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Seasonal dynamics of mats composed by green alga *Zygnema* (Zygnematophyceae)

Sezónní dynamika nárostů řas rodu *Zygnema* (Zygnematophyceae)

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Doctoral thesis

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Author's declaration

I hereby declare that I have written thesis independently using listed references. I have submitted neither this thesis nor its parts to acquire any other academic degree.

Prohlášení autorky

Čestně prohlašuji, že jsem nepředložila práci ani její části k získání jiného nebo stejného akademického titulu a že jsem práci zpracovala samostatně za použití citované literatury.

In Prague Kateřina Trumhová

Contents

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Papers included in the thesis

I hereby declare that I have substantially contributed to all papers included in the thesis. My contributions to particular papers are as follows:

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MP designed the study; MP and AH sampled algae; MP cultivated the algae; DR and KT isolated DNA and did PCR; MP, JK and KT did molecular phylogenetic analyses; AH did electron microscopy; TŠ described and determinated zygospores; YN did confocal microscopy; MP, AH and YN prepared the manuscript, and all the authors edited the manuscript.

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KT, MP and AH designed the study; KT and MP prepared material; KT performed freezing experiments, staining, measurements, light microscopy; KT and GN performed cry-microscopy; KT prepared samples for electron microscopy; SO did electron microscopy; KT, MP and AH analyzed the data; KT wrote the manuscript, and all the authors edited the manuscript.

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In Prague Martina Pichrtová

Abstract

The mats formed by *Zygnema* spp. are well known and frequently found in streams, puddles, seepages, and ponds. Owing to the low and inconsistent water quantity of these biotopes, algae are periodically subjected to stresses like desiccation, freezing, or strong solar radiation. Recent studies have revealed that *Zygnema* survives various stresses in pre-akinetes, modified resistant vegetative cells. However, earlier experiments mostly aimed at osmotic stress and desiccation. Moreover, the mats formed by these algae have seasonal dynamics that have been observed several times, not only in temperate conditions but also in the Arctic. Field observations revealed that most of the biomass is periodically lost, and new mats are formed every year. Simultaneously, we know little about the genetic composition of the mats and how it changes over time; previous work has focused mainly on the mats as an entirety.

Therefore, this work mainly aimed to subject the mats formed by the algae *Zygnema* spp. to long-term observation and subsequent experiments. We selected a region in the Austrian Alps, which we visited several times a year, and sampled two sites with different water regimes. Further research in collaboration with colleagues occurred in the Arctic. *Zygnema* spp. strains were subsequently isolated from the samples and genetic diversity within the mats was compared. Simultaneously, we focused on the type of cells forming the mats and water availability during observations. In the experimental part, we compared for the first time the tolerance of young vegetative cells and pre-akinetes to typical seasonal stress factors: Ultraviolet (UV) radiation and freezing. We tested the effect of photosynthetically active radiation in combination with UV-A and UV-B. Furthermore, we also tested the freezing tolerance of these two cell types to determine the limits of their survival.

We discovered a considerable diversity of *Zygnema* spp. in both the studied regions. Mats were mostly composed of several genotypes of this alga, and we found up to five of them growing together. Although the composition of the mats changed seasonally, the occurrence of algae at the site is continuous and we found the same genotypes even after four years. Moreover, the proportion of young vegetative cells and pre-akinetes changes dramatically throughout the year. Young vegetative cells dominate in the spring and summer and have a higher resistance than the pre-akinetes to excessive irradiation because of their phenolic compounds. However, their frost resistance is low compared with that of the pre-akinetes. Pre-akinetes, which dominate the mats in autumn, can withstand freezing temperatures down to -70 °C. Simultaneously, we observed that even during favorable conditions, the presence of a small percentage of resistant pre-akinetes ensures the survival of the algae in case of sudden changes in conditions and exposure to stress. The results of this work indicate that *Zygnema* spp. is well adapted to various stress factors in vegetative state without the need to produce specialized life cycle stages. We also demonstrated that Zygnematophyceaen mats should not be considered as single populations of one species. Therefore, future studies on field material should always apply molecular methods to test for probable hidden diversity.

Key words: seasonality, stress resistance, Zygnematales, diversity, filamentous algae

Abstrakt

Nárosty tvořené řasami rodu *Zygnema* jsou dobře známé a často je nalézáme v potocích, tůních, loužích a jezírkách. Vzhledem k malému, a navíc nestálému množství vody v těchto biotopech jsou tyto řasy během roku cyklicky vystaveny různým stresům, jako je vysychání, zamrzání a silné sluneční záření. Recentní studie ukázaly, že *Zygnema* různé stresy přežívá v podobě modifikovaných odolných vegetativních buněk, tzv. pre-akinet, experimenty se však zaměřovaly především na osmotický stres a vysychání. Navíc, nárosty tvořené touto řasou mají sezónní dynamiku, která byla pozorována již mnohokrát, a to nejen v temperátních podmínkách, ale i v Arktidě. Pozorování v terénu ukázala, že většina biomasy pravidelně odumírá a nové nárosty se vytvářejí každý rok znovu. Zároveň chybí informace o genetickém složení porostů a jejich sezónní dynamice, dosavadní práce zatím nárosty braly jako celek.

Hlavním cílem této práce proto bylo podrobit nárosty tvořené řasami *Zygnema* spp. dlouhodobému pozorování a následně také experimentům. Vybrali jsme lokalitu v rakouských alpách, kterou jsme navštěvovali několikrát do roka a odebírali vzorky ze dvou míst, která měla odlišný vodní režim. Další výzkum nárostů proběhl ve spolupráci s kolegy v Arktidě. Ze vzorků byly následně izolovány kmeny *Zygnema* spp. a byla vyhodnocena genetická diverzita. Zároveň jsme se během pozorování zaměřili na typ buněk tvořící nárosty a dostupnost vody na lokalitě. V experimentální části jsme poprvé porovnali stresovou odolnost mladých vegetativních buněk a pre-akinet proti dalším typickým sezónním stresovým faktorům: UV záření a zamrzání. Zaměřili jsme se na vliv fotosynteticky aktivního záření v kombinaci s UV-A a UV-B a dále jsme otestovali odolnost těchto dvou typů buněk proti mrazu s cílem najít limit jejich přežití.

V obou studovaných regionech se podařilo odhalit značnou genetickou diverzitu v rámci nárostů tvořených *Zygnema* spp. Zjistili jsme, že nárosty jsou většinou tvořeny několika genotypy této řasy, našli jsme až pět genotypů rostoucích spolu. Ačkoliv se složení nárostu měnilo každou sezónu, výskyt řas na lokalitě je trvalý a našli jsme stejné genotypy i po čtyřech letech. Také poměr mladých vegetativních buněk a pre-akinet se mění během roku. Mladé vegetativní buňky převažují v nárostu na jaře a v létě a díky obsahu fenolických látek vykazují vyšší odolnost proti nadměrnému ozáření než pre-akinety. Naopak jejich odolnost proti mrazu je v porovnání s pre-akinetami malá. Pre-akinety, které převažují před nástupem zimy, jsou schopné odolat zamrznutí až do – 70 °C. Zároveň jsme pozorovali výskyt malého procenta odolných pre-akinet i během příznivých podmínek, což zajišťuje jejich přežití při náhlé změně podmínek a vystavení stresu. Výsledky této práce dokládají, že *Zygnema* spp. je dobře přizpůsobena různým stresovým faktorům ve vegetativním stavu, bez nutnosti tvorby specializovaných stadií. Zároveň jsme ukázali, že nárosty spájivek není možné brát jako populaci jednoho druhu a další studie na přírodním materiálu by proto měly vždy zahrnovat molekulární metody za účelem odhalení skryté diverzity.

Klíčová slova: sezonalita, stresová odolnost, Zygnematales, diverzita, vláknité řasy

1 Introduction

1.1 Zygnematophycean green algae

Zygnematophycean green algae are widely distributed, morphologically, and ecologically diverse, and monophyletic group belonging to the phylum Streptophyta. They are part of the ZCC clade, also known as advanced charophytes, together with classes Charophyceae and Coleochaetophyceae (de Vries et al., 2016). Zygnematophyceae have recently been recognized as the immediate sister lineage to Embryophytes (Becker & Marin, 2009; Wodniok et al., 2011; Timme et al., 2012; Becker, 2013; Zhong et al., 2014; de Vries & Archibald, 2018; Domozych & Bagdan, 2022). Several studies have suggested the colonization of moist habitats in the proximity of water by the charophyte algal ancestor of land plants and its gradual transition to dry land (Fig. 1) (Becker & Marin, 2009; de Vries & Archibald, 2018; Cheng et al., 2019; Fürst-Jansen et al., 2020). Therefore, they are studied as prerequisites for terrestrial life together with other charophycean algae (Cheng et al., 2019; Leebens-Mack et al., 2019; de Vries & Rensing, 2020; Fürst-Jansen et al., 2020).

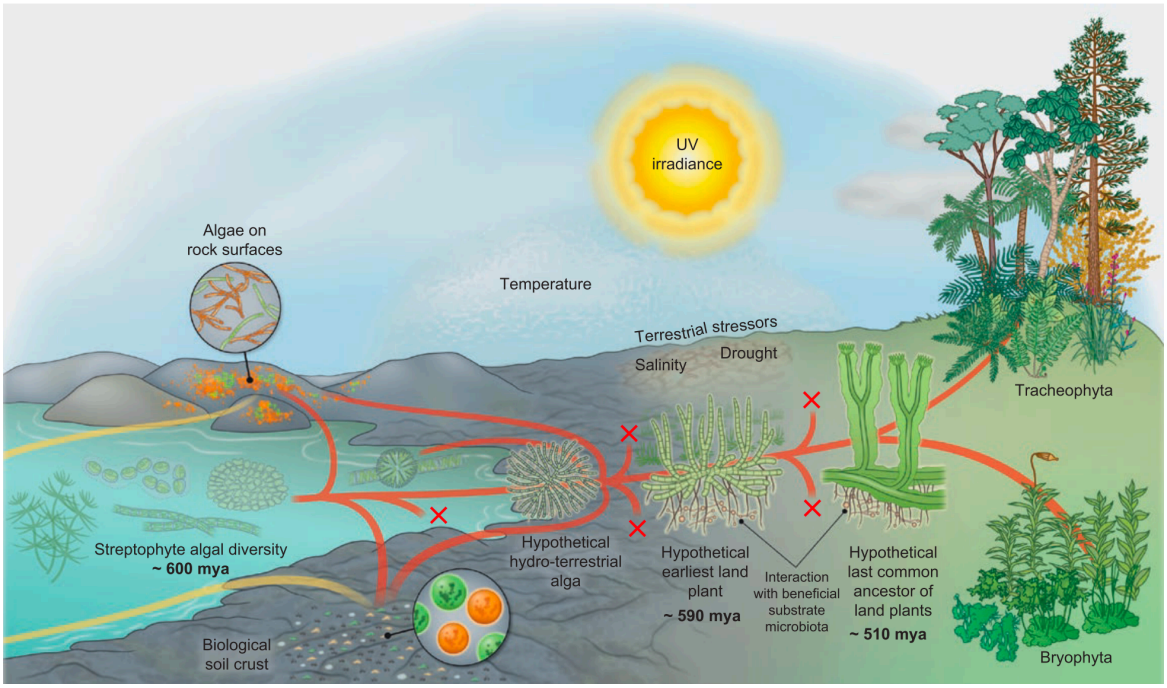

Fig 1: The evolutionary scenario of the conquest of land by streptophytes. The last common ancestor of land plants had traits of algal ancestry. Although streptophyte algae are the only photosynthetic eukaryotes from which the macroscopic land flora evolved (red lines), numerous lineages failed during this process (x labels). Algae from various other lineages also colonized land (yellow lines). Reprinted from Fürst-Jansen et al. (2020).

Representatives of this class are structurally simple, with coccoid or filamentous thallus. Although Zygnematophyceae are occasionally considered unbranched, they form holdfasts and undergo branching (Stancheva et al. 2014; Delwiche & Cooper 2015). They are also characterized by their special type of sexual reproduction, where zygote formation occurs as a result of conjugation without the presence of flagellate gametes (Guiry, 2013). Their morphological diversity and beauty have attracted scientific study for decades.

Zygnematophyceae are the most species-rich group of charophyte algae, with greater than 4000 described species (Gerrath, 2003). Algae of this class were recently divided according to the phylogenetical study of 326 genes into orders Desmidiales, Spirogyrales, Zygnematales, Serritaeniales, and Spirogloeales (Fig. 2) (Hess et al., 2022).

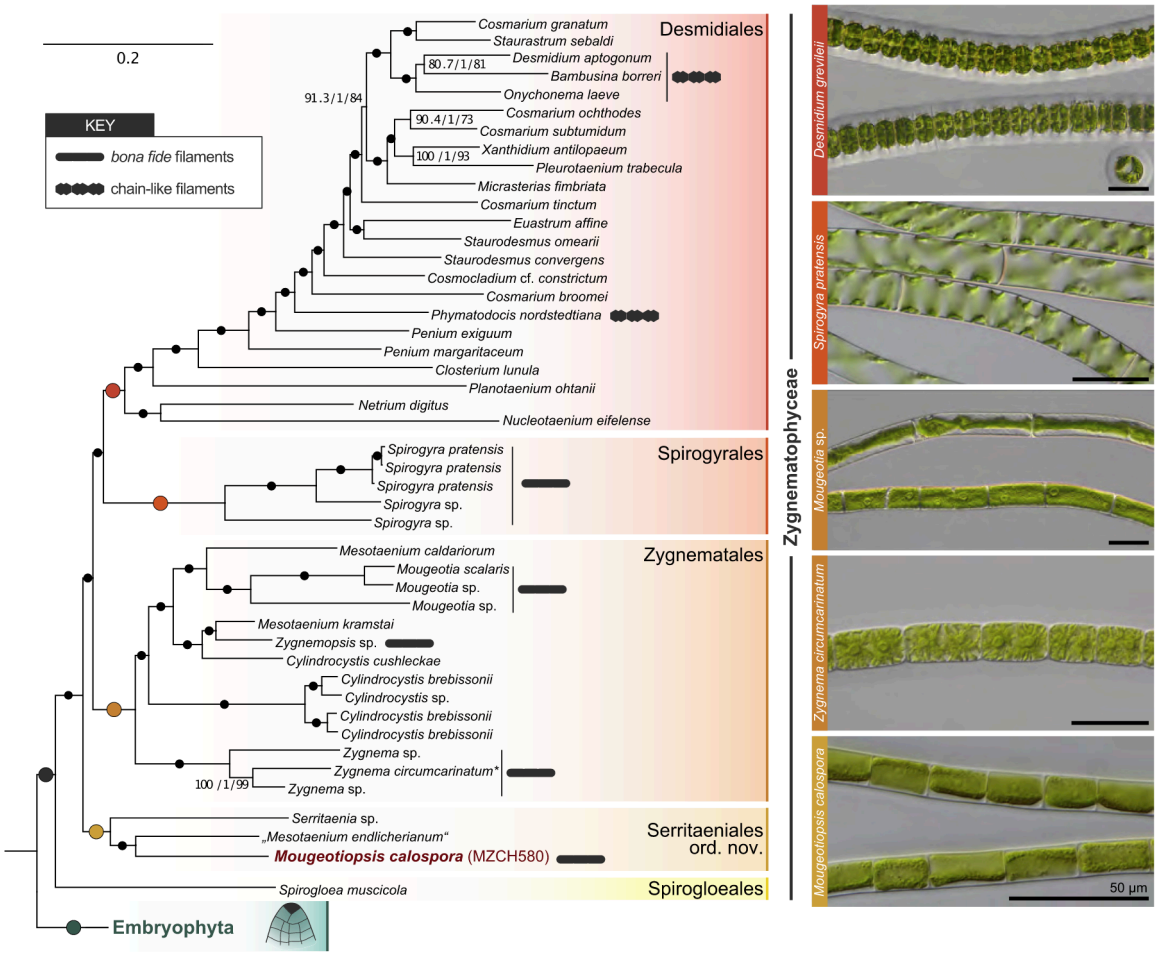

Fig. 2: Phylogenetic tree illustrating zygnematophycean phylogeny based on 326 genes. Zygnematophyceae comprises five deep-branching clades, which are defined as orders here. Gray symbols highlight zygnematophytes that form chain-like and bona fide filaments. Scale bars in all micrographs are 50 mm. Scale bar for phylogeny is 0.2 expected substitutions per site. Reprinted from Hess et al. (2022).

Zygnematophycean algae inhabit a wide range of environments. They are most diversely found in oligotrophic freshwater wetlands. However, they are also found in several other habitats, such as lakes, rivers, pools, and streams. Although all the members of this class still depend on water supply, hydro-terrestrial and semi-terrestrial species also exist, which typically live in small pools and seepages that periodically dry out. Well-known representatives of this class are beautiful desmids that frequently inhabit peat bogs, lakes, and rivers (Brook, 1981). The filamentous species of the order Zygnematales and Spirogyrales, for example, the genera *Spirogyra*, *Zygnema,* and *Mougeotia*, form typical and familiar extensive mats in streams and ponds (Kadlubowska, 1984). Certain Zygnematophycean algae have even been reported in extremely inhospitable places such as glacier surfaces (Remias et al., 2012a; Procházková et al., 2021; Winkel et al., 2022).

1.2 Annual development of the filamentous Zygnematophytes

The annual development of algae comprises alternating periods between favorable rapid exponential growth and unpleasant conditions. Seasonality is driven by geographical location and climate, with an additionally pronounced effect in certain places and less in others, entailing various periodic abiotic stresses. Filamentous Zygnematophycean algae frequently live in water or hydro-terrestrial habitats. Their transition to the land and the consequent life cycle is associated with the development of various adaptations that allow them to cope with stress. In addition to the semi-terrestrial way of life itself, the constantly changing conditions associated with the changing seasons entail various stresses. Weather changes frequently cause water scarcity, high/low temperatures, and changing levels of illumination. Algae living in such habitats develop complex life cycles with different seasonal stages to ensure their long-term survival. Patterns in changes of abundancies have been observed not only for Zygnematophycean algae but also for several algal genera across the entire tree of life (Wehr et al., 2015). The seasonality of filamentous Zygnematophyceae has been studied mainly in the previous century; however, recent studies are quite scarce (Pichrtová et al., 2016b; Volkova et al., 2018).

