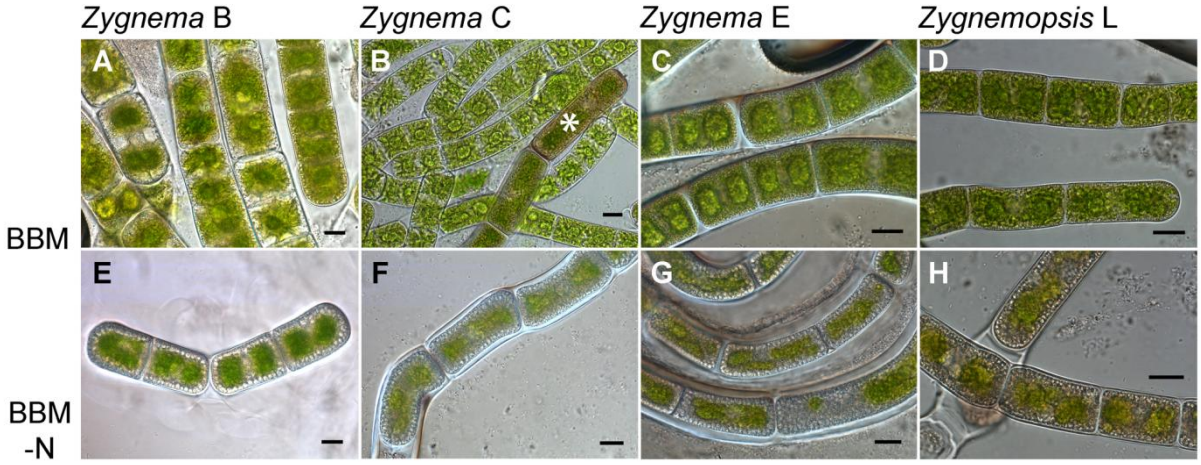

Figure 2: Light micrographs of the strains pre-cultivated on agar medium for 9 weeks. A–D: cultures grown on regular BBM medium (A BBM); **E–H:** cultures grown on BBM without nitrate (A BBM-N). The pictures were taken prior to the desiccation experiments; a 'true' *Zygnema* akinete with a brown mesospore is marked with an asterisk. Scale bars: 10 µm.