This phenomenon was studied by Transeau (1916), who recognized four different periods in the life of freshwater algae: germination, vegetative development, reproduction, and dormancy. Although algae germinate, grow vegetatively, and produce permanent stages every year, they can be divided into several groups based on their life strategies: winter annuals, spring annuals, summer annuals, autumn annuals, ephemerals, and perennials (Transeau, 1916). Although filamentous algae of the group Zygnematophyceae can belong to most of these groups, several of them are winter, spring, and summer annuals, which means their maximum development and reproduction periods span from April to August (Fig. 3). A few Zygnematophycean algae are also perennials; therefore, their vegetative cycle may continue through the year, mainly in permanent streams and ponds (Transeau, 1916).

The seasonality of filamentous Zygnematophycean algae, mainly *Spirogyra*, *Zygnema*, and *Mougeotia*, has been observed several times in field conditions and considerable attention has been paid to long-term observations (Transeau, 1916; Sheath & Burkholder, 1985; Hawes, 1988; Hoshaw & McCourt, 1988; Simons & van Beem, 1990; Cambra & Aboal, 1992; Graham et al., 1995). Similar attention was also paid to unicellular representatives of Zygnematophyceae, especially desmids (Lund, 1971; Biswas, 1992; Coesel & Blokland, 1994; Huszar & Reynolds, 1997; Machová-Černá & Neustupa, 2009; Neustupa et al., 2012; Svoboda et al., 2014).

These observations confirmed that the occurrence of filamentous algae is mostly seasonal and depends on the season and current conditions. Mass occurrence of filamentous Zygnematophycean algae starts in spring. Algal mats start their development at the bottom of water bodies from spores (Hillebrand, 1983). Hillebrand (1983) observed the first mats in February in Netherlands, just after the end of frost. Zygnematophycean algae mats typically grow rapidly at the beginning of the season. *Spirogyra* can triplicate its own biomass in only three days and even earlier (Cambra & Aboal, 1992). Algal biomass is then transported to the surface by oxygen bubbles entrapped between filaments (Hillebrand, 1983; Graham et al., 1995). Other movements were also observed in experiments with strains of *Spirogyra*, which is known for its ability to move actively toward light (Kim et al., 2005).

The algal biomass volume generally peaks between May and September in temperate conditions (Fig. 4; Lund, 1971; Cattaneo & Kalff, 1978; Hillebrand, 1983; Simons & van Beem, 1990; Coesel & Blokland, 1994). Although algal mats typically become compact during the summer, and disappear during August or September, a small amount of biomass remains until winter (Hillebrand, 1983).

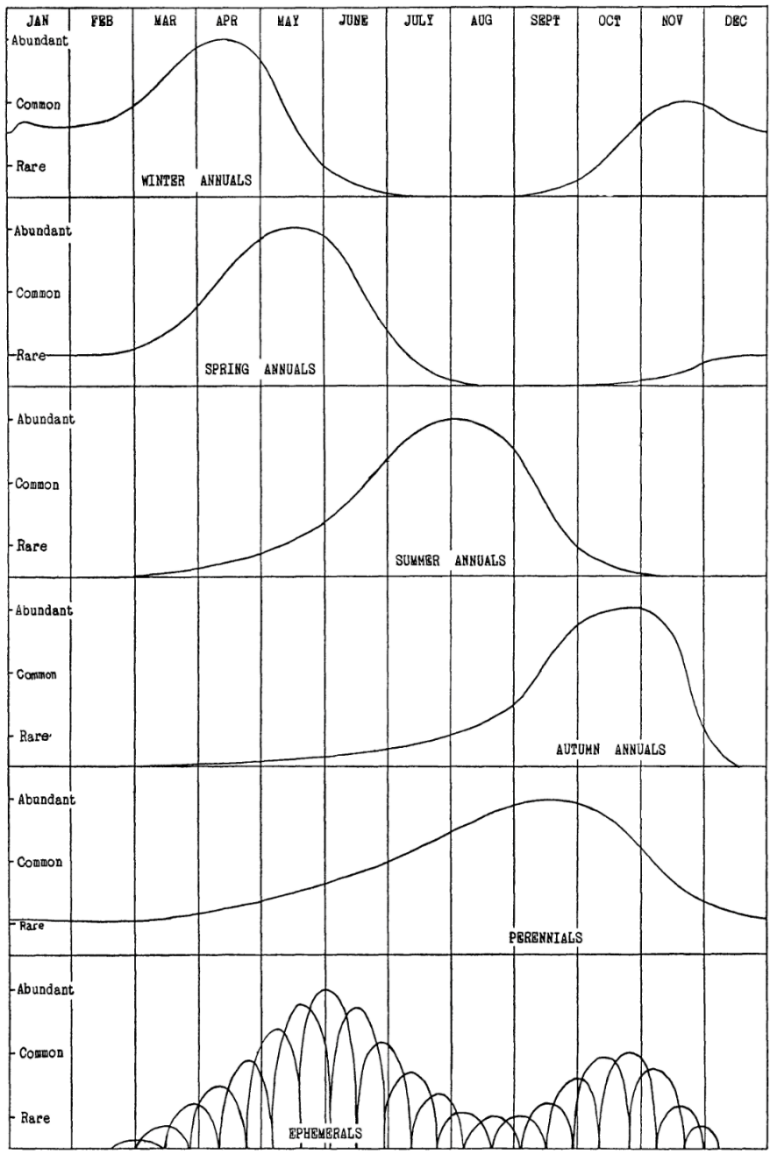

Fig 3: Frequency curves of the six ecological algae groups. Reprinted from Transeau (1916).

Although algal mats in the Arctic and Antarctic (Fig. 4) also exhibited higher abundances in spring and summer, the period is noticeably shorter (Hawes, 1988; Pichrtová et al., 2016b). Volkova et al. (2018) studied *Spirogyra* mats in Lake Baikal (Russia) and its tributaries over a period of five years. They identified total 15 morphotypes inhabiting this area and studied their abundance at different times. In the southern tributaries of the lake, the greatest increase in biomass was observed from July to September, while in the other areas it was only from the second half of summer to late autumn. The morphotypes that dominated in June and July were then replaced by other morphotypes growing from August to November. The authors even found *Spirogyra* growing under ice in March at temperatures below 4 °C, which is different from observations in other areas. The mats disappeared in Antarctica during

periods of frozen water levels and less sunshine (Hawes, 1988). Moreover, Pichrtová et al. (2016b) reported rapid growth of *Zygnema* sp. mats at the beginning of the spring in Spitzbergen. A part of the population even overwintered under snow and ice and resumed their growth immediately upon return of favorable conditions.

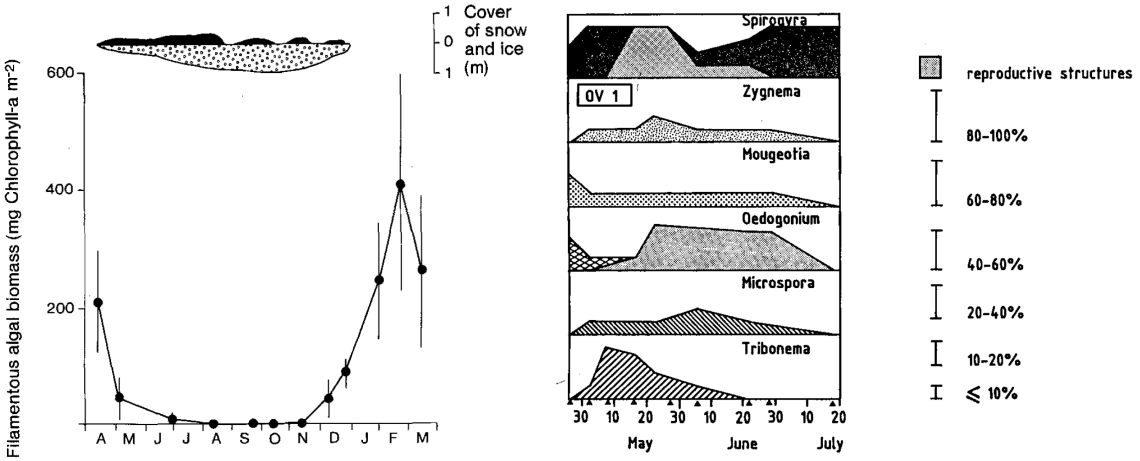

Fig. 4: Seasonal variations in algal abundances in different conditions and sampling sites. Left: Changes in the chlorophyll-a amount of *Spirogyra* in the Antarctic lake (Hawes, 1988). Right: Seasonal dynamics of the main components in floating mats from pool at Oostvoome (Netherlands). Scales on the right indicates the percentage of covered surface. Reprinted from Hawes (1988) and Simons and van Breem (1990).

Algae have endogenous rhythms of growth capacity and photosynthesis (Fogg & Thake, 1987; Lüning, 1994; Roenneberg & Mittag, 1996). Longer days and higher irradiance lead to rapid growth, especially in sufficient nutrient availability conditions. Total water surface coverage by floating algal mats in spring can reach 70–80% during suitable conditions (Simons & van Beem, 1990). Reproduction also generally occurs during the spring and summer periods (Transeau, 1916). In the case of *Spirogyra* species observed in the Netherlands (Simons & van Beem, 1990), sexual reproduction was observed during April to August. Sexual reproduction and zygospore formation of *Mougeotia* was observed from May to September in Tyrol (Austria) (Permann al., 2021a). Mats formed by filamentous algae are frequently composed of multiple genera growing together. In addition to the typical representatives of the class Zygnematophyceae (*Zygnema*, *Spirogyra*, *Mougeotia*), the genera *Oedogonium* (Chlorophyceae), *Tribonema* (Xanthophyceae), *Cladophora* (Ulvophyceae), *Chara* (Charophyceae), *Hydrodiction* (Chlorophyceae) and others are frequently found in the mats (Hillebrand, 1983; Simons & van Beem, 1990).

1.3 Specialized cells in life cycles

The formation of stress-resistant stages, generally called cysts or spores, is a widespread strategy for the long-time survival of algae and other groups of protists. They are characterized by decreased metabolic activity and formation of a thick multilayered cell wall. These characteristics enable them to resist unfavorable or extreme conditions that would be lethal for vegetative cells (Coleman, 1983). Resistant stages with unique morphology can be also found in advanced Charophytes, classes Charophyceae, Coleochaetophyceae,

and Zygnematophyceae (Brook, 1981; Kadlubowska, 1984; Delwiche et al., 1989; Leliaert et al., 2012).

Two major types of specialized resting cells with thick cell walls are observed among Zygnematophyceae. The first are spores of non-sexual origin. Akinetes are created by the thickening of the original cell wall far more than in metabolically active vegetative cells. They may also incorporate additional types of wall material into the original wall (Fritsch, 1935). Classic examples of this type are the akinetes of *Zygnema* (Kadlubowska, 1984; Stancheva et al., 2012). Aplanospores are dormant stages that can be found, for example, in the genus *Zygogonium*. They are formed inside the mother cells and germination has been observed after a dormant phase of 30 days (Stancheva et al., 2014). The aplanospores of *Zygogonium ericetorum* have a simple and unornamented cell wall (Stancheva et al. 2014), unlike those of the genus *Zygnema*. The aplanospores of genus *Zygnema* have greater similarity to the zygospores of this genus (Stancheva et al., 2012). The third type of resting vegetative cells are parthenospores, which are a result of incomplete conjugation (Stancheva et al. 2012).

The second type of thick-wall specialized cell is a spore with sexual origin, called a zygospore (dormant spore or dormant zygote). The fusion of gametes produces a zygote, which gradually matures into a zygospore. The result of such a process is a zygospore with a new, heavy, and decorated cell wall (Brook, 1981; Kadlubowska, 1984). Before germination, meiosis occurs in the zygospores, and the newly germinated filament already contains recombined genetic information (Haig, 2010). Although the recorded survival time of zygospores is extremely impressive, it is limited by a lack of old material for experiments. Zygnematophycean zygospores were observed to be viable after 20 years (Coleman, 1983). Zygospore cell walls are composed of three major layers: endo-, meso- and exospore with different chemical compositions. Recent studies of zygospores from *Spirogyra* and *Mougeotia* have revealed the composition of each layer (Permann et al., 2021a, 2021b; Permann et al., 2022b). The exospore and endospore layers are both composed of polysaccharides (Poulíčková et al., 2007; Permann et al., 2021a, 2021b; Permann et al., 2022b) The middle mesospore layer contains a sporopollenin-like material and is suggested as efficient protection against ultraviolet (UV) and desiccation stress. The ornamentation and color of this layer also defines the zygospore surface structure (Permann et al., 2021a, 2021b; Permann et al., 2022b). The zygospores of *Mougeotia* have an additional fourth lipid-like layer between the endo- and mesospore (Permann et al., 2021a). Permann et al. (2022b) reported cellulose fibrils arranged in a helicoidal pattern in the endospore and exospore layers of *Spirogyra*. This newly discovered unique three-dimensional arrangement of microfibrils and the integration of highly resistant components in the cell wall are not found in the vegetative state of this alga.

Sexual reproduction of these algae may be crucial to develop tolerance to extreme stresses, such as high temperature and desiccation (Permann et al., 2021b). Considering the extremely long period of time for which the zygospores can be viable (Coleman, 1983), it can be concluded that their production ensures the long-term survival of Zygnematophycean algae.

1.4 Survival of harsh conditions in a vegetative stage

Several algae, including filamentous Zygnematophyceae, do not form any specialized stages, such as akinetes or dormant zygotes, in their life cycles and can resist environmental stresses in their vegetative form (Agrawal, 2009). The filaments of Zygnematophycean algae

can aggregate into colonies, forming biofilms or multilayered mats (Hillebrand, 1983; Hawes, 1988). For example, *Zygogonium ericetorum* forms mats composed of two types of cells: so-called green and purple morphotypes. The upper layer formed by the purple morph protects the lower green morph from high irradiation (Aigner et al., 2013). Investigation of the mats of *Zygnema* from Svalbard (High Arctic) revealed different transcriptomic activity in the top and bottom layers. The top layer is transcriptionally extremely active (>6500 upregulated genes covering energy metabolism, photosynthesis, photoprotection, and protection from oxidative stress as well as cell wall modifications), unlike the bottom layer. The bottom cell layer is thus protected against stresses. Moreover, metabolic turnover is generally higher in the top layer. The upregulation of protection mechanisms in the top layer appears to be an immediate response to stress (Rippin et al., 2019). Similarly, biofilms formed by members of the class Klebsormidiophyceae on aeroterrestrial substrata provide self-shading and photoprotection to cells (Karsten et al., 2013).

Stress-resistant vegetative cells differ from normal cells in several characteristics. They generally accumulate storage products, reduce their physiological activity, cease cell division, and thicken their cell walls. Such cells were traditionally called winter forms, mature cells, or pre-akinetes. Zygnematophycean algae *Micrasterias* and *Zygnema* have been investigated multiple times for their resistance in a vegetative state (Meindl et al., 1989; Fuller, 2013; Pichrtová et al., 2014a, 2014b; Herburger et al., 2015; Pichrtová et al., 2016a). Lack of any type of spores was also observed for overwintering *Spirogyra* in a lake in Antarctica. Although the algae were surviving in vegetative form, their biomass volume strongly decreased (Hawes, 1988). Zygnematophycean algae also grow in extreme habitats such as glaciers. *Ancylonema nordenskioeldii* and *Mesotaenium bergrenii* were studied in connection to a reduction in glacial albedo by the darkening of its surface (Yallop et al., 2012; Stibal et al., 2017). Both species grow in the top layer of ice and cryoconite without any need to form specialized cells even for overwintering (Remias et al., 2012a).

1.5 Coping with seasonal abiotic stresses

Freshwater Zygnematophycean algae have developed different ways to challenge stress, even after being limited by their unicellular or filamentous thallus. Transition to terrestrial habitats relates to frequent exposure to naturally occurring abiotic stresses such as desiccation, freezing, and high photosynthetically active radiation (PAR) and UV radiation (Fig. 5). Development of specialized cells and structural changes in vegetative cells have enabled them to deal with several stresses (Holzinger & Lütz, 2006; Holzinger & Karsten, 2013; Karsten & Holzinger, 2014; Permann et al., 2022a).

Higher summer temperatures generally lead to increased evaporation and stress connected with limited water availability. Poikilohydric organisms such as algae cannot actively regulate their water content (Kranner et al., 2019), which can easily lead to desiccation. Pre-akinetes formed by *Zygnema* (Zygnematophyceae) were found to be resistant to desiccation (Pickett-Heaps, 1975; Pichrtová et al., 2014b; Herburger et al., 2015; Rippin et al., 2017) and osmotic stress (Kaplan et al., 2013; Pichrtová et al., 2014a). Herburger et al. (2019) reported accumulation of homogalacturonan in mature *Zygnema* sp. cells to increase their water holding capacities. Rippin at al. (2017) found several crucial pathways after analyzing organic osmolytes (sucrose) in *Zygnema* sp. after desiccation stress using a transcriptomic approach. Arc et al.

(2020) compared the differences between young *Zygnema* cells and pre-akinetes using a metabolomic approach and found an overall reduction in primary metabolites, consistent with a lower metabolic turnover, while they accumulated lipids and oligosaccharides. The transition to pre-akinetes involves change of photosynthetically fixed energy into storage instead of growth.

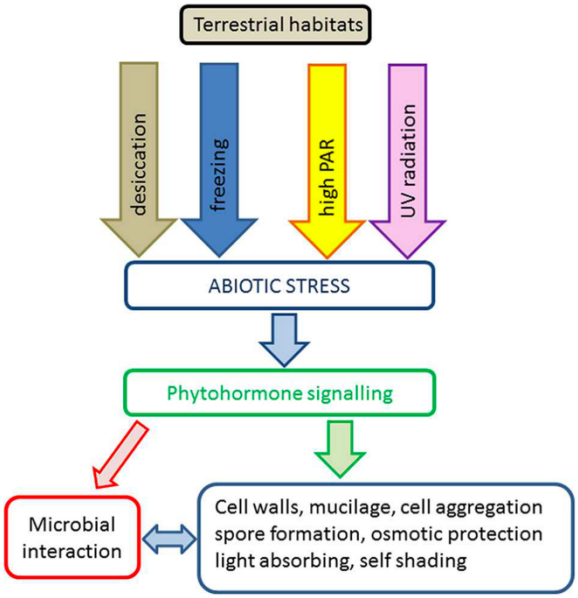

Fig 5. Scheme of abiotic stress factors influencing green algae. Reprinted from Holzinger & Pichrtová (2016).

Algae survive water deprivation by using structural adaptation such as the incorporation of callose (β-D-1,3-glucan) into cell walls. Callose increases the flexibility of the walls and thus reduces the effects of desiccation damage. This adaptation has been observed in *Zygnema* and in *Klebsormidium* (Klebsormidiophyceae) (Herburger & Holzinger, 2015). The effects of desiccation have also been studied on the other representatives of charophycean algae from different classes. Strains of *Entransia* and *Hormidiella* (Klebsormidiophyceae) were both found to be resistant to desiccation, and *Entransia* can cope with drought even better than other freshwater algae (Herburger et al., 2016). Moreover, the most known representative of the same class, *Klebsormidium* (Klebsormidiophyceae), has been found to have a high desiccation tolerance (Elster et al., 2008; Karsten et al., 2010; Holzinger et al., 2011), which well explains its abundant occurrence in alpine soil crusts (Karsten et al., 2010). Although the genus *Klebsormidium* is unrelated to *Zygnema*, they are frequently found together in the same location, occasionally in the same mats (personal observation).

In addition to desiccation, algae living in hydro-terrestrial habitats may also experience heat stress, especially during the summer season. Heat stress can damage cells by changing protein activity and reducing chlorophyll production and the fluidity of membranes (Hu et al., 2020). Effects of this stress were recently studied on different genera of filamentous Zygnematophyceae. DeVries et al. (2020) observed pronounced phenotypic alterations in plastids of *Spirogyra* and *Mougeotia* after exposure to 37 °C for 24 h. Their response to heat stress revealed a set of genes that encode proteins known as regulators of stress response in land plants. High temperatures were measured directly in natural populations of *Spirogyra*. Temperature increased by up to 32 °C on the mat surface, indicating the common prevalence of this stress (Hillebrand, 1983). Graham et al. (Graham et al., 1995) studied population

of *Spirogyra* sp. in a lake in Indiana. Algae exhibited positive net photosynthesis at temperatures from 5 °C to 35 °C. Similarly, lowland- and alpine-origin strains of *Zygnema* sp. exhibited maximum oxygen production at 35 °C and 30 °C, respectively, during photosynthesis, and the production greatly decreased at higher temperatures (Herburger et al., 2015). However, *Zygnema* strains originating in subpolar and polar habitats reached maximum photosynthetic oxygen production at significantly lower temperatures (20 °C and 15 °C, respectively; Permann et al., 2022c), and exposing them to 25 °C for two weeks led to decrease in photosynthesis. However, these strains dissipated absorbed energy (non-photochemical quenching; Permann et al., 2022c). Results of the mentioned studies indicate that filamentous algae of the class Zygnematophyceae are well adapted to higher temperatures. The strains of *Zygnema* sp. used in these studies (Herburger et al., 2015; Permann et al., 2022c) were collected from different habitats. However, the differences in their heat stress response cannot be explained based on their relationship according to the phylogeny from Stancheva et al. (2012), considering that strains belonging to the same lineage reacted quite differently. Similarly, a study on desmid *Cosmarium* sp. found a relationship between the habitat from which the strain was isolated and its physiological characteristics (Stamenković & Hanelt, 2013).

Another abiotic stress connected mainly with the summer season is high irradiation, both PAR and UV radiation. These stresses have been thoroughly studied in green algae, including filamentous Zygnematophyceae (Holzinger & Lütz, 2006; Karsten & Holzinger, 2014; Holzinger & Pichrtová, 2016; Permann et al., 2022a). Unlike other groups of algae that use mycosporine-like amino acids (Karsten, 2008; Hartmann et al., 2016) or secondary carotenoids as photoprotectants (Remias et al., 2005), Zygnematophyceae use phenolic compounds that are soluble in water and stored in vacuoles (Holzinger & Pichrtová, 2016). Exposure to higher levels of UV radiation led to their accumulation in genera *Zygnema* (Pichrtová et al., 2013), *Zygogonium* (Aigner et al., 2013), *Spirogyra* (Cannell & Alio, 1988), and *Zygnemopsis* (Figueroa et al., 2009). Germ et al. (2009) studied the effects of UV-B radiation on *Zygnema* isolates from a mountain lake (1,880 m a.s.l.) and demonstrated no damage to the optimal quantum yield of photosystem II (PSII). Similarly, Arctic *Zygnema* sp. mats exposed to high irradiation revealed no impairment of photosynthesis or ultrastructural changes (Holzinger et al., 2009). In contrast to these findings, the results of UV-B stress on polar *Zygnema* revealed different levels of damage (Pichrtová et al., 2013). Moreover, Antarctic *Zygnema* sp. exhibited negative effects on PSII and poor recovery after UV-B exposure (Prieto-Amador, 2016). Pierangelini et al. (2017) observed lower photoprotection capacity and less tolerance to high light intensity in *Zygnema* sp. than in two *Klebsormidium flaccidum* isolates.

In glacier-inhabiting algae, *Ancylonema nordenskiöldii* (Remias et al., 2012a) and *Mesotaenium berggrenii*, UV-absorbing phenolic compounds of purple to brown color were found and characterized as purpurogallin-derived secondary pigments (Remias et al., 2012b). In *Zygogonium ericetorum*, these substances were also observed absorbing in the visible waveband. This was a red glycosylated derivative of gallic acid complexed with ferric iron (Aigner et al., 2013; Herburger, Remias, et al., 2016). Purple morphotype of *Zygogonium ericetorum*, located in the upper layer of the mat, contains higher amount of UV-absorbing phenolic substances (Aigner et al., 2013; Herburger et al., 2016b). Moreover, *Zygnema* sp. can differentiate the function of distinct layers in the mat and the bottom layer provides

photoprotection to the bottom layers (Holzinger et al., 2009; Rippin et al., 2019). Mats are frequently formed by more than one genus of filamentous algae (Hillebrand, 1983; Simons & van Beem, 1990) or morphotype (Volkova et al., 2018), and thus shading may be provided by other components of the mat.

In desmids, extracellular mucilage produced in a large amount may play a role in UV protection. *Micrasterias* cells exhibited resistance to harsh experimental UV treatments, and only wavelengths lower than 284 nm harmed the cells and induced observable changes in the ultrastructure (Meindl & Lütz, 1996; Lütz et al., 1997). Further, the mucilage of *Serritaenia* was studied for its light-absorption capability. High concentrations of extracellular pigment observed in the field material suggest that the extracellular mucilage of *Serritaenia* not only extends the active phases of this alga owing to its water-holding capacity, but also represents an effective 'broadband' sunscreen (Busch & Hess, 2022).

Although the cell walls of filamentous Zygnematophyceae are generally colorless, representatives of the genera *Spirogyra*, *Zygnema, Mougeotia*, and *Zygnemopsis* produce zygospores with blue, brown, or reddish spore walls (Stancheva et al., 2012, 2013; Pichrtová et al., 2018; Takano et al., 2019; Permann et al., 2021a, 2021b). The photoprotective function of pigments is most likely present in zygospores although it has not yet been investigated.

Winter is the most significant period of the year in the meaning of the presence of stresses. During freezing and thawing, cells are exposed to a radically changing environment, which leads to cryoinjuries (damages caused by frost). Depending on the cooling rate, two different damaging mechanisms can occur: freezing cytorrhysis (collapse of a cell wall owing to the loss of water through osmosis) caused by considerable cell dehydration, or mechanical disruption of membranes (Acker & McGann, 2003). Hawes (Hawes, 1990) studied frost tolerance of *Zygnema* sp. in the Antarctica. Most of the cells were able to resist freeze-thaw temperature cycles slightly below freezing point; however, only a few could resist down to -20 °C. Another filamentous Zygnematophycean alga *Spirogyra* also exhibited the ability to survive winter without the formation of any resistant cells. However, most of the population died beneath the ice cover of the lake (Hawes, 1988). Recently, Permann et al. (2022c) reported the adaptation of (sub-)polar *Zygnema* strains to cold environmental conditions and sensitivity of the photosynthetic apparatus to higher temperatures. These results indicate the adaptation of distinct strains of *Zygnema* sp. to the inhabited environment. Effects of freezing were also investigated in charophycean alga *Klebsormidium* (Klebsormidiophyceae; Elster et al., 2008; Nagao et al., 2008). Strains from different geographical regions have been observed to share the same freezing tolerance (Elster et al., 2008). Interestingly, tolerance to freezing can be significantly improved by cold acclimation. This process was also connected with the accumulation of polysaccharides, enlargement of chloroplasts and starch grains, and reduction of vacuoles (Nagao et al., 2008a). In contrast to the described tolerance strategies, certain Zygnematophycean algae found a way to avoid frost. Although *Micrasterias denticulata* has little frost tolerance, it can survive in frozen waterbodies (Steiner et al., 2021). Snow and ice on the top of the water create dark conditions; however, the environment is protected from frost. *Micasterias denticulata* also exhibited reorganization at the cellular level. Cold stress led to increased organelle contacts (Steiner et al., 2021).

1.6 Genus *Zygnema*

We chose the genus *Zygnema* as a model organism for the study of seasonality in the order Zygnematales because of its known but poorly investigated trends in annual development. The oldest known paleontological record of the alga with a morphology of *Zygnema* sp. was found in Cretaceous amber from southern Germany (Dörfelt & Schäfer, 2000). Fossil *Palaeozygnema spiralis* has unbranched filaments with cells ≤ 20 μm long and 14 μm wide with preserved chloroplasts and zygotes. The morphology is extremely similar to recent *Zygnema* species; however, we can only speculate about the real level of congeniality. The algae classified as species of *Zygnema* comprise commonly unbranched filaments of cylindrical cells with two stellate chloroplasts and a large pyrenoid in each of them (Kadlubowska, 1984; Stancheva et al., 2012). They measure 10–50 μm in diameter, with most of the species within 20–30 μm. Their length is equal to or quite higher than the width. Filaments are covered with a mucilage envelope of variable thickness; varying from those barely visible under the microscope to those thicker than the cells themselves. Fuller (2013) observed formation of secondary pectic layers in *Zygnema irregulare* during prolonged cultivation on solid medium. The nucleus is located in the cytoplasmatic bridge between the chloroplasts, with haploid chromosome numbers varying from 19 to 82 (Prasad & Godward, 1966). DNA content variability in the genus *Zygnema* is likely to be higher than previously known. While the previously measured range was 0.49 to 1.5 pg (0.50 to 1.54 Gbp) (Kapraun, 2005; Mazalová et al., 2011), Čertnerová (2021) found a DNA content of 1.11 to 2.86 pg (1.09 to 2.80 Gbp) in the three *Zygnema* strains. Further, Feng et al. (2021) reported a large difference in the nuclear genome sizes of two *Zygnema* sp. strains.

Representatives of the genus *Zygnema* reproduce both sexually and asexually. Their life cycle is haplobiontic and conjugation is mostly scalariform. Most of the species is anisogamous (one gamete is ameboid, the other stationary), with a few isogamous species (both gametes are motile). Zygospores (Fig. 6) are formed inside one cell wall of conjugating cells (anisogamous) or inside a conjugation tube (isogamous). They are spherical to ovoid, with a three-layer cell wall and suture. The exospore is thin, smooth, and colorless. The middle layer, called the mesospore, is thicker and colored, typically blue, brown, or yellowish with keels, ridges, and small depressions. The inner endospore is thin and barely visible using light microscopy (Pickett-Heaps, 1975; Kadlubowska, 1984).

Asexual spores of *Zygnema* sp. (Fig. 6) can be formed in different ways. Parthenospores are formed as a result of incomplete conjugation (Kadlubowska, 1984), and are extremely similar to aplanospores, which develop from shrunken protoplasts of cells (Stancheva et al., 2012). Akinetes have a rectangular shape and develop directly from a vegetative cell (Kadlubowska, 1984; Stancheva et al., 2012). All of them (together with zygospores) need to germinate to form a new filament (Pickett-Heaps, 1975; Kadlubowska, 1984; Stancheva et al., 2012) (Fig. 6). Morphology and occurrence of both asexual and sexual spores are crucial characteristics for traditional species delimitation (Kadlubowska, 1984; Stancheva et al., 2012).

A special type of vegetative cell found in mats formed by *Zygnema* sp. is a pre-akinete (Fig. 6). They are formed during stress when the cell division is stopped owing to lack of nutrients. Pre-akinetes have accumulated storage material, thickened cell walls, and reduced chloroplasts.

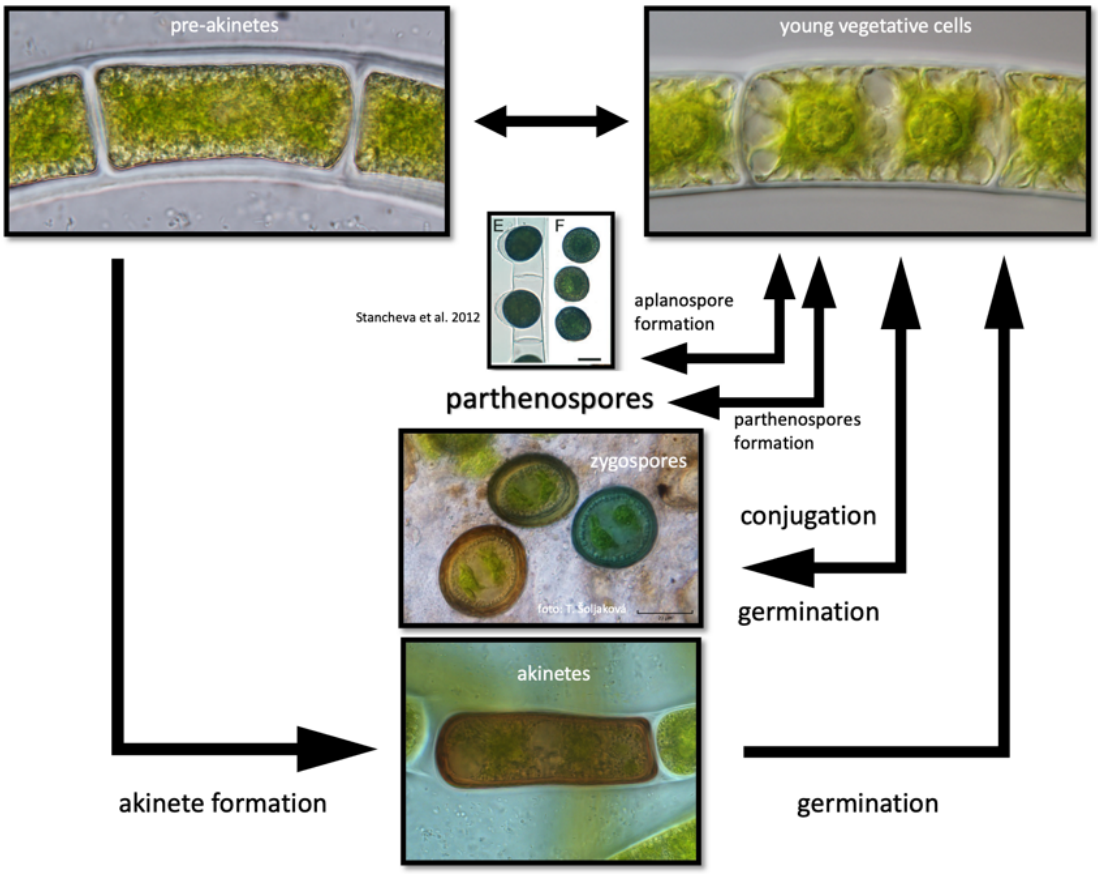

Fig. 6: Different specialized stages observed in genus *Zygnema*. According to Pickett-Heaps 1975; Kadlubowska 1984; Stancheva et al. 2012; and my own observations.

Their formation is characteristic for *Zygnema* species from different climatic conditions (McLean & Pessoney, 1970; Stancheva et al., 2012; Herburger et al., 2015). Filaments of pre-akinetes easily disintegrate into individual cells as a possible way of spreading onto new localities (personal observation). There is a difference between pre-akinetes and other spores in terms of further development. Zygospore, parthenospores, aplanospores, and akinetes need to germinate by rupturing the cell wall to form a new vegetative filament (Kadlubowska, 1984). Pre-akinetes can continuously transform into vegetative cells and vice versa (Herburger et al., 2015).

Genus *Zygnema* is a well-supported monophyletic clade (Hall et al., 2008). Its representatives were traditionally divided into four sections; Pectinarum, Leiosporum, Collinsianum, and Cylindricum (Kadlubowska, 1984). According to recent knowledge, this division does not correspond with phylogenetic analyses (Stancheva et al., 2012). Currently, greater than 200 species are accepted taxonomically in the algal taxonomic database Algaebase (Guiry & Guiry, 2022). However, only a minority of sequences have been assigned to a traditionally described species. Traditional taxonomy of the genus is based on morphological characteristics connected with sexual reproduction; the color of mesospore, ornamentation, and position of the zygospore in tubes or gametangia. Poulíčková et al. (2007) observed that the size of spores is not a helpful characteristic for species identification.

Structure, ornamentation, and coloration of aplanospores, parthenospores, and akinetes are important for defining certain asexual species (Kadlubowska, 1984; Stancheva et al., 2012).