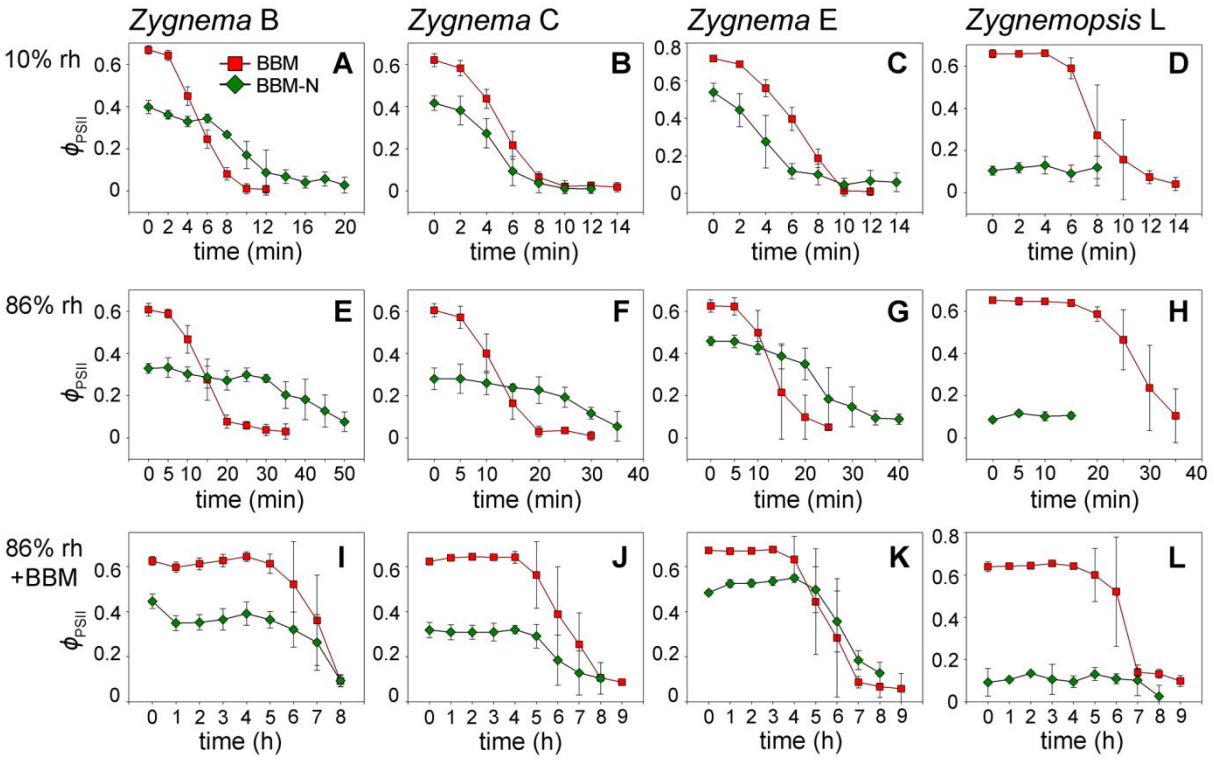

Figure 3: Effective quantum yield (Φ_{PSII}) under different desiccation scenarios. A–D: rapid desiccation, approximately 10% relative humidity (rh); **E–H:** slow desiccation, 86% rh; **I–L:** very slow desiccation, 86% rh plus additional moistening of samples with 10 µl BBM. All strains shown were pre-cultivated on regular agar medium (A BBM) or on medium without nitrate (A BBM-N). Results are means ± standard deviations of four independent experimental replicates.

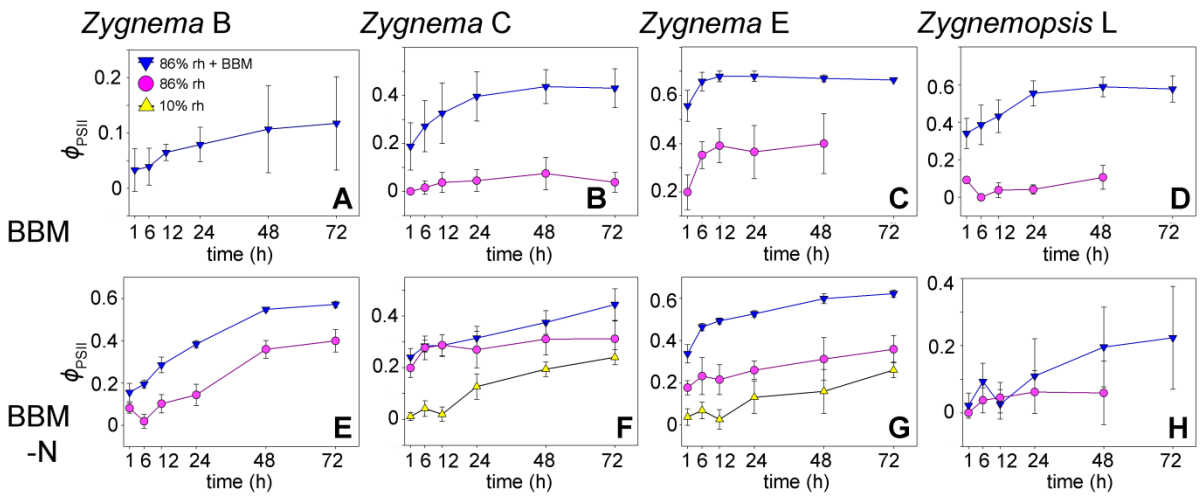

Figure 4: Recovery of effective quantum yield (Φ_{PSII}) during rehydration after 24 hours desiccation. Each panel compares the performance of the same culture desiccated under different conditions; no plots were constructed for those regimes that were lethal for all of the cells. Samples were collected from cultures pre-cultivated on agar media: **A**–**D:** cultures grown on regular agar medium (A BBM); **E**–**H:** cultures grown on BBM without nitrate (A BBM-N). Results are means ± standard deviations of four independent experimental replicates.

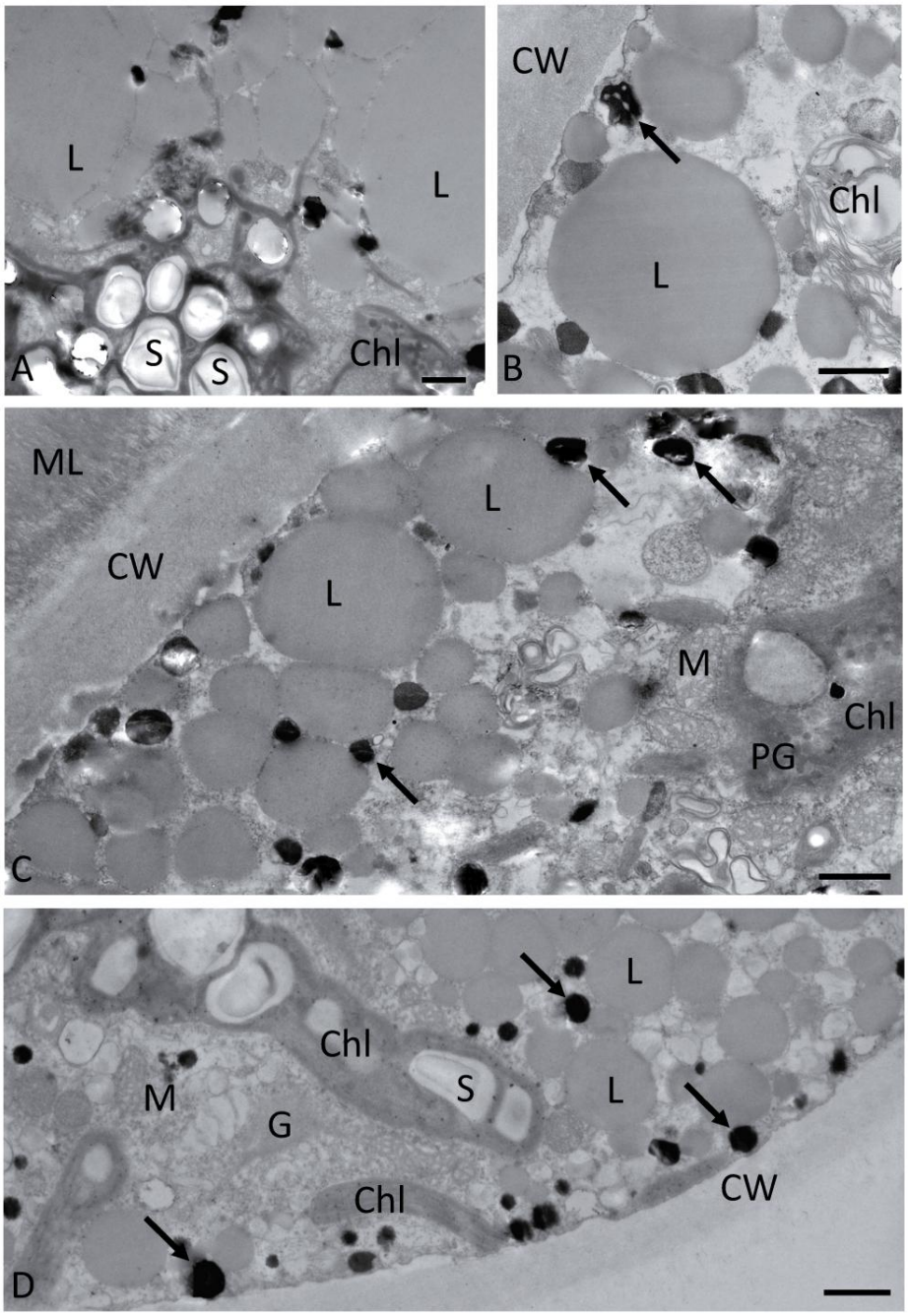

Figure 5: Transmission electron micrographs of pre-akinetes prior to desiccation. A: *Zygnema* sp. B. Large lipid bodies and chloroplast with starch grains are indicated. **B:** *Zygnema* sp. C. Lipid bodies and electron-dense particles (arrow) are indicated. **C:** *Zygnema* sp. E. Lipid bodies in the cell periphery, electron-dense particles (arrows) and cell walls with a fibrillose mucilage layer are marked. **D:** *Zygnemopsis* sp. L. Chloroplast lobes with starch grains, electron-dense particles (arrows), and lipid bodies are marked. All cultures were cultivated on agar medium without nitrate (A BBM-N) for 9 weeks. Abbreviations: Chl: chloroplast; L: lipid body; M: mitochondrion; ML: mucilage layer; PG: plastoglobules: and S: starch. Scale bars: 1 µm.