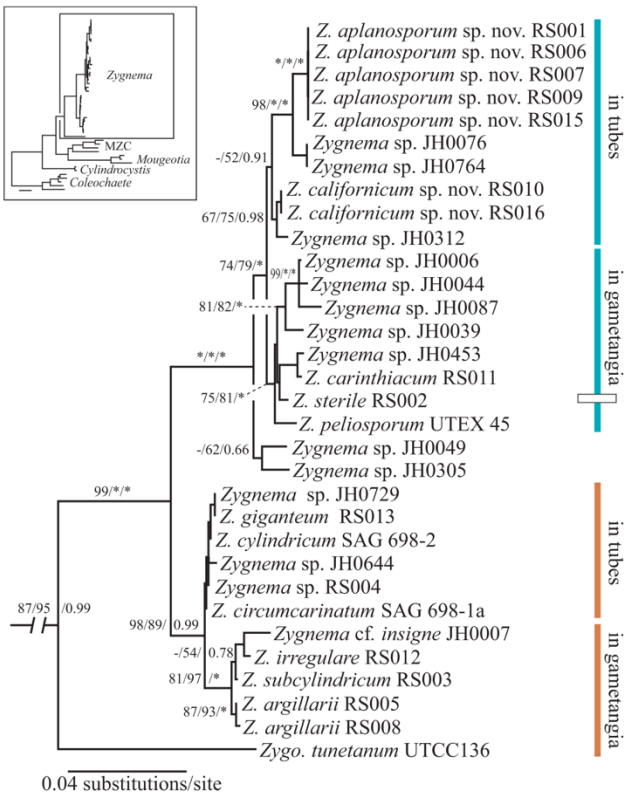

Fig. 7: Phylogeny of *Zygnema* based on *rbc*L data. Numbers above the branches are bootstrap values from parsimony and maximum likelihood analyses and posterior probabilities from a Bayesian analysis. An asterisk indicates bootstrap support of 100 or a posterior probability of 1.0. A dash indicates bootstrap support of <50 or a posterior probability <0.5. Mesospore color is indicated by the color of the bar on the right. From Stancheva et al. (2012).

Stancheva et al. (2012) conducted a phylogenetical study and divided the genus *Zygnema* into two well-supported clades. The color of the mesospore appears to be a promising characteristic corresponding with molecular phylogeny (Fig. 7). However, the study was focused on strains from a restricted area and the occurrence of conjugation is still required for species determination. Sterile filaments cannot be classified on a species level without the occurrence of characters connected with sexual reproduction. As the prevailing method of reproduction can be influenced by the nutrition composition of media (Poulíčková et al., 2007), this characteristic is likely to be dependent on environmental characters and not on phylogenic position. During the revision of the genus *Zygogonium*, a third clade was added to the genus *Zygnema*, representing the species *Zygnema tunetanum* (formerly *Zygogonium tunetanum*; Stancheva et al., 2014).

Genus *Zygnema* is an ecologically important inhabitant of hydro-terrestrial habitats and water bodies all over the world. Habitats range widely from stagnant to running and fresh to brackish water. They can be found in slow streaming water, ponds, puddles, and ditches, and were also observed in a shoreline zone on the coast of the Baltic sea (Hawes, 1990; Aboal, 1991; Novis, 2004; Holzinger et al., 2009; Stancheva et al., 2012; Skácelová et al., 2013; Pichrtová et al., 2013b, 2016b; Herburger et al., 2015; Holzinger & Pichrtová, 2016). Filaments of the genus *Zygnema* generally form free-floating masses called mats that can cover several square meters. They frequently grow together with other filamentous representatives of the order Zygnematales; mostly *Spirogyra* and *Mougeotia* (Simons & van Beem, 1990).

Blooms of *Zygnema* occur in littoral zones of soft water lakes in early stages of acidification (Turner et al., 1991) and several species have been recently used as a bioindicator of acidification (Schneider & Lindstrøm, 2011); however, their usage for this purposes is limited by problems with species identification.

Mats formed by *Zygnema* sp. encounter environmental stress during the entire year. Constantly changing water levels lead to their cyclic desiccation, and they are exposed to high irradiation and frost during the winter. Recent studies revealed the unexceptionable resistance of pre-akinetes to different stresses. They are resistant to desiccation (Pickett-Heaps, 1975; Pichrtová et al., 2014b; Herburger et al., 2015; Rippin et al., 2017), osmotic stress (Kaplan et al., 2013; Pichrtová et al., 2014a), and UV radiation (Holzinger et al., 2009). Mats formed by *Zygnema* sp. are characterized by their seasonal dynamics and differ according to the geographic origin; for example, massive losses in biomass volume have been observed in early spring in the Arctic (Pichrtová et al., 2016b) or during hot summers in temperate conditions (personal observations). Their reported frost resistance (Hawes, 1990) would imply an ability to survive winter in high abundances, which is not in accordance with field observations (Pichrtová et al., 2016b).

We lack a complex view on the effects of stresses on their seasonal dynamics, genetic composition of the mats, and persistency of populations in one location. Moreover, nothing is known about the resistance of different cell types to frost or cold. Winter is clearly the crucial season affecting their populations; however, other stresses could also play an important role in a chance for long-term survival.

2 Research objectives and methods

This thesis mainly aimed to provide an insight into the life strategy of filamentous Zygnematophycean alga *Zygnema* sp. with respect to the environmental stresses they encounter during their annual development. The seasonal dynamicity of the mats formed by these algae has been known to experts for a considerable period (Hillebrand, 1983; Sheath & Burkholder, 1985; Simons & van Beem, 1990; Pichrtová et al., 2016b). Mats of *Zygnema* sp. have an annual character and they generally disappear during the winter (Pichrtová et al., 2016b). However, no study to date has focused on the long term observation of mats; especially, nothing is known about their diversity and dynamics: it is still unclear whether the same genotypes remain persistently at the same locations.

Therefore, in this work we aimed at discovering if there are changes in the composition of mats across seasons and years. In addition, the focus was on stress resistance mechanisms that enable the algae to overcome periodical stresses and survive from one season to another. Several previous studies discovered unexpected resistance of hardened cells (pre-akinetes) of *Zygnema* sp. (Pickett-Heaps, 1975; Pichrtová et al., 2014b; Herburger et al., 2015a; Rippin et al., 2017). We investigated these cells during our field observations and also tested their stress tolerance in laboratory experiments, comparing them with young vegetative cells.

The aims of this thesis can be summarized through several questions, which I decided to answer together with my colleagues:

1. **What is the diversity within *Zygnema* sp. mats?** (Paper I, IV) Can we find greater number of genotypes in one mat? Does one of them prevail? Can we find endemic *Zygnema* lineages inhabiting Alpine or polar regions?

2. **Are there any changes in the diversity of mats across several years and seasons?** (Paper I, IV) Are the populations in the locality stable or changing?

3. **Is there any adaptation to UV-B stress in young cells and pre-akinetes of *Zygnema* sp.?** (Paper II) What is the importance of this adaptation for surviving the stresses associated with the changing seasons?

4. **How resistant are the cells of *Zygnema* sp. to frost?** (Paper III) How does frost damage different cell types? Can we observe any changes in the morphology and ultrastructure of the cell after freezing? Is repeated freezing additionally harmful?

To find the answers, we combined field observations of natural populations with a series of laboratory experiments. Molecular and morphological diversity of *Zygnema* sp. mats was investigated in Spitzbergen (Paper I) and the Tyrolean Alps (Paper IV). We used molecular markers *rbcL* (Paper I, IV) and *trnG* (Paper IV) to uncover diversity. The dataset was supplemented by sequences of the genus *Zygnema* available in the GeneBank database (Paper I, IV). Long-term field observation was performed in the Alps from August 2018 to May 2020 (Paper IV). We chose an accessible location, which was visited 3–4 times per year. We sampled

two habitats with different water regimes. First was a tributary area of a stream flowing into a small lake. Water was available year round and conditions were relatively stable because the algal mats were submerged most of the year. The second location was a small streamlet on a meadow. Water levels strongly fluctuated at this location, from almost complete desiccation to a high amount of streaming water. The mats in both locations were exposed to direct sunlight most of the year. We performed UV experiments on *Zygnema* sp. strains with distinct geographical origins. Untreated and UV-exposed strains of different ages were investigated for changes in pigments and phenolic compounds using high-performance liquid chromatography (HPLC; Paper II). In the second experimental part of the work, we used freezers with controlled regimes to reveal limiting survival temperatures for young vegetative cells and hardened pre-akinetes (Paper III). LT_{50} temperatures were established based on chlorophyll fluorescence measurements and fluorescent vital staining after the experimental freezing (Paper III). Direct effect of freezing was observed via cryo-microscopy and changes in the ultrastructure were investigated using light microscopy and transmission electron microscopy (TEM; Paper III).

3 Original papers

Paper I

Molecular and morphological diversity of *Zygnema* and *Zygnemopsis* (Zygnematophyceae, Streptophyta) from Svalbard (High Arctic)

European Journal of Phycology

Molecular and morphological diversity of *Zygnema* and *Zygnemopsis* (Zygnematophyceae, Streptophyta) from Svalbard (High Arctic)

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Running title: Diversity of Arctic *Zygnema* and *Zygnemopsis*

Abstract

Filamentous conjugating green microalgae (Zygnematophyceae, Streptophyta) belong to the most common primary producers in polar hydro-terrestrial environments such as meltwater streamlets and shallow pools. The mats formed by these organisms are mostly composed of sterile filaments with *Zygnema* morphology, but the extent of their diversity remains unknown. Traditional taxonomy of this group is based on reproductive morphology, but sexual reproduction (conjugation and formation of resistant zygospores) is very rare in extreme conditions. In the present study we gave the first record of zygospore formation in Svalbard field samples and identified conjugating filaments as *Zygnemopsis lamellata* and *Zygnema* cf. *calosporum*. We applied molecular phylogeny to study genetic diversity of sterile *Zygnema* filaments from Svalbard in the High Arctic. Based on analysis of 143 *rbc*L sequences, we revealed a surprisingly high molecular diversity: 12 Arctic *Zygnema* genotypes and one *Zygnemopsis* genotype were found. In addition, we characterized individual Arctic genotypes based on cell width and chloroplast morphology using light and confocal laser scanning microscopy. Our findings highlight the importance of molecular approach when working with sterile filamentous Zygnematophyceae, as hidden diversity might be very beneficial for adaptation to harsh environmental conditions, and experimental results could be misinterpreted when hidden diversity is neglected.

Key words: Arctic, chloroplast shape, cryptic diversity, microscopy, molecular phylogeny, *rbc*L, Svalbard, *Zygnema*, *Zygnemopsis*

Introduction

Conjugating green algae (class Zygnematophyceae) are the most species-rich group of charophyte algae, with more than 4000 described species (Gerrath, 2003). They are morphologically diverse and visually attractive. Unravelling the diversity and evolution of this group is an important research topic in current phycology, because conjugating green algae are considered to be the closest algal relatives of land plants (Wickett *et al.*, 2014; Zhong *et al.*, 2015), and the genetic identity of the vast majority of morphospecies is still unknown (however see, e.g. Drummond *et al.*, 2005; Gontcharov & Melkonian, 2005; Stancheva *et al.*, 2014). Zygnematophyceae are also important ecological dominants in certain habitat types. For example, unicellular desmids typically occur in benthic communities of peat bogs (Coesel & Meesters, 2007) or ephemeral freshwater pools (Šťastný, 2008). Their species composition reflects abiotic conditions; hence they are used as bioindicators (Coesel, 2001) and model organisms in ecological studies (Svoboda *et al.*, 2014). Filamentous Zygnematophyceae often dominate various freshwater habitats where they can quickly produce large amounts of biomass. They are particularly important in polar hydro-terrestrial habitats where they form extensive mats and are among the main primary producers influencing mineral cycling and primary colonization and development of soils (Elster, 2002). Such mats are present in both the Arctic (Sheath *et al.*, 1996; Kim *et al.*, 2008, 2011; Pichrtová *et al.*, 2016) and Antarctic (Hawes, 1989; Davey, 1991; Skácelová *et al.*, 2013).

These polar hydro-terrestrial algal mats are often formed by algae of the genus *Zygnema* C. Agardh that are very well-adapted to harsh conditions of polar climate, as demonstrated by various ecophysiological studies (Hawes, 1990; Holzinger *et al.*, 2009; Pichrtová *et al.*, 2013, 2014a, 2014b; Vilumbrales *et al.*, 2013; Choi *et al.*, 2015). Nevertheless, both floristic and ecophysiological publications refer only to "*Zygnema* spp." because species diversity within these mats remains unexplored. Consequently, comparison of results across different studies is practically impossible. Moreover, the lack of knowledge of species diversity precludes the testing of any ecological or biogeographical hypotheses.

The main reason for genus level identification of *Zygnema* specimens is the fact that traditional taxonomy of the genus is based on morphological characteristics connected with sexual reproduction (conjugation) such as wall colour and ornamentation of zygospores or sporangial shape (Kadlubowska, 1984; Stancheva *et al.*, 2012). Besides zygospores, other specialized cell types are known in some species of *Zygnema*: parthenospores that result from incomplete conjugation, aplanospores that are formed inside vegetative cells and akinetes that develop directly from vegetative cells by thickening of the cell wall (Kadlubowska, 1984; Stancheva *et al.*, 2012). Structure, ornamentation and coloration of such cells is important for definition of some asexual species (Kadlubowska, 1984; Stancheva *et al.*, 2012, 2014). Conjugation usually takes place only occasionally. In extreme environmental conditions, it is rare or never occurs, which is usually explained as the result of a trade-off between sexual reproduction and growth (Holzinger *et al.*, 2009), a phenomenon widespread in vascular plants (Eckert, 2002). With the only exception of *Zygnema* cf. *leiospermum* De Bary from Ellesmere Island in Canada (Elster *et al.*, 1997), zygospores of *Zygnema* have not been reported from polar regions.

The situation regarding species identification is different for closely related unicellular desmids, in which species are traditionally defined by vegetative morphological characteristics

such as shape, cell wall ornamentation, number of pyrenoids and other features (Coesel & Meesters, 2007). However, modern molecular phylogenetic studies repeatedly show that traditional taxonomy does not reflect the actual relationships, and reveal the existence of cryptic species (Gontcharov, 2008). For example, cryptic lineages that occur sympatrically across Europe were identified within the morphologically well-defined species *Micrasterias truncata* (Corda) ex Bréb (Nemjová *et al.*, 2011).

The application of molecular phylogenetic methods is thus also essential for obtaining insight into the diversity of sterile populations of filamentous Zygnematophyceae. The only study investigating molecular phylogeny of the genus *Zygnema* was based on *rbc*L and *cox*1 genes and showed that the genus is split into two major clades, representing distinct differences in reproductive morphology, and that existing taxonomic concepts are not consistent with phylogeny (Stancheva *et al.*, 2012). Molecular methods have also been used in two recent experimental studies of polar *Zygnema* strains. The results showed sufficient variability in *rbc*L gene sequences among strains isolated from Svalbard and Antarctica (Pichrtová *et al.*, 2013, 2014*b*).

In some other algal groups from polar hydro-terrestrial or terrestrial mats, the application of molecular methods has already brought insight into their diversity and biogeography. *Prasiola crispa* (Lightfoot) Kützing (Trebouxiophyceae, Chlorophyta) is very common in both the Arctic and Antarctica. Recent molecular investigations showed that the Antarctic morphospecies *Prasiola crispa* in fact comprises three cryptic species (Moniz *et al.*, 2012). Moreover, a similar study in the Arctic even revealed cryptic genera among Prasiolales (Heesch *et al.*, 2016). Richter *et al.* (2016) reported that all studied populations of *P. crispa* from a region of Svalbard belong to a single clade (based on *rbc*L phylogeny), although their data also indicate diversification on a population level. In Antarctic Tribonemataceae (Stramenopiles), phylogenetically distant, yet ecologically and morphologically similar filamentous freshwater algae, cryptic genera were also revealed (Rybalka *et al.*, 2009), and no biogeographical limitation of individual clades and endemism was revealed (Rybalka *et al.*, 2009). By contrast, most lineages of filamentous green algal genus *Klebsormidium* (Streptophyta) have a geographically limited distribution, and only one cosmopolitan lineage was reported in both Arctic and Antarctic isolates (Ryšánek *et al.*, 2016).

In the present study, we explored the genetic (*rbc*L sequences) and morphological (width of filaments and chloroplast shape) diversity of *Zygnema* mats from different localities on Svalbard. Key questions were how many different *Zygnema* genotypes could be found within a small sampling region in central Svalbard, and whether individual mats consist of single or multiple genotypes. Additionally, we wondered whether polar *Zygnema* strains form a monophyletic clade or are closely related to non-polar strains. We searched for sexual reproduction and zygospore formation to identify species, and for vegetative morphological features, that could potentially be used to distinguish individual genotypes.

Materials and methods

Origin and cultivation of strains

Hydro-terrestrial algal mats were sampled at 18 different locations in Central Spitzbergen (1–17) and close to Ny Ålesund (18; Svalbard archipelago; Fig. 1). Sampling sites were shallow pools or slow running streams supplied by permafrost or snow meltwater.

At most locations, several independent mats (39 in total) covering a surface area ranging from several square decimetres to several square metres were sampled. In addition, during 2015 sampling, temperature, pH and conductivity were measured using a portable meter WTW pH/Cond 340i. The natural samples were transported to the department of Botany, Charles University, using a cooling box. From each mat several individual filaments were isolated and cultured (Tab. 1). To cover the largest possible area, other available *Zygnema* strains from Svalbard were also included, namely, strains 353-10, 354-10, 355-10, 356-10, 357-10 and 358-10 from the CCCryo culture collection isolated in Hornsund (19) by Thomas Leya in 2010. Additionally, three Antarctic *Zygnema* strains were also investigated, namely CCCryo 280-06, CCCryo 279-06 and the strain MB1 (courtesy of Miloš Barták). All cultures were grown in liquid BBM medium (Bischoff & Bold, 1963) and maintained at 15°C with continuous irradiance of 35 μmol m^{-2} s^{-1}. In addition, we tried to induce conjugation by slow desiccation on agar plates, nitrogen starvation and cultivation in a crossed gradient of light and temperature.