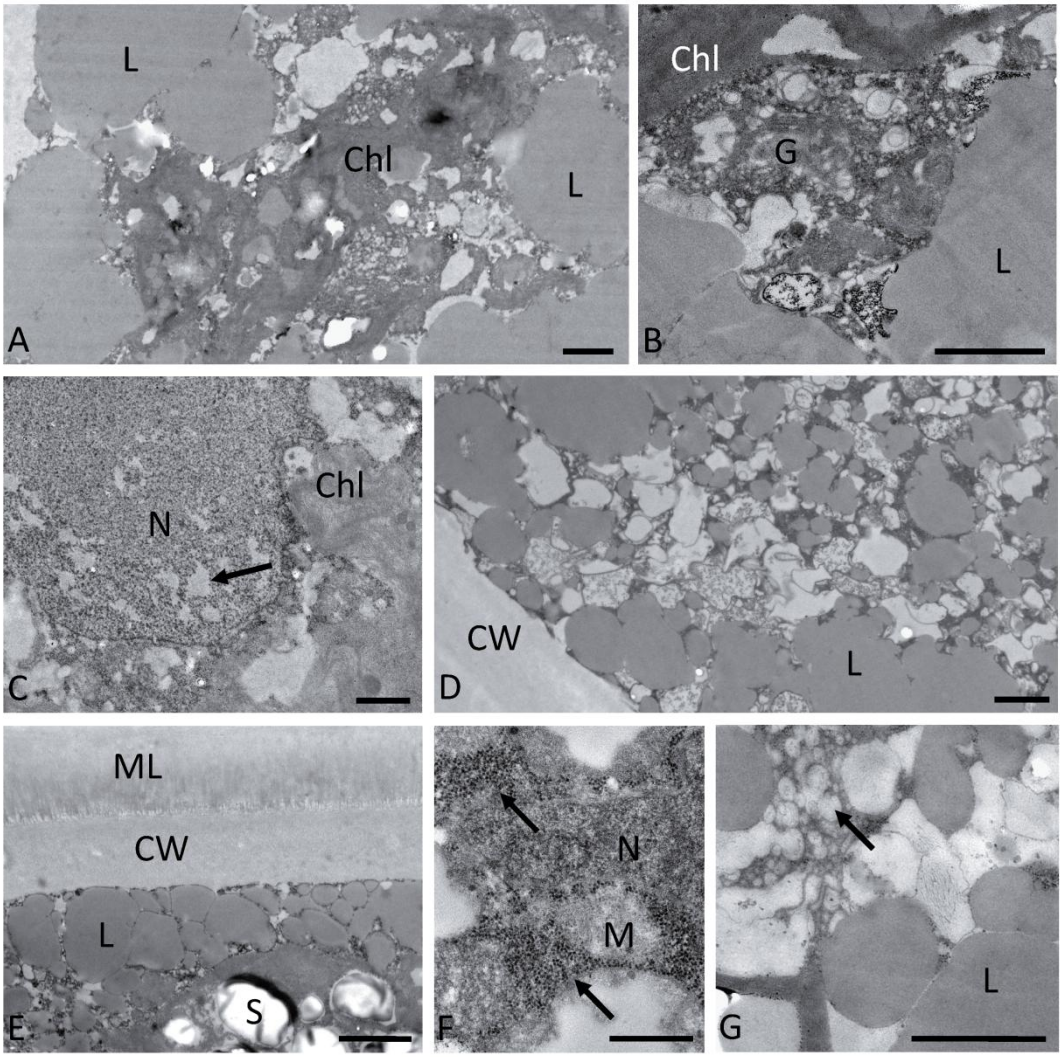

Figure 6: Transmission electron micrographs of pre-akinetes following desiccation for 2.5 hours at 86% rh. A–C: *Zygnema* B; **D:** *Zygnema* C; **E–F:** *Zygnema* E; and **G:** *Zygnemopsis* L. Note the following features: **A:** dense structure of the chloroplast and fusions of lipid bodies; **B:** dense structure of Golgi body and chloroplast; **C:** nucleus with less electron-dense areas of heterochromatin (arrow); **D:** accumulation of lipid bodies in the cell periphery; **E:** starch grains, lipid bodies in the cell periphery and the cell wall covered by a fibrillose mucilage layer; **F:** accumulations of ribosomes (arrows) next to the nucleus; and **G:** accumulation of vesicles and small electron translucent compartments next to the lipid bodies. Abbreviations: Chl: chloroplast; G: Golgi body; L: lipid body; M: mitochondrion; ML: mucilage layer; N: nucleus; S: starch. Scale bars: A–D and G: 1 µm; E: 2 µm; and F: 0.5 µm.

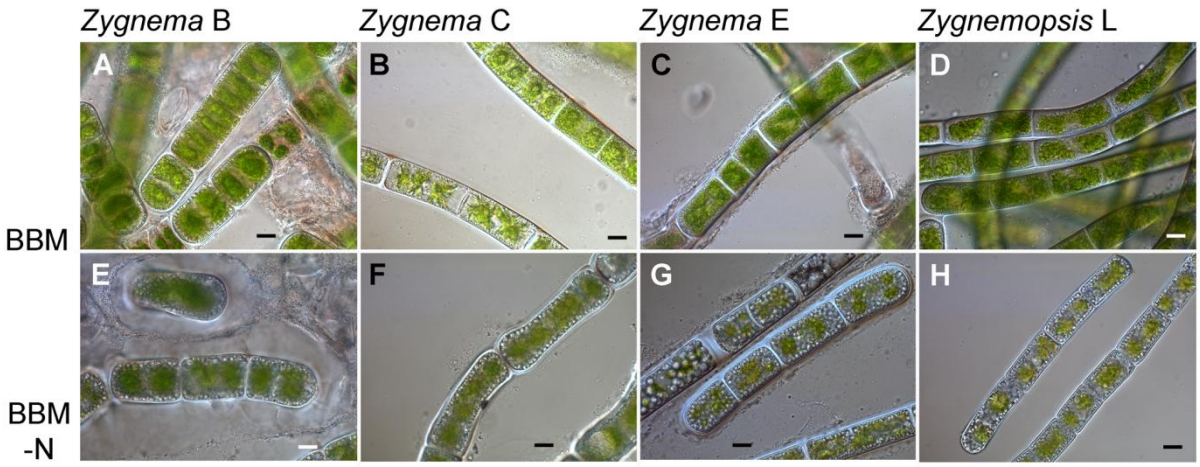

Figure S1: Light micrographs of the experimental strains pre-cultivated in liquid medium for 9 weeks. A–D: cultures grown in regular L BBM medium; and **E–H:** cultures grown in L BBM-N medium. The pictures were taken prior to the desiccation experiments. Scale bars: 10 µm.