DNA isolation, PCR and phylogenetic analyses

DNA was isolated from vegetative filaments in log-phase of growth according to the protocol of Ryšánek *et al.* (2015), but with 50 μl of Instagene Matrix instead of 100 μl (Bio-Rad Laboratories, USA). Primers RH1 and 1385R (McCourt *et al.*, 2000), and newly designed primers ZygF (5'TATGTCAACCACAAAC3') and ZygR (5'GTATCAAATTCAAATTTA3'), were used for amplification of the *rbc*L gene in reactions containing 13.9 μl of sterile Milli-Q water, 2 μl of $MgCl_2$ (25 mM), 2 μl of AmpliTaq Gold 360 Buffer (Applied Biosystems, Carlsbad, CA, USA), 0.4 μl of dNTP mix (10 mM), 0.25 μl of forward and reverse primer (25 pmol ml^{-1}), 0.2 μl of AmpliTaq Gold 360 DNA Polymerase and 1 μl of DNA (10 ng μl^{-1}). Cycling was performed with an initial denaturation for 10 min at 95°C, followed by 35 amplification cycles of 1 min denaturation at 94°C, 1 min annealing at 48°C, and 2.5 min extension at 72°C, and a final extension at 72°C for 10 min. Purification of PCR products and DNA sequencing were performed by Macrogen Inc. (Seoul, South Korea). The obtained unique sequences were assigned capital letters A, M, N, O, P, R, S, U and V, and submitted to GenBank under accession numbers MG818336 to MG818344. Capital letters were assigned to individual genotypes based on our recent publications on polar *Zygnema* spp. (Kaplan *et al.*, 2013; Pichrtová *et al.*, 2013, 2014 *b*).

A 1290 nucleotide alignment of 63 sequences (Supplementary Material 1) was created using nine of the obtained sequences, as well as our previously published Arctic and Antarctic *Zygnema* spp. and *Zygnemopsis* sp. sequences (Pichrtová *et al.*, 2013, 2014 *b*), other unique *rbc*L *Zygnema* and *Zygnemopsis* sequences available in the GenBank database, and several closely related Zygnematophyceae sequences (based on BLAST searches and Hall *et al.* 2008). The alignment also included Arctic *Zygnema* strain CCAC 1384B (ASW 07067), which was isolated from Möllerhafen in 1992 and sequenced by Gontcharov *et al.* (2004). This strain is included in the map and the list of sampling sites (no. 20; Fig. 1; Tab. 1). A list of all strains used in the final phylogenetic tree and their GenBank accession numbers can be found in the Supplementary Material 2.

Three different phylogenetic analyses were performed: Bayesian inference (BI), maximum likelihood (ML) and weighted parsimony (wMP, character-weighted). Sequence

evolution models were determined as GTR+gamma for the first codon position, JC+I for the second position and GTR+I+gamma for the third position using MrModel Test 2.3 (Nylander, 2004) with the Akaike Information Criterion. The BI phylogenetic tree was constructed using MrBayes 3.2.6 (Ronquist & Huelsenbeck, 2003). Two parallel Markov chain Monte Carlo runs were carried out for 3,000,000 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, and the value was 0.003835. Trees and parameters were sampled every 100 generations, and trees from the initial 1000 generations were discarded using the sumt burnin function. Bootstrap analysis was performed by ML in Garli 2.0 (Zwickl, 2006) and PAUP∗ Portable version 4.0b 10 (Swofford, 2002). ML analyses consisted of rapid heuristic searches (100 pseudoreplicates) 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 (number limited to 10,000 for each replicate). The Bayesian tree was midpoint-rooted and further processed using Mega 6 (Tamura *et al.*, 2013) and Adobe Illustrator CS3 (Adobe Systems, San Jose, CA, USA).

Light microscopy and cell measurements

Three-week-old cultures were used for light microscopy observations using a Zeiss Axiovert 200M microscope equipped with a 63×1.4 NA objective lens (Carl Zeiss Microscopy GmbH, Jena, Germany). Images were captured with an Axiocam MRc5 camera and Zeiss Axiovision software. Sixty cells for each genotype were randomly chosen for width measurements in ImageJ 1.50i software (http://imagej.nih.gov/ij). Where possible, two or three strains per genotype were used, in which case 30 cells per strain were measured (20 respectively).

Levene's test for homogeneity of variance confirmed that variation in cell width among genotypes was not homogenous ($F = 22.70333$, $p < 0.0001$). Therefore, differences in cell width among individual genotypes were tested by non-parametric Kruskal-Wallis tests and subsequent Mann-Whitney pairwise comparisons with Bonferroni correction using PAST 2.17c (Hammer *et al.*, 2001).

Conjugation and zygospore formation were recorded in a natural sample. The sample was stored in the habitat water diluted with distilled water 50:50 and incubated under optimal growth conditions, 17°C and continuous light. Morphological variability during conjugation and all zygospore developmental stages were continuously monitored and photodocumented using an Olympus BX51 light microscope (Nomarski differential contrast, phase contrast) with Olympus Camedia C-5060Z digital microphotographic equipment (Olympus, Tokyo, Japan). Portions of samples were fixed with 1.5% glutaraldehyde for later determination, and portions of mature samples were treated with 10% KOH to distinguish spore wall layers (Stancheva *et al.*, 2012). After completion of zygospore germination, the species was determined using the traditional literature (Transeau, 1951; Randhawa, 1959; Gauthier-Lièvre, 1965; Kadlubowska, 1984).

Confocal laser scanning microscopy

Chloroplast morphology was investigated using a Leica TCS SP2 laser scanning confocal microscope (Leica Microsystems, Wetzlar, Germany) equipped with an Argon-Krypton laser. Several *Zygnema* filaments were transferred to a drop of distilled water and mounted together with a piece of solidified agar medium to prevent movement during scanning. A 488 nm excitation wavelength and an AOBS filter-free system (Leica Microsystems) collecting emitted light between 498 and 700 nm were used. A Leica DM IRE2 inverted microscope (Leica Microsystems) was used to visualise a series of chloroplast optical sections for three-dimensional (3D) morphology reconstruction. Chlorophyll autofluorescence was exploited for visualisation of chloroplast structure, and chloroplast 3D morphology reconstructions were produced using ImageJ version 1.50 with the Fiji image processing package (Schindelin *et al.*, 2012).

Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) was essentially performed as previously described (Holzinger *et al.*, 2009). The whole fixation and embedding procedure was performed immediately in Svalbard. Freshly harvested samples were fixed in 2.5% glutaraldehyde in 50 mM cacodylate buffer for 2 h, washed in the same buffer, then embedded in 3% agarose. Samples were then post-fixed in 1% OsO_4 at 4°C for 12 h, followed by dehydration using increasing ethanol concentrations, transferred to propylene oxide, embedded in modified Spurr's embedding resin and heat polymerised. Samples were further processed in the laboratory in Innsbruck, and ultrathin sections were prepared with a Leica Ultracut microtome, and counterstained with uranyl acetate and Reynold's lead citrate. Sections were viewed using a Zeiss Libra 120 TEM instrument at 80 kV, and images were captured with a TRS 2k SSCCD camera and further processed with Adobe Photoshop Elements 11 software (Adobe Systems, San Jose, CA, USA).

Results

Molecular diversity and occurrence of Zygnema mats

Chloroplast-encoded *rbc*L sequences were obtained for 143 Arctic strains with vegetative *Zygnema*-like morphology isolated from 39 different mats at 20 different localities on Svalbard (Fig. 1; Tab. 1). Phylogenetic analyses revealed 12 different *Zygnema* genotypes (A, B, G, J, M, N, O, P, R, S, U and V) from the Arctic and one member of the genus *Zygnemopsis* (L; Fig. 2). The identified genotypes are evenly distributed among genotypes of non-polar origin in the two main clades of the genus, and do not form any monophyletic clusters (Fig. 2). Only genotypes G and R, which differ by 5 bp within the investigated region, form a separate well-supported lineage (Fig. 2). Some of the Arctic genotypes are very closely related to others, such as *Zygnema* sp. S that differs only at one site (without altering the translation product) from the Austrian strain *Zygnema* sp. SAG 2418. An Arctic genotype J was even identical to previously described *Z. cylindricum* from the Czech Republic (strain SAG 698-2). The genus *Zygnemopsis* forms a monophyletic clade only distantly related to the genus *Zygnema*, but closely related to another filamentous genus (*Mougeotia*).

Additionally, we also investigated all Antarctic *Zygnema* strains currently available, consisting of three newly sequenced strains (CCCryo 280-06, 279-06 and MB1) and published sequences of strains *Zygnema* sp. C, D and E (Kaplan *et al.*, 2013; Pichrtová *et al.*, 2013). All share an identical *rbc*L sequence, and are closely related to other strains: Their *rbc*L sequence differs with only 1 bp from *Zygnema irregulare* Krieger isolated in California (strain RS012, Stancheva *et al.*, 2012) and with 4 bp from the most common Arctic genotype B. Nevertheless, all these sequences encode an identical translation product.

The frequency of individual genotypes differed; four genotypes were found at a single site only, whereas genotype B was the most common (present in 41 out of 143 strains, and in 15 out of 39 mats; Tab. 1). Notably, the only *rbc*L sequence of Svalbard *Zygnema* obtained from public databases, strain M1384 isolated in Möllerhafen, is virtually identical with our B genotype (*rbc*L sequences differ at only one site, where M1384 has Y instead of T); hence both are referred to as genotype B throughout the paper.

Surprisingly, a certain level of diversity was revealed within individual mats, which were presumed to be formed by a single species. Fourteen out of 39 mats included more than one genotype. One site, mat no. 1 at locality 9 Petuniahytta (Elster & Rachlewicz, 2012) with an area of 1 m^2, was selected for more detailed investigation. We isolated and sequenced 25 *Zygnema* strains from this location revealing four different genotypes (Tab. 1).

Environmental parameters are available for several mats sampled in August 2015. Water temperature did not exceed 12°C in most cases, pH was neutral to alkalic and conductivity ranged between 37 and 880 μS.cm-1 (Supplementary Material 3).

Sexual reproduction and zygospore formation

We never observed formation of asexual spores (aplanospores, akinetes) in this study, sexual reproduction was observed only twice. First, we observed conjugation and formation of zygospores of *Zygnema* in material collected in 2013 at the Garmaksla sampling site (no. 5; Fig. 3, 4). Unfortunately, this sample was small and did not provide sufficient information for species determination, nor were we able to establish a culture from conjugating filaments for DNA analysis. Based on the few images without the final stage of cell wall development, the zygospores best resemble *Z. calosporum* C.-C. Jao. This species was originally described from China, with spherical or almost spherical brown zygospores sized from 29x35 to 32x38 μm and with filament width range 20-26 mm.

In 2015, massive conjugation occurred in a single mat (mat 4) from the Petuniahytta sampling site (no. 9). The water at the sampling site had neutral pH and conductivity 409 $\mu S.cm^{-1}$ (Supplementary Material 3). Field material was collected, and development of zygospores was successfully tracked in laboratory conditions (Fig. 5–9). Scalariform conjugation was observed exclusively, and zygospores were generally quadrangular pillow-shaped, quadrately ovoid, and laterally compressed (Fig. 5, 6), and only rarely globose. An early zygospore was formed in the conjugation tubes, which was fully filled, mostly protruding to both gametangia. The zygospore was already surrounded by a lamellate wall (exospore) after zygospore formation, and this was surrounded by a pectic-cellulosic appendage with a wing-like shape (Fig. 5, asterisk). The original filaments often fell apart soon afterwards. A two-layered mesospore developed as a colourless wall that turned blue-green very quickly (Fig. 6, 7). The inner mesospore layer evolved slowly into a yellow-brown layer

with scrobiculate ornamentation (Fig. 8). The external layer became thinner and undulate, often poorly visible, but still blue (Fig. 8). In the final stage, the blue-coloured layer was generally very thin or completely absent, and zygospores became darker (Fig. 9). Based on these morphological features, the species was identified as *Zygnemopsis lamellata* Randhawa. The original description comprises zygospores with 44-52 μm in diameter, the same pattern of their formation and appearance and vegetative filaments ranging 15-21 μm in width. In transmission electron micrographs, zygospores at different stages of development were observed and characterised from field material that was fixed immediately for TEM analysis at the collection site on Svalbard. The cell lumina of zygospores contained huge quantities of lipids, and chloroplasts occupied only a small area (Fig. 10, 11). Zygospores remained in the central parts of the copulation channel (Fig. 10), and due to the orientation of the sectioning, only one gametangium (original filament) was visible. Gametangia were almost entirely filled by a pectic-cellulosic appendage that covered the exospore (Fig. 10, 12). Within the appendage, the loose arrangement of cellulose fibrils was clearly visible (Fig. 13). The structure of the 'mother cell wall' of gametangia was clearly distinct from these layers (Fig. 14), and zygospores were surrounded by an exospore and two distinct mesospore layers. The outer mesospore layer (Me1, Fig. 14) was electron-translucent and thin, while the inner mesospore layer (Me2) was irregular and electron-dense, indicating differences in chemical composition. The endospore was of intermediate electron density, and composed of several highly symmetrically arranged fibrillary layers (Fig. 14).

Vegetative morphology

The genera *Zygnema* and *Zygnemopsis* share a very similar vegetative morphology, even though they are not closely related. Using light microscopy, they can be easily confused (for *Zygnema* genotypes, see Fig. 15–26; for *Zygnemopsis lamellata*, Fig. 27). However, when the morphology of the chloroplast was observed using confocal laser scanning microscopy, the differences were quite clear (Fig. 28–39 for *Zygnema* spp., Fig. 40 for *Zygnemopsis*). *Zygnemopsis* possesses two compact lobate chloroplasts per cell (Fig. 40), with the nucleus placed between chloroplasts in the middle of the cell. The terminal lobes are wide, flat and rounded in shape, with relatively deep incisions. Chloroplasts occupy most of the cell's interior, and they are porous or spongy inside (not shown), with small, irregular cavities on the surface that give a granular appearance. The flat lobes are often appressed to the cell wall, and this feature seems to be important for distinguishing *Zygnemopsis* from *Zygnema*.

Confocal laser scanning microscopy was also applied to characterise individual genotypes of *Zygnema* based on the morphology of their star-shaped chloroplasts. When observed in exponentially growing cultures, chloroplast shape remained stable. Generally, within the studied *Zygnema* strains, four groups of plastids were recognised: (i) chloroplasts with very delicate and thin lobules as shown for genotypes N (Fig. 30), J (Fig. 34), V (Fig. 36), O (Fig. 37) and M (Fig. 38); (ii) chloroplasts with lobes wider at the base and terminated by a blunt tip as shown for A (Fig. 28), R (Fig. 32) and B (Fig. 39); (iii) chloroplasts with flat, wide terminal lobes as shown for U (Fig. 29) and S (Fig. 31); (iv) chloroplasts with prolonged lamellate lobes as shown for G (Fig. 33) and P (Fig. 35). However, chloroplast shape does not appear to reflect the phylogenetic position of the strains; unrelated genotypes may possess

a similar chloroplast type and, conversely, genotypes from one lineage (e.g., R and G; Fig. 32, 33) can differ considerably in chloroplast morphology.

In addition to chloroplast shape, we also characterised genotypes according to filament width, and mean cell width differed significantly among nearly all genotypes (Kruskal-Wallis test and Mann-Whitney pairwise comparisons with Bonferroni correction; Fig. 41). However, the measured values strongly overlapped among individual genotypes, large differences in cell width variance were observed (Fig. 41) and congruence between filament width and chloroplast morphology was not observed (the results of statistical analyses can be found in Supplementary Material 4).

Discussion

Diversity of sterile 'Zygnema / Zygnemopsis' mats on Svalbard

The diversity of conjugating green algae on Svalbard displaying vegetative *Zygnema* morphology was surprisingly high. Phylogenetic analyses based on the *rbc*L gene revealed 12 different *Zygnema* genotypes and one *Zygnemopsis* genotype among 143 strains with almost uniform vegetative appearance. Until recently, such algae collected from Svalbard were always reported only as *Zygnema* sp. (e.g. Kim *et al.*, 2008, 2011; Holzinger *et al.*, 2009). In this study, we confirmed the occurrence of *Zygnemopsis* (Pichrtová *et al.*, 2014 *b*), and further determined the species as *Zygnemopsis lamellata* by morphological characterisation of a fertile sample observed in the field.

Traditional taxonomy of *Zygnema* and *Zygnemopsis* is based on morphological characteristics connected with sexual reproduction. Consequently, studies describing the diversity of these algae are limited to records of fertile samples (Novis, 2004; Poulíčková *et al.*, 2007; Kim *et al.*, 2012; Stancheva *et al.*, 2012). Novis (2004) reported only one fertile specimen (i.e., one species) of *Zygnema* from New Zealand. Poulíčková *et al.* (2007) reported various zygospores but identified only one species from a sampling site in the Czech Republic. Kim *et al.* (2012) reported two species of *Zygnema* in Korea despite more than 3 years of collecting. Finally, Stancheva *et al.* (2012) reported eight species (including two new to science) from streams in California, one of which was assigned as *Z. sterile* Transeau based on akinetes, because the reproduction of this species is unknown (Transeau 1951). These researchers also reported numerous sterile samples that were not investigated molecularly (Stancheva *et al.*, 2012).

However, a few recent ecophysiological studies indicated that there is diversity hidden within non-reproducing field populations of *Zygnema* or *Zygnemopsis* (Kaplan et al. 2013, Pichrtová et al. 2013, 2014b). In another filamentous conjugating green algal genus, *Spirogyra*, molecular diversity analysis of sterile strains from Germany and Austria revealed 53 different SSU rDNA genotypes within 130 isolated strains, and the genus was split into eight independent lineages (Chen *et al.*, 2012). Similarly, Stancheva et al. (2013) characterized by morphological and molecular methods 15 *Spirogyra* species from streams in California. In general, application of molecular methods in morphologically uniform protists usually reveals hidden diversity on various levels, depending on the selected marker(s) (e.g. Nemjová *et al.*, 2011; Moniz *et al.*, 2012; Heesch *et al.*, 2016).

Despite many recent studies, the taxonomy and phylogeny of many Zygnematophyceae genera have not yet been revised. It has been repeatedly shown that morphology is not congruent with phylogeny (Gontcharov & Melkonian, 2005; Chen *et al.*, 2012; Stancheva et al. 2013). Currently, both *Zygnema* and *Zygnemopsis* genera belong to order Zygnematales and family Zygnemataceae (Guiry, 2013). Gontcharov (2008) mentioned 139 species of *Zygnema*, but 212 species are currently listed as taxonomically accepted in AlgaeBase (www.algaebase.org). Nevertheless, many were observed only once, making it highly probable that their "unique" morphology reflects phenotypic plasticity, and that the number of species has been overestimated. To date, only 13 species in public databases have been assigned based on both morphological determination and *rbc*L sequence analysis (Fig. 2). Moreover, due to the lack of taxonomic revision and/or hybridisation experiments, the number of base pair changes sufficient for species-level discrimination has not been determined. Therefore, we use the term "genotype" instead of "species". The R and G pair are the most closely related Arctic genotypes, and they differ at five sites, while two well-defined species, *Z. cylindricum* and *Z. giganteum* Randhawa, differ at only two sites.

A recent phylogenetic study of *Zygnema* split the genus into two main well-supported clades (Stancheva *et al.*, 2012), which corresponded to the zygospore colour in *Zygnema*, i. e. blue vs. brown. A new understanding of the phylogenetic position of the genus *Zygogonium* was recently proposed based on *atpB*, *psbC* and *rbc*L genes, as well as morphological features (Stancheva *et al.*, 2014). Some of the former *Zygogonium* species were transferred to *Zygnema*, based on combination of chloroplast structure and reproductive features. Thus, our third clade 3 (Fig. 2) contains *Zygnema tunetanum*, species originally described as *Zygogonium tunetanum*. Consistent with this previously published phylogeny, our genotypes were distributed within the two largest clades (Fig. 2). It can be hypothesized that strains A, U, N, S, R and G would produce spores with blue mesospore layer and genotypes J, P, V, O, M and B with yellow or brown (Stancheva et al. 2012).

Zygnemopsis is a small genus with only 43 described species. It is not a sister lineage to *Zygnema*, since they are only distantly related. There are only four public *rbc*L sequences that form a monophyletic clade, including one *Cylindrocystis* strain, which was most likely incorrectly determined using morphological methods alone.

Occurrence of fertile specimens

Although most specimens were sterile, we also observed conjugation and zygospore formation in our field samples, representing the first record of sexual reproduction in *Zygnema* and *Zygnemopsis* from Svalbard. Production of zygospores is energetically demanding, because they develop a three-layered cell wall with a middle layer containing sporopollenin-like material (Poulíčková *et al.*, 2007). Moreover, the short vegetation period and water loss of mats might prevent complete development of zygospores.

Based on the morphology of zygospores and gametangia in field samples found at site 4 in 2015, we assigned our *Zygnemopsis* sp. L sample as *Z. lamellata*. Its phylogenetic position has already been determined in our previous work (Pichrtová *et al.*, 2014 *b*). Zygospores of *Zygnemopsis* were distinguishable from those of *Zygnema* because they have four lamellate and solid, wing-like appendages attached (Transeau, 1951), and the zygospores themselves are quadrangular pillow-shaped, as is the case for *Zygnemopsis lamellata*. We confirmed the presence

of wing-like lamellate appendages surrounding the exospore using light microscopy (Fig. 5–9), as well as by TEM (Fig. 10–14), which clearly showed that these structures are cellulosic-pectic, with a loose fibrillary appearance (Fig. 13). The function of the 'wings' remains unclear, although pectic layers are common in Zygnematophyceae, and usually related to the water holding capacity, suggesting that the lamellate appendages could be beneficial when water availability is scarce during zygospore formation. *Zygnema*/*Zygnemopsis* mats have been frequently found to dry out during the vegetative season (Holzinger *et al.*, 2009; Pichrtová *et al.*, 2014 *a*), which could result in incomplete zygospore development. The appendages may therefore help to overcome this problem in *Zygnemopsis lamellata*.

Our light microscopy observations of spore layers were consistent with the differences in the electron density of the two mesospore layers observed by TEM: while the outer layer (Me1) is electron-translucent and thin (Fig. 14), the inner layer is electron-dense and irregular, hence the scrobiculate appearance. Endospores exhibited a highly organised fibrillary structure of medium electron density (Fig. 14), consistent with the translucent appearance in light microscopy observations.

Unfortunately, *Zygnema* zygospores from a natural sample collected in 2013 could not be determined precisely, but some features of the zygospore wall indicate that it could possibly be *Zygnema* cf. *calosporum*.

The distribution and the ecological characterisation of *Zygnema calosporum* and *Zygnemopsis lamellata* are not sufficiently described. There are some records of *Z. lamellata* from India (Randhawa, 1937) and Norway (Kadlubowska, 1984). *Z. calosporum* is known from China, Algeria (Kadlobowska, 1984) and is also reported from The Netherlands (Simons, 1987).

Biogeographical and ecological patterns in polar microalgae

The 12 Arctic *Zygnema* genotypes investigated in this study cluster into two main clades of the genus (Fig. 2) and are intermixed with, and in some cases closely related to strains isolated from different regions. The difference between the Antarctic strains and the Californian strain of *Z. irregulare* is so small (1 bp) that they could be considered a single species, and similarities in morphology (formation of akinetes) and stress tolerance support this theory (Fuller, 2013; Pichrtová *et al.*, 2014 *b*). Similarly, other protist genera comprise cosmopolitan lineages where even Arctic and Antarctic strains cluster together (Heesch *et al.*, 2016; Hodač *et al.*, 2016; Ryšánek *et al.*, 2016).

This is not surprising, since the ubiquity theory assuming cosmopolitan distribution and unlimited dispersal of protists was proposed as an explanation of the worldwide distribution of protists (Finlay *et al.*, 1996; Finlay, 2002). However, this theory has been tested many times, and numerous examples of protist species with limited distribution are known. Another theory of protist distribution, the moderate endemicity model, was proposed that admits the existence of endemic species (Foissner, 1999, 2006). For example, Antarctic and Arctic endemites are common within diatom species complexes (Souffreau *et al.*, 2013; Kociolek *et al.*, 2017; Pinseel *et al.*, 2017). Antarctic microchlorophytes (De Wever *et al.*, 2009) or *Prasiola* (Moniz *et al.*, 2012) also possess endemic lineages. Typically, different species within a single protist genus may exhibit contrasting distribution patterns (Heesch *et al.*, 2016; Ryšánek *et al.*, 2016). Polar *Zygnema* strains do not form any clear endemic lineage, but we cannot exclude the possibility that some of the reported Arctic genotypes are endemic to the Arctic region.

To support this hypothesis, however, more extensive molecular data from other regions are needed, because even putative endemites can be redetected elsewhere, depending only on sampling effort.

This study was not specifically designed to test ecological differences between regions, but some patterns were nonetheless observed. For example, genotypes U and V were found only in Longyearbyen, and were never observed in Petuniabukta where most samples were taken. The sampling site in Longyearbyen was located high up a mountain beneath a snow field (conductivity 40-60 μS.cm-1; Supplementary Material 3), whereas sites in Petuniabukta were located near the seashore and therefore rich in minerals (Komárek *et al.*, 2012). Also, climatic conditions differed between Petuniabukta and Longyearbyen (Láska *et al.*, 2012). Ecological conditions may therefore play an important role in determining the distribution and abundance of some genotypes. The lineages of another streptophytic algal genus, *Klebsormidium*, also showed ecological preferences (Škaloud & Rindi, 2013). Similarly, individual species of polar Tribonemataceae were defined ecologically rather than biogeographically (Rybalka *et al.*, 2009), and the diversity of *Prasiola* from Svalbard was also correlated with environmental conditions (Richter *et al.*, 2016).

Possible role of seasonal changes in local diversity

Molecular investigations also revealed that 14 out of 39 comparably sized mats were composed of two or more genotypes of *Zygnema* or *Zygnemopsis lamellata* and the mat chosen for a more thorough investigation consisted of four genotypes. The coexistence of different genotypes indicates that they share similar ecological preferences. Similarly, up to three genotypes of *Spirogyra* were detected at a single site (Chen *et al.*, 2012; Stancheva et al. 2013), and sympatric occurrence of cryptic (or pseudocryptic) species has been described for other microalgal assemblages such as diatoms (Vanormelingen *et al.*, 2008) and desmids (Nemjová *et al.*, 2011). Nevertheless, it should be considered that our genotypes may not represent true 'cryptic species', because we could not use all distinguishing features due to the absence of zygospores. Moreover, the co-occurrence of various well-defined species of a single genus at one site is a relatively common phenomenon in Zygnematophyceae (Nováková, 2002; Štěpánková *et al.*, 2012).

Co-occurrence of various genotypes is supported by the annual characteristics of mats, since new biomass rapidly develops every year from a small inoculum, and the time for competition is rather limited (Pichrtová *et al.*, 2016). Interestingly, sites that were investigated repeatedly (sites 10, 11 and 15, and mat 2 at site 9; Fig. 1) retained the same genotypes, suggesting that survival of cells from the previous season, together with their rapid spring growth, is more important for colonisation of the pool than allochthonous transport of new genotypes from other sites. In the absence of zygospores, the role of surviving cells is played by pre-akinetes, which are hardened, old vegetative cells resistant to various environmental stresses (Holzinger *et al.*, 2009; Pichrtová *et al.*, 2014 *a*, 2014 *b*, 2016; Herburger *et al.*, 2015).

Vegetative morphology and indications of polyploidy

The very rare occurrence of zygospores on Svalbard precludes the morphological determination of individual species in field conditions. Moreover, despite the experimental effort, induction of zygospore formation in culture conditions was not successful. Therefore, we decided to test whether our genotypes could be discriminated based solely on their vegetative morphology, even though it is well known that the molecular diversity of filamentous Zygnematophyceae is far greater than indicated by the lack of morphological variation (Chen *et al.*, 2012). Despite being based on generative morphological features, traditional species description of filamentous Zygnematophyceae usually includes information on cell diameter, and particularly chloroplast features including shape, length of protrusions, globularity and compressed appearance (Kadlubowska, 1984; Stancheva *et al.*, 2012, 2013).

To study chloroplast shape, we applied confocal laser scanning microscopy because this method generates 3D chloroplast images based on autofluorescence without disturbing signals from other cellular structures. This method was previously applied in other green algae, e.g. in *Trebouxia* and *Jenufa*, and differences in chloroplast ontogeny (changes during cell growth and autosporogenesis) were described (Škaloud & Radochová, 2004; Škaloud & Peksa, 2008; Němcová *et al.*, 2011). We compared the plastid shape of young vegetative cells in fresh exponentially growing cultures (3 weeks after inoculation) for all genotypes, and this was stable in cells grown under controlled laboratory conditions. Vegetative filaments of *Zygnemopsis* are usually indistinguishable from those of *Zygnema* using light microscopy, and the two genera can be separated only by features observable during sexual reproduction (Transeau, 1951). However, based on 3D chloroplast morphology, these two genera were clearly discernible; chloroplasts of *Zygnemopsis lamellata* are porous, and include wide, thick lobes with deep incisions, some of which are appressed to the cell wall, whereas *Zygnema* chloroplasts are not deeply dissected, and where wide lobes are formed they are flat and do not contact the cell wall. In addition, chloroplasts of *Zygnemopsis* have a more granular appearance. However, it should be noted, that the chloroplasts in different species of *Zygnemopsis* are variable in shape, which may complicate the separation of *Zygnema* and *Zygnemopsis* based on vegetative morphology (Transeau, 1951).

In the investigated *Zygnema* genotypes, differences in chloroplast shapes were apparent. We did not record any phylogenetic signal in chloroplast shape, and assume that chloroplasts of similar shape could have arisen repeatedly during evolution. Chloroplast shape in *Zygnema* is influenced by various factors. For example, during maturation and pre-akinete formation, chloroplasts lose their complex shape with lobes and become smaller (Fuller, 2013; Pichrtová *et al.*, 2014 *b*; Herburger *et al.*, 2015). Thus, all our strains were investigated during exponential growth to avoid chloroplast shape changes caused by life cycle differences or suboptimal conditions. Although chloroplast shape itself is not sufficient for species determination, it may serve as an additional morphological feature upon which to characterise and define species identity. It provides better discriminatory power at genus level to distinguish vegetative filaments of *Zygnema* and *Zygogonium* (Stancheva et al. 2014) for example.

In addition, we observed significant differences in cell width among individual genotypes, but they do not reflect phylogeny as previously suggested (Herburger *et al.*, 2015). Moreover, the extent of variation in cell width was very high in some genotypes such as *Zygnema cylindricum* (genotype J), which indicates that cell width may not be a reliable feature

for discrimination. Cellular diameter is influenced by additional factors such as culture age (Herburger *et al.*, 2015) and environmental conditions (Miller & Hoshaw, 1974; Stancheva *et al.*, 2012). Doubt was first cast on filament width as a taxonomic character of *Zygnema* by Miller & Hoshaw (1974), who suggested that this feature could be influenced by polyploidy. Polyploidy within the genus *Zygnema* has been proposed several times (Miller & Hoshaw, 1974; McCourt *et al.*, 1986), and our cell width data may also support this possibility, but it has not yet been proven experimentally. Polyploidy therefore remains an open question with important implications for taxonomy because the existence of species complexes complicates species delimitation. For example, the number of *Spirogyra* species is widely regarded as exaggerated, because different ploidal levels within complexes can interbreed (Wang *et al.*, 1986).

Conclusions

In this study, we identified 12 different *Zygnema* genotypes from sampling sites in Svalbard, representing unexpectedly high genetic diversity within putative *Zygnema* mats. Surprisingly, a degree of diversity was also revealed within individual mats that were believed to be formed by a single species. Although the mats appeared uniform, slight gradients in environmental conditions could create microhabitats preferred by distinct genotypes with different biochemical and physiological adaptations. High genetic differentiation may also contribute to the ecological success of *Zygnema* in environments where unpredictable changes might occur. The investigated genotypes were evenly distributed among genotypes of non-polar origin, and did not form any monophyletic polar clusters.

Although most specimen were sterile, we observed conjugation and zygospore formation, representing the first record of sexual reproduction in *Zygnema* and *Zygnemopsis* from Svalbard. Although conjugation is believed to be extremely rare in polar regions, it seems that at least some species benefit from investment in zygospore production. Although molecular methods are clearly essential for studying diversity in sterile vegetative filaments, our 3D reconstruction of chloroplast morphology using confocal laser scanning microscopy provided sufficient information to distinguish *Zygnema* and *Zygnemopsis* genera. However, chloroplast shape did not appear to reflect the phylogenetic position of *Zygnema* strains. We therefore assume that chloroplasts of a similar shape could have arisen repeatedly during the course of evolution.

In the extreme habitats of Svalbard, *Zygnema* mats contribute substantially to primary production. Knowledge on their species composition, life cycles and survival strategies (including spore formation) may help us to understand their importance at the ecosystem level. Moreover, any structural and functional knowledge could help to resolve the diversity and evolution of Zygnematophyceae, the most species-rich charophyte lineage. Furthermore, this knowledge will improve the use of these algae as model organisms in experimental studies, and facilitate comparisons while reducing the risk of misinterpretation.

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Table 1. Complete list of sampling localities and number of cultures isolated from each mat and genotype

Locality				Genotype												
No. on map	**description**	**mat**	**GPS** (if available)	**B**	**G**	**S**	**L**	**R**	**J**	**A**	**O**	**M**	**V**	**U**	**P**	**N**
1	Longyearbyen	1	78°13.143'N; 15°35.28'E	-	-	-	-	-	-	-	-	-	-	7	-	-
		2	78°13.153'N; 15°35.088'E	-	-	-	-	-	-	-	-	-	4	-	-	-
		3		-	-	2	-	-	-	-	-	-	-	-	-	1
2	Björndalen	1	78°13.585'N; 15°19.393'E	-	-	2	-	-	-	-	-	-	-	-	-	-
		2	78°13.071'N; 15°19.664'E	1	-	-	-	-	-	-	1	-	-	-	-	-
		3	78°12.957'N; 15°20.003'E	-	-	2	-	-	-	-	-	-	-	-	-	-
3	Colesdalen	1		-	-	-	-	-	-	-	2	-	-	-	-	-
4	Skansbukta	1		1	-	-	-	-	-	-	-	-	-	-	-	-
5	Garmaksla	1		3	-	-	-	-	-	-	-	-	-	-	-	-
6	Pyramiden	1		4	-	-	-	-	-	-	-	-	-	-	-	-
		2		2	-	-	-	-	-	-	-	-	-	-	-	-
		3		-	-	-	1	-	-	-	-	-	-	-	-	-
		4		-	-	-	-	-	-	3	-	-	-	-	-	-

		5		1	-	1	-	-	1	-	-	-	-	-	-	-
7	Bertilbreen moraine	1		-	-	-	2	-	-	-	-	-	-	-	-	-
8	path to Pyramiden	1	78°39.96'N; 16°25.6333'E	-	1	-	-	-	-	-	-	-	-	-	2	-
9	Petuniahytta	1	78°40.842'N; 16°27.487'E	13	-	8	3	1	-	-	-	-	-	-	-	-
		2	78°40.84'N; 16°27.512'E	-	1	-	1	-	-	6	-	-	-	-	-	-
		3	78°40.838'N; 16°27.523'E	-	1	1	-	-	-	-	-	-	-	-	-	-
		4		3	-	-	3	-	-	-	-	-	-	-	-	-
		5		-	1	-	-	-	-	4	-	-	-	-	-	-
		6		2	-	-	-	-	-	-	-	-	-	-	-	-
10	old path in Petuniabukta	1	78°41.35'N; 16°26.818'E	-	5	-	-	-	-	-	-	-	-	-	-	-
		2		1	-	-	-	-	-	-	-	-	-	-	-	-
11	automatic weather station	1	78°42.11'N; 16°27.64'E	4	-	-	-	-	-	-	-	-	-	-	-	-
12	open top chambers	1		-	2	-	-	-	-	-	-	-	-	-	-	-
		2		-	-	-	-	4	-	-	-	-	-	-	-	-
13	Svenbreen moraine	1		1	-	-	-	1	-	-	-	-	-	-	-	-
14	Hørbyebreen moraine	1		-	2	-	-	-	-	-	-	-	-	-	-	-
15	Fortet	1		-	3	-	-	-	-	-	-	-	-	-	-	-
16	Brucebyen	1		-	-	-	-	5	1	-	-	-	-	-	-	-

17	Mathiesondalen	1	-	1	-	1	-	-	-	-	-	-	-	-	-
		2	-	-	-	-	-	-	-	-	2	-	-	-	-
		3	3	-	-	-	-	1	-	-	-	-	-	-	-
		4	-	1	-	2	-	-	-	-	1	-	-	-	-
		5	-	-	-	-	-	2	-	-	-	-	-	-	-
18	Blomstrand island	1 78°57.816'N; 12°2.964'E	1	-	-	-	-	-	-	-	-	-	-	-	-
19	Hornsund[a]	1	-	-	6	-	-	-	-	-	-	-	-	-	-
20	Möllerhafen[b]	1	1	-	-	-	-	-	-	-	-	-	-	-	-
	Number of mats where the genotype was present		**15**	**10**	**7**	**7**	**4**	**4**	**3**	**2**	**2**	**1**	**1**	**1**	**1**
	Total number of isolated strains per genotype		41	18	22	13	11	5	13	3	3	4	7	2	1