4 Summary and conclusions

Zygnema spp. typically form extensive mats in hydro-terrestrial environments of both the Arctic and Antarctic. These habitats are regarded as extreme and unstable, and microalgae in these environments are exposed to multiple stresses. This thesis summarizes recent findings about the stress resistance of polar *Zygnema* spp. and provides new insights into the questions presented in the Introduction.

Diversity of polar Zygnema *spp. mats*

There are several reports of *Zygnema* sp. from both the Arctic and Antarctic, but its diversity had remained unknown largely due to the lack of sexual reproduction. The investigation of molecular diversity within *Zygnema* mats was not a primary aim of this thesis. Nevertheless, as species could not be determined according to morphological traits, molecular phylogenetic analysis was performed to assess the positions and relationships of the isolated strains used in the experiments. Thus, data were gathered that provided the first insights into the hidden diversity of polar *Zygnema*. Altogether, more than 80 strains from Svalbard were obtained, and five separate lineages within *Zygnema* and one lineage of *Zygnemopsis* were discerned using the chloroplast gene *rbc*L. However, only three of these genotypes, B, G and L, were used in the experiments presented in this thesis, while a thorough account of the diversity of these microalgae has not yet been published (the publication is currently in preparation). *Zygnema* strains B and G are not closely related; each of these belongs to different main clades of the genus *Zygnema* (Stancheva et al. 2012), whereas both Antarctic strains (C and E) share identical *rbc*L sequences. The discovery of *Zygnemopsis* sp. was rather surprising. The two genera have similar vegetative morphology, even though they are not very closely related among the Zygnematophyceae (Hall et al. 2008).

This thesis demonstrates that the diversity of *Zygnema* mats is greater than expected. To my knowledge, this is the first report of *Zygnemopsis* from the Arctic. It is, however, possible that it was already observed before but was mistaken for *Zygnema*. Moreover, it is highly likely that the genetic diversity of *Zygnema* spp. mats, at least in part, explains the differences in the ecophysiological performance of individual strains. A similar situation arose in the study of Antarctic *Prasiola*, where molecular methods revealed three distinct species with different ecological preferences (Moniz et al. 2012). Therefore, future studies of vegetative *Zygnema* spp. from polar regions should also include molecular data.

Mechanisms of UV resistance

I showed that polar strains of *Zygnema* produce several phenolic substances with wide absorption maxima that screen UV-A, UV-B and (partially) PAR irradiation. These phenolics are produced constitutively, but their increase after experimental UV exposure was also observed. Phenolics are water-soluble compounds that are usually stored in vacuoles. I anticipate that the enigmatic electron-dense vesicles that resemble brown algal physodes may also contain phenolics. They accumulate at the cell periphery, which is advantageous for the screening and protection of chloroplasts. In addition, other mechanisms of stress resistance are probably involved. For example, mat-forming growth provides good protection of the lower layers of another polar hydro-terrestrial alga, *Prasiola* sp. (Post & Larkum 1993). Moreover, mucilage is also believed to provide protection against UV (Holzinger & Lütz 2006).

Formation of specialized cells

Very rarely, zygospores or traces of conjugation were observed in samples from Svalbard, but unfortunately, the material did not provide sufficient information for species determination. Also, very rarely, "true" *Zygnema* akinetes with brown-colored cell walls were present in the culture of Antarctic *Zygnema* sp. C.

On the contrary, pre-akinetes were observed in all natural samples and cultures. At the end of the summer, the natural *Zygnema* spp. mats in the Arctic consisted predominantly of pre-akinetes in all types of habitats, from permanent pools to the naturally desiccated soil surface. We hypothesized that their formation was induced by starvation because their extensive growth in relatively small water bodies may lead to the depletion of nutrients and the cessation of growth. Under laboratory conditions, we confirmed that pre-akinete formation could be induced by nitrogen limitation whereas mild dehydration stress simulated by cultivation on agar plates had no such effect.