[a] Cultures obtained from the CCCryo collection
[b] Strain sequenced by Gontcharov et al. (2004)

Figures

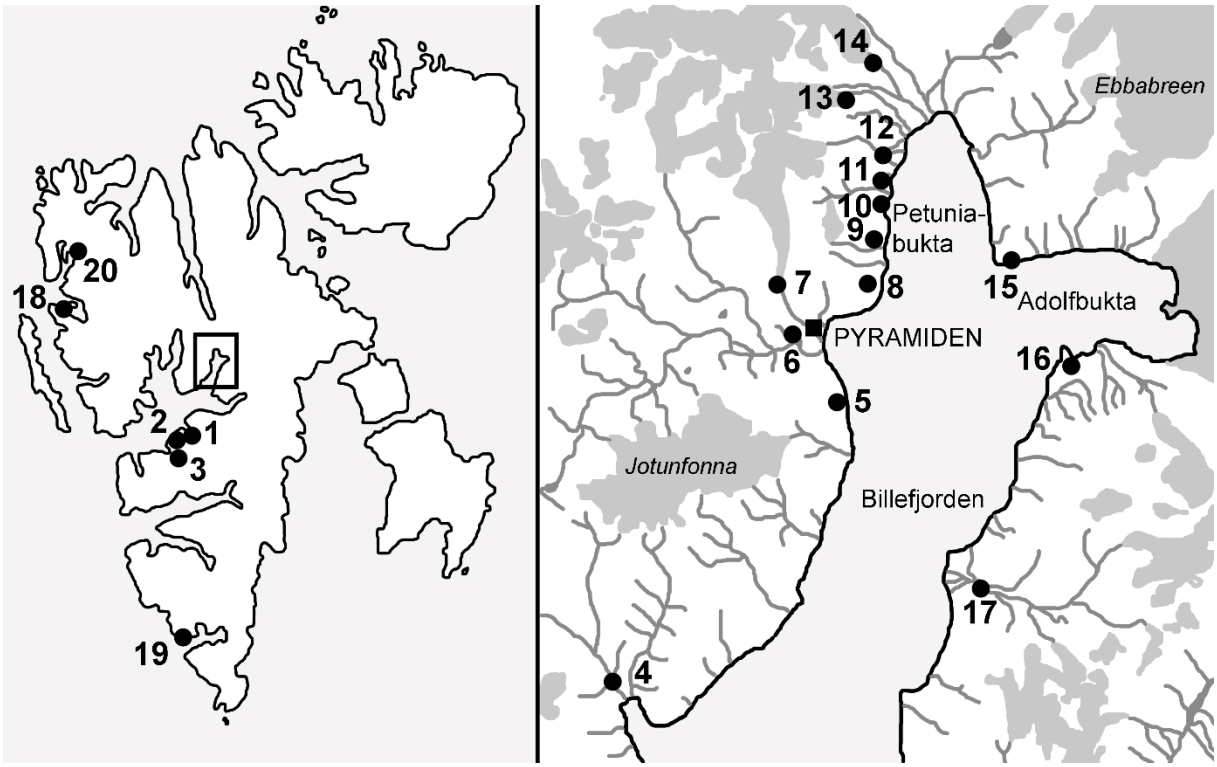

Fig. 1. Map showing the locations of original sampling sites of strains investigated in this study. Numbers correspond with Table 1. Dark grey areas indicate glaciers.

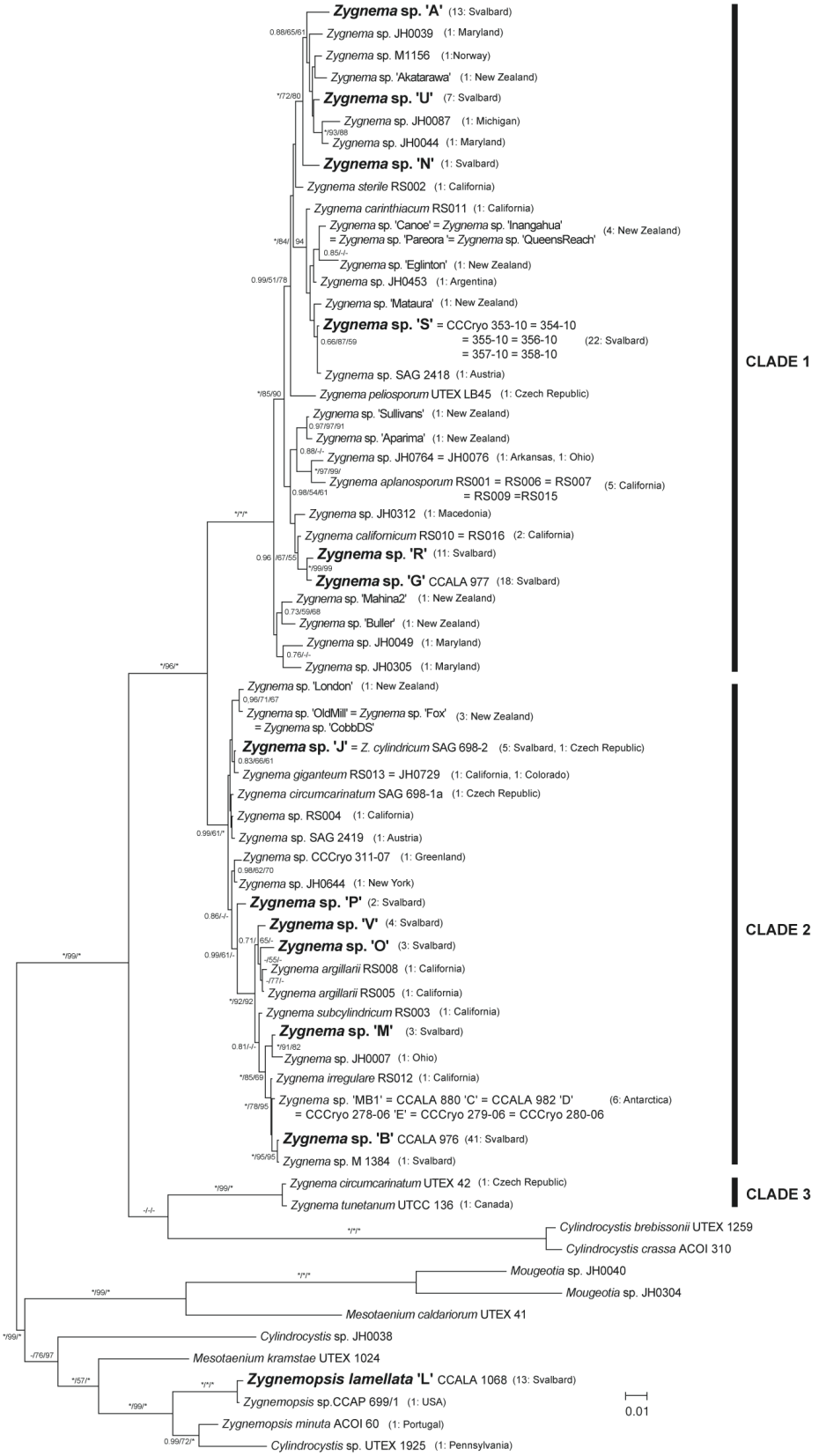

Fig. 2. Phylogenetic tree of genera *Zygnema, Zygnemopsis*, and other closely related Zygnematophyceae. A midpoint-rooted Bayesian tree of *rbcL* sequences is shown. Genotypes from Svalbard are in bold. Values at branches indicate Bayesian posterior probabilities (BI PP), maximum likelihood (ML) and 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. The number of strains isolated for each Zygnema/Zygnemopsis genotype and their geographical origin are given in brackets.

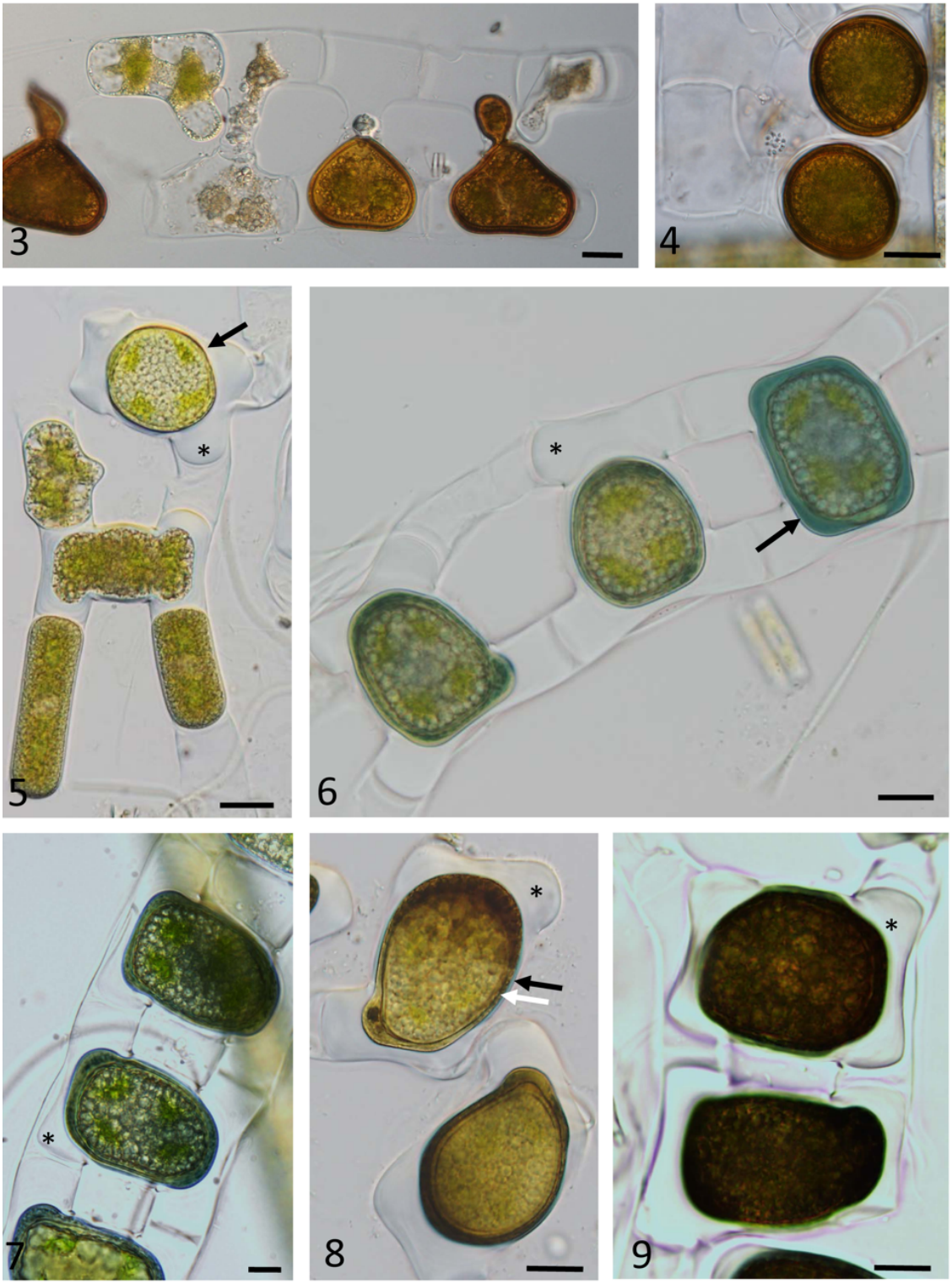

Fig. 3–9. Light microscopy images of zygospores of *Zygnema* cf. *calosporum* (Fig. 3, 4) and *Zygnemopsis lamellata* (Fig. 5–9) collected in Svalbard. Fig. 3, conjugating filaments, with partially developed zygospores; Fig. 4, mature zygospores; Fig. 5, conjugating stage (arrow) and fully fused zygospore (arrow) with massive appendages (asterisk); Fig. 6, zygospores with blue mesospore (black arrow) at different stages of development; Fig. 7, zygospores still in conjugating filaments, positioned in the middle, with appendages (asterisk); Fig. 8, zygospores with a two-layered mesospore clearly visible, comprising an outer layer in blue (black arrow), and an inner layer scrobiculate (white arrow), with appendages marked by an asterisk; Fig. 9, dark appearance of fully developed zygospores, showing appendages of the exospore (asterisk); Scale bars: Figs 3–9, 20 μm.

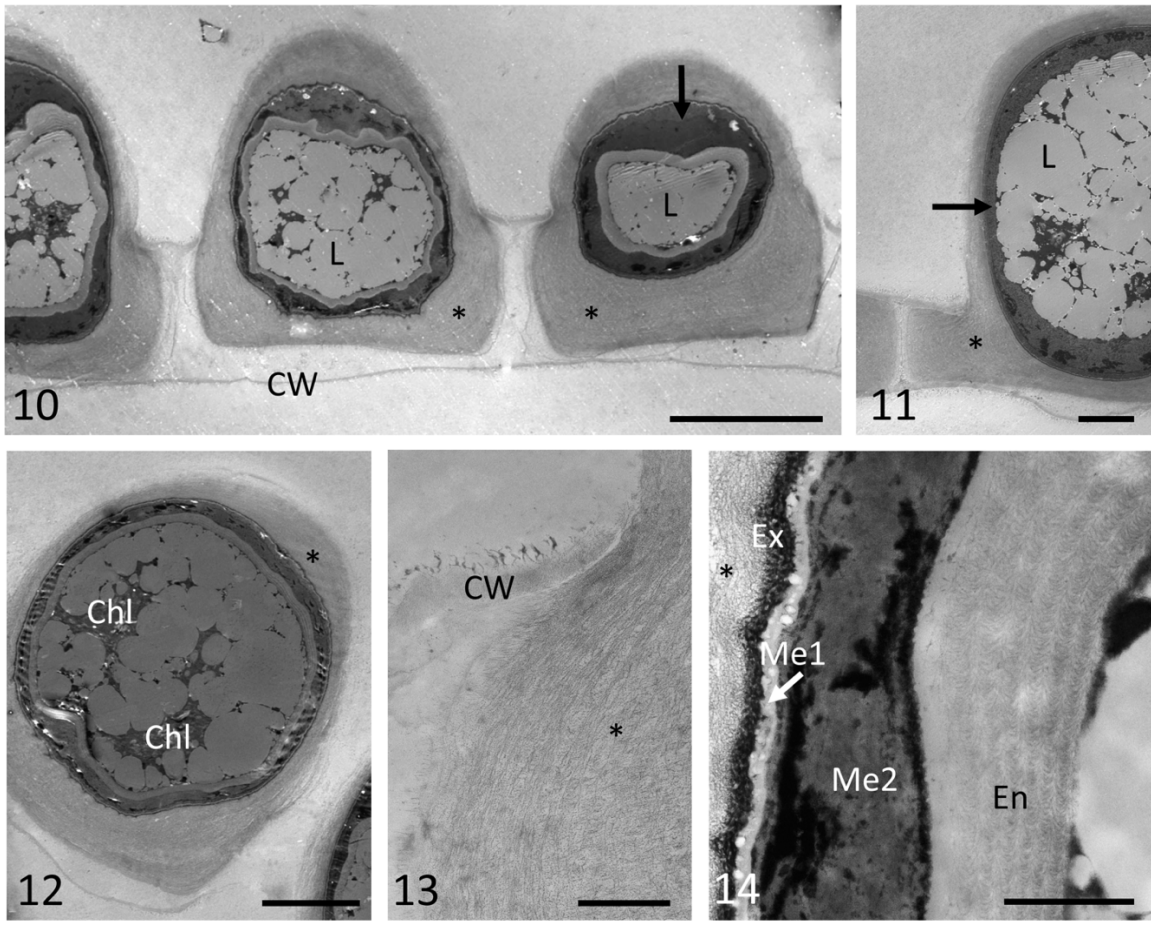

Fig. 10–14. Transmission electron micrographs of zygospores of *Zygnemopsis lamellata* collected in the field. Fig. 10, several zygospores still in the original filaments, showing an electron-dense inner mesospore layer (black arrow), and exospore surrounded by pectic-cellulosic appendages ('wings') marked with an asterisk; Fig. 11, electron-dense mesospore layer (arrow), with lipid bodies inside the zygospores, and appendages marked by an asterisk; Fig. 12, chloroplasts in zygospores; Fig. 13, pectic-cellulosic appendage within the gametangium; Fig. 14, exospore (Ex), two-layered mesospore (Me1 = electron-translucent, Me2 = electron-dense and irregular) and highly sculptured endospore (En). Abbreviations are Chl = chloroplast, CW = cell wall of the mother cell, Ex = exospore, L = lipids, Me1 = outer mesospore layer, Me2 = inner mesospore layer, En = endospore. Scale bars: Fig. 1, 20 μm; Figs 11-12, 10 μm; Fig. 13, 2 μm; Fig. 14, 1 μm