On the ultrastructural level, the pre-akinetes were characterized by large accumulation of lipids, and their chloroplasts had somewhat reduced lobes. The accumulation of carbohydrates and lipids occurs when the rate of photosynthesis exceeds that necessary for cell growth and division, which can occur as a consequence of stress factors, e.g., nutrient limitation (Hawes 1988; Solovchenko 2012). In an eustigmatophyte *Nannochloropsis oculata*, the lipid content changed markedly over the course of the season, even with constant nutrient availability, showing the importance of other environmental factors, mainly temperature and light (Olofsson et al. 2012). Nevertheless, the neutral lipids that accumulated under stress

conditions are not merely storage products but are actively involved in protection mechanisms (Solovchenko 2012). Thus, the pre-akinete can also be perceived as simply a stressed or old vegetative cell that becomes stress-resistant due to the synthesis and accumulation of compounds that would not be produced during the growth phase.

Osmotic and desiccation stress tolerance of pre-akinetes

While all natural populations consisted of pre-akinetes regardless of their water status, significant differences were revealed in their osmotic stress tolerance. Therefore, we speculated that hardening during slow desiccation was required for the pre-akinetes to become stress-resistant. This assumption was later confirmed using cultures under experimental conditions. In contrast to vegetative cells, pre-akinetes were able to survive desiccation. However, only after previous acclimation by slow desiccation (induced either by controlled desiccation at high relative humidity or by pre-cultivation on agar), the pre-akinetes were able to survive rapid desiccation in air at a relative humidity of 10%. This is a rather important finding because although there are numerous reports of algal akinete formation and stress tolerance, to my knowledge, the need for such hardening to greatly improve performance under stress conditions has not previously been indicated.

General implications for stress tolerance studies

As discussed above, rather than specialized stages, pre-akinetes can be considered to simply represent modified vegetative cells, which are typical of old or stressed cultures or natural populations. This finding has an important consequence for laboratory experiments that investigate algal stress tolerance. Such studies are usually performed using fresh cultures but with the goal of explaining the organism's occurrence in extreme habitats. However, it has been known for a long time that the morphology of microalgae under natural conditions often resembles akinetes (Morison & Sheath 1985; Darling et al. 1987; Hoppert et al. 2004) and, therefore, it is different from that under optimal culture conditions. Moreover, morphology and even cell wall composition can markedly differ between cultures grown on agar and in liquid medium (Graham et al. 2012). I showed that in addition to the drying rate (Gasulla et al. 2009), duration of desiccation (Karsten & Holzinger 2012) and light conditions during desiccation (Gray et al. 2007), the age and state of the culture also play an important role in survival and recovery after desiccation. Therefore, it seems insufficient to assess an alga's stress tolerance capacity from experiments performed only with log-phase, non-hardened

cultures. Such results should always be compared with the results of experiments employing cultures that better resemble the state of the microalgae under natural conditions.

Concluding remarks

From the results described above, I can conclude that naturally hardened pre-akinetes play a key role in the survival of *Zygnema* spp. and *Zygnemopsis* sp. in the hydro-terrestrial environment of polar regions. As desiccation and freezing have similar physiological effects, I assume that hardened akinetes are also important for survival in the winter. Such ability to withstand prolonged freezing in the vegetative state has been observed in other Arctic tundra microalgae as well (Sheath et al. 1996). To date, there are no experimental data that support the freezing tolerance of *Zygnema* pre-akinetes, but we directly observed viable pre-akinetes in frozen winter samples in Svalbard (unpublished result). This is in contrast to the notion that such cells are able to survive only short-term stress events during the summer (Genkel & Pronina 1979). However, the authors investigated *Z. stellinum* from Belarus, which may have a different strategy for winter survival from that of the polar strains because other specialized cells such as zygospores, parthenospores and "true" akinetes were common in their samples (Genkel & Pronina 1979).

Due to their mat-forming growth and mucilage production, polar *Zygnema* (and *Zygnemopsis*) species are well-protected from harsh environmental conditions, and it can be assumed that the rapid desiccation applied in our experiments does not appear under natural conditions. Nevertheless, high desiccation tolerance is very important for the survival of short fragments or single-celled akinetes that are no longer protected within mats but instead serve as airborne propagules (Marshall & Chalmers 1997).

Various authors have reported high winter mortality in polar eukaryotic microalgae from hydro-terrestrial and terrestrial habitats in the Arctic and Antarctic (Davey 1991b; Elster 2002). From our observations, we suppose that the bottleneck is not freezing during the winter but rather cyclical freeze–thaw events that occur during the spring, when *Zygnema* spp. have already started growing and thus consist of vegetative, sensitive, de-hardened cells. During such events, it is important that at least a few cells retain their stress resistance and survive (Hawes 1988, 1990; Tashyreva & Elster 2012). Although it appears that *Zygnema* spp. and *Zygnemopsis* sp. are well-adapted to surviving the harsh conditions of the polar hydro-terrestrial environment, the scarcity (and probably complete absence in the Antarctic) of sexual reproduction indicates that these microalgae actually live at their physiological limits.

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6 Curriculum Vitae

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Study and practice

Since 2010:	Research assistant at the Charles University in Prague, Faculty of Science, Department of Botany – part time position
Since 2010:	Doctoral student at the Academy of Sciences of the Czech Republic, Institute of Botany, Třeboň – part time position
Since 2009:	PhD study in Botany: Charles University in Prague, Faculty of Science, Department of Botany
2007–2009:	Master study in Botany: Charles University in Prague, Faculty of Science, Department of Botany Thesis topic: Shape dynamics of silica structures in synurophytes
2004–2007:	Bachelor study in Biology: Charles University in Prague, Faculty of Science Thesis topic: The impact of environmental factors on morphological variation of silica structures in chryophytes (Synurophyceae)

Stays abroad

University of Innsbruck, Austria: (Dr. Andreas Holzinger)	**May 2013** (1 month) **March-June 2011** (4 months)
Field research (Svalbard, Norway):	**April 2012** (1 week) **August 2011** (3 weeks) **June-July 2010** (4 weeks)

British Antarctic Survey, Cambridge, United Kingdom: **May 2010** (2 weeks)
(Dr. Roger Worland)

Publications in SCI journals

PICHRTOVÁ, M., HÁJEK, T. & ELSTER, J. (2014): Osmotic stress and recovery in field populations of *Zygnema* sp. (Zygnematophyceae, Streptophyta) on Svalbard (High Arctic) subjected to natural desiccation. – FEMS Microbiology Ecology 89(2): 270–280.

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PICHRTOVÁ, M., ŘEZÁČOVÁ-ŠKALOUDOVÁ, M. & ŠKALOUD, P. (2007): The silica-scaled chrysophytes of the Czech-Moravian Highlands. – Fottea 7 (1): 43–48.

Abstracts, posters and presentations

PICHRTOVÁ, M., HÁJEK, T., KULICHOVÁ, J. & ELSTER, J. (2014): Stress resistance of filamentous conjugating green algae (Zygnematophyceae) from polar regions. – 62nd Annual Meeting of the British Phycological Society, Galway, Ireland, 25. – 27.6.2014.

PICHRTOVÁ, M., KULICHOVÁ, J., HÁJEK, T., HOLZINGER, A. & ELSTER, J. (2013): Diversity and stress resistance of *Zygnema* and *Zygnemopsis* in polar regions. – 54. Conference of the Czech Phycological Society, Třeboň, Czech Republic, 16. – 18.9.2012.

PICHRTOVÁ, M., VESELÁ, J., HOLZINGER, A. & HÁJEK,T. (2013): Diversity and desiccation tolerance of *Zygnema* (Zygnematophyceae, Streptophyta) on Svalbard (High Arctic). Phycologia 52: 87. – 10th International Phycological Congress, Orlando, Florida, USA, 4. – 10.8.2013

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PICHRTOVÁ, M. & NĚMCOVÁ, Y. (2012): Effect of temperature on shape and size of synurophyte silica scales - a geometric morphometric approach. - 8th International Chrysophyte Symposium, Prague, Czech Republic, 12. – 17. 8. 2012.

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