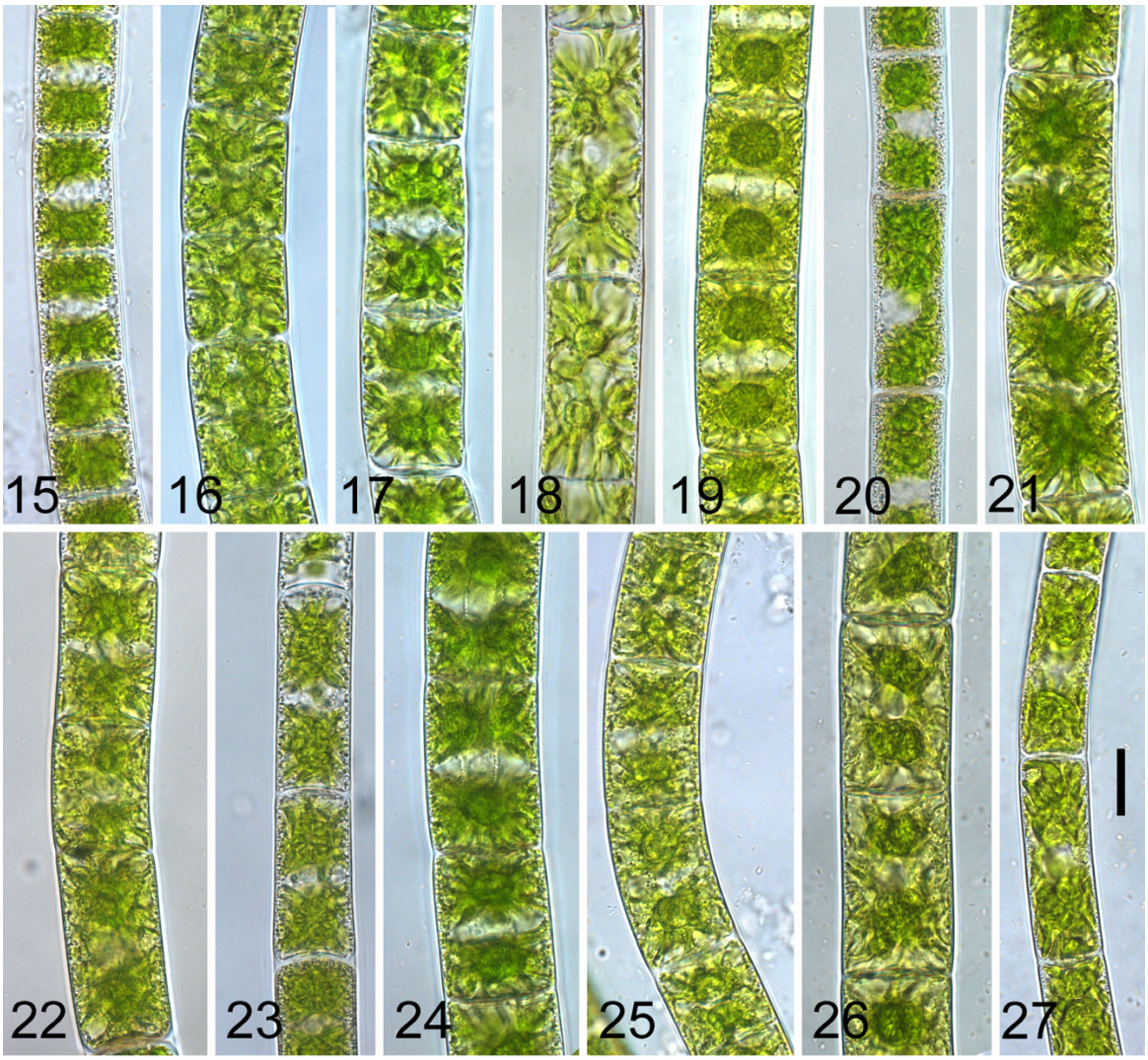

Fig. 15–27. Young vegetative cells (3 weeks after transfer to fresh medium) of the investigated genotypes. Fig. 15, genotype A; Fig. 16, U; Fig. 17, N; Fig. 18, S; Fig. 19, R; Fig. 20, G; Fig. 21, J; Fig. 22, P; Fig. 23, V; Fig. 24, O; Fig. 25, M; Fig. 26, B; Fig. 27, L. Scale bar = 20 μm in all images. Genotypes are ordered according to phylogeny described in Fig. 2.

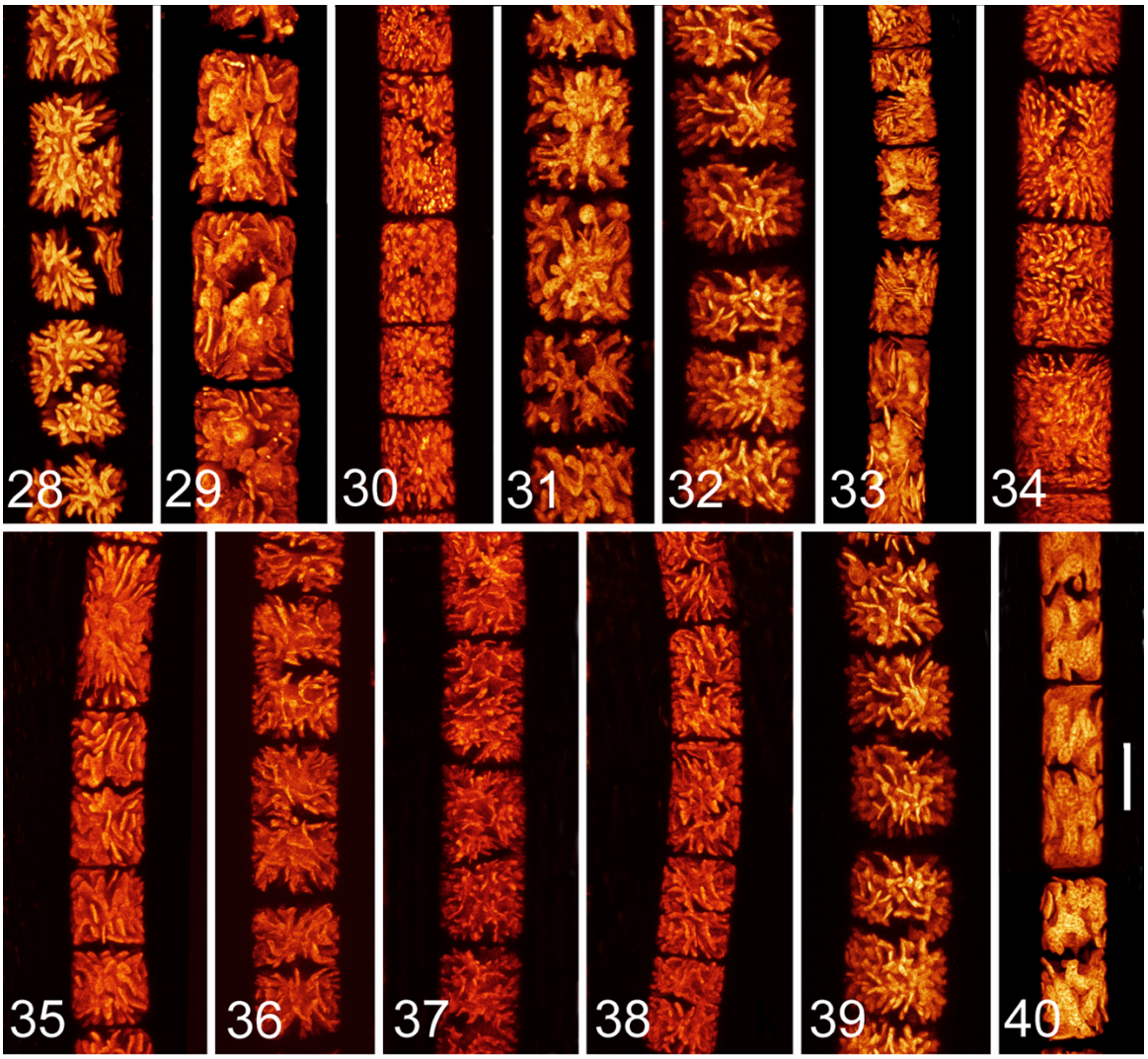

Fig. 28–40. Confocal laser scanning microscopy of young vegetative cells of the investigated genotypes. Fig. 28, genotype A; Fig. 29, U; Fig. 30, N; Fig. 31, S; Fig. 32, R; Fig. 33, G; Fig. 34, J; Fig. 35, P; Fig. 36, V; Fig. 37, O; Fig. 38, M; Fig. 39, B; Fig. 40, L. Scale bar: 20 μm in all images. Genotypes are ordered according to phylogeny described in Fig. 2.

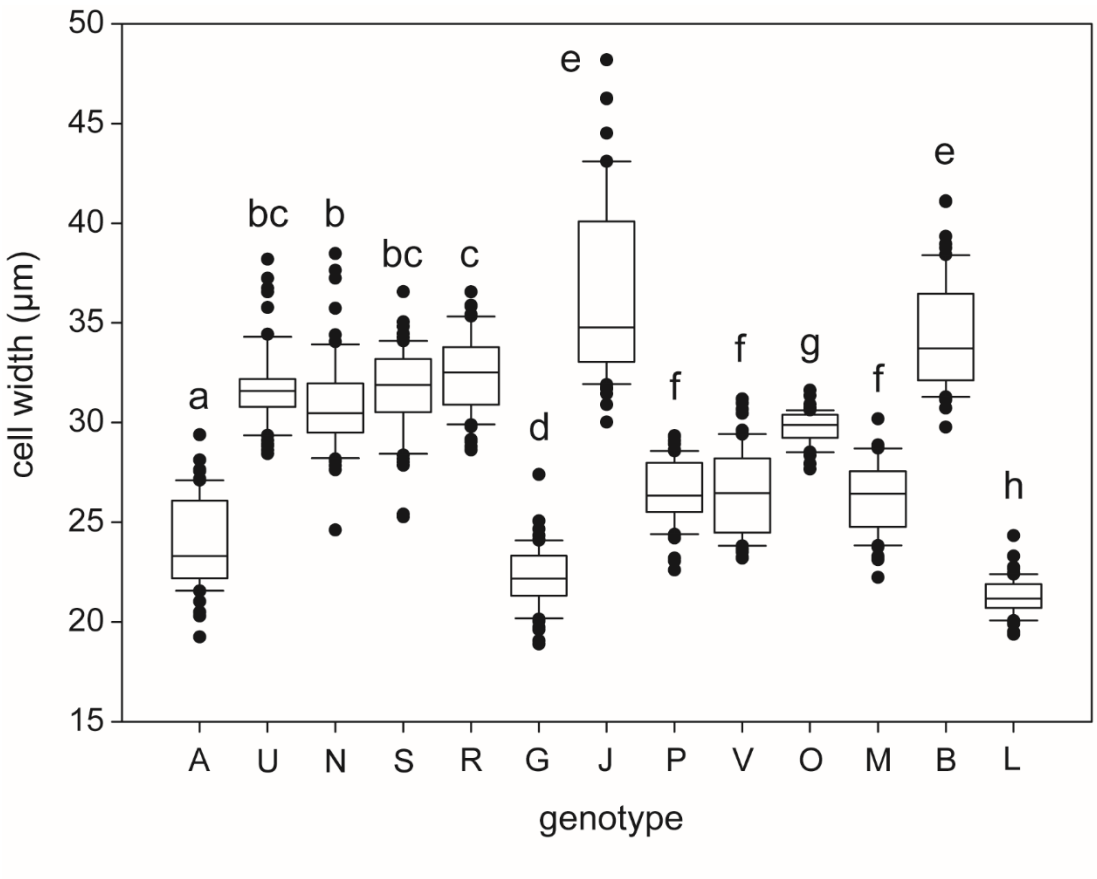

Fig. 41. Cell width of all investigated genotypes (n = 60). The line within the box marks the median, boundaries indicate the 25th and 75th percentiles, error bars indicate the 10th and 90th percentiles, and individual points denote outliers (samples with values outside this range). Genotypes that do not significantly differ from each other share at least one letter, while those significantly different from each other do not share any letters ($p < 0.05$; Kruskal-Wallis test with multiple comparisons).

Paper II

Arctic, Antarctic, and temperate green algae *Zygnema* spp. under UV-B stress: vegetative cells perform better than pre-akinetes.

Protoplasma

Arctic, Antarctic, and temperate green algae *Zygnema* spp. under UV-B stress: vegetative cells perform better than pre-akinetes

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Abstract

Zygnema spp. forms macroscopically visible mats in polar and temperate terrestrial habitats where they are exposed to environmental stresses. We investigated three previously characterized isolates (Arctic *Zygnema* sp. B, Antarctic *Zygnema* sp. C and temperate *Zygnema* sp. S) for their tolerance to experimental UV radiation. We exposed samples of young vegetative cells (one month old) and pre-akinetes (6 months old) to photosynthetically active radiation (PAR, 400 – 700 nm, 400 μmol photons m^{-2} s^{-1}) in combination with experimental UV-A (315-400 nm, 5.7 W m^{-2}, no UV-B), designated as PA or UV-A (10.1 W m^{-2})+UV-B (280-315 nm, 1.0 W m^{-2}) designated as PAB. The experimental period lasted for 74 h, the radiation period was 16 h PAR/UV-A per day, or with additional UV-B for 14 h per day. The effective quantum yield, generally lower in pre-akinetes, was mostly reduced during the UV treatment, and recovery was better in young vegetative cells vs. pre-akinetes during the experiment. Analysis of the primary pigment composition revealed a statistically significant ($p<0.05$) increase of the deepoxidation state of the xanthophyll cycle pigments in *Zygnema* sp. C and S. UV absorbing phenolic compounds were significantly higher ($p<0.05$) in young vegetative cells when compared to pre-akinetes and showed a tendency to increase after PA and PAB treatment. In young vegetative *Zygnema* sp. S, a significant increase ($p<0.05$) of these phenolic compounds upon PA and PAB was found. Transmission electron microscopy showed an intact ultrastructure with massive starch accumulations at the pyrenoids. A tentative increase in electron dense bodies in PAB treated cells and the occurrence of cubic membranes in the chloroplasts are likely protection strategies. Metabolite profiling by non-targeted RP-UHPLC-qToF-MS allowed a clear separation of the investigated strains, but could not detect changes due to the PA and PAB treatments. 617 distinct molecular masses were detected,

from which around 200 could be annotated from databases. It can be concluded, that particular young vegetative cells can adapt well to the experimental UV-B stress.

Key words: UV-A, UV-B, UV simulation, green algae, ultrastructure, metabolomics

Introduction

The effects of UV radiation on green algae have been studied extensively (reviewed by e.g. Holzinger and Lütz 2006, Karsten and Holzinger 2014, Holzinger and Pichrtová 2016), which was mainly due to the detection of stratospheric ozone-holes over the polar regions, which will result in increased UV-B radiation. This could lead to destructive effects on chloroplasts and DNA, which in turn would influence algal development and distribution. Different avoidance and protection mechanisms have been described, particularly in groups that live in terrestrial habitats.

UV shielding and protecting substances came in focus of research, which are largely varying in different groups of green algae. In Zygnematophyceaen green algae, unusual phenolic compounds with UV absorbing capacities have been found in *Spirogyra* sp. or *Zygnema* sp. (e.g. Nishizawa et al. 1985, Cannel et al. 1988, Pichrtová et al. 2013). These phenolic substances may even absorb in the visible waveband, like the red vacuolar pigment in *Zygogonium ericetorum* that turned out to be glycosylated derivative of gallic acid, complexed with ferric iron (e.g. Aigner et al. 2013, Herburger et al. 2016). In the ice-algae *Ancylonema nordenskiöldii* (Remias et al. 2012a) and *Mesotaenium berggrenii*, purple to brown visible and UV absorbing compounds were found, the latter characterized as purpurogallin derived secondary pigment (Remias et al. 2012b). Several, particularly chlorophytic green algae contain different UV-absorbing compounds, as mycosporine-like amino acids (MAAs; e.g. Karsten et al. 2007, Hartmann et al. 2016). They were also found in basal streptophytic green algae where they had slightly different absorption spectra with a peak at 324 nm (Kitzing et al. 2014). Other chlorophytes are protected by secondary carotenoids, pigments of the astaxanthin family, giving them a red appearance (e.g. Remias et al. 2005). Zygnematophyceae possess neither MAAs nor secondary carotenoids, therefore we focused our investigations on phenolic compounds.

Several studies have been undertaken to investigate the effects of UV-radiation in Zygnematophycean green algae (e.g. Meindl and Lütz 1996, Lütz et al. 1997, Holzinger et al. 2009, Germ et al. 2009, Pichrtová et al. 2013, Stamenkovic and Hanelt 2014, Prieto-Amador et al. 2016, Stamenkovic and Hanelt 2017). In a previous study the changes in phenolic compounds in three *Zygnema* spp. derived either from arctic or Antarctic habitats were investigated (Pichrtová et al. 2013). The species were *Zygnema* sp. B (also included in the present study), *Zygnema* sp. G and *Zygnema* sp. E, which showed a significant increase in total phenolic compounds (Pichrtová et al. 2013). For the present study, we selected the Antractic *Zygnema* sp. C, which is identical to the previously investigated *Zygnema* sp. E by means of *rbc*L analysis (Pichrtová et al. 2014). According to Stancheva et al. (2012), the genus *Zygnema* is divided into two major clades. The here investigated strains all belong to the same clade, where *Zygnema* sp. B and C are closely related to *Z. irregulare* (Pichrtová et al. 2014) and *Zygnema* sp. *S* to *Z. circumcarinatum* (Herburger et al. 2015). All investigated strains were previously characterized concerning their physiological and ultrastructural parameters (Kaplan et al. 2013, Pichrtová

et al. 2013, 2014; Herburger et al. 2015). In *Zygnema* sp. S, hyperspectral characterization was preformed that allowed to acquire a total absorption spectrum in the range of 400-900 nm (Holzinger et al. 2016).

The possibilities in UV simulation under experimental conditions are limited. In cultured *Zygnema* spp. we used previously a UV simulation that was described as a predominantly UV A treatment (Pichrtová et al. 2013). Therefore, the 'sun simulation system' at the Helmholtz Center in Munich is used that allows to create a realistic PAR to UV scenario (Remias et al. 2010, Hartmann et al. 2015). Hartmann et al. (2015) exposed the chlorophyte green algae *Pseudomuriella engadiensis* and *Coelastrella terrestris* in the same sun simulation device used in the present study; by exposing the cells to 13.4 Wm^{-2} UV-A and UV-B up to 2.8 Wm^{-2}, they found an enhancement of some primary metabolites, mainly aromatic amino acids, nucleic bases and nucleosids (Hartmann et al. 2015). In a study by Remias et al. (2010) applying this sun simulator, the chlorophytic snow alga *Chlamydomonas nivalis* and a terrestrial alga from a polar habitat were investigated by relatively high PAR of 724 μmol photons m^{-2} s^{-1} that was combined with UV-A values of 15.9 Wm^{-2} and UV-B values of up to 1.43 Wm^{-2} (Remias et al. 2010). A study on different strains of the desmid *Cosmarium* used 700 μmol photons $m^{-2}s^{-1}$ in combination with 27.5 Wm^{-2} UV-A or 28.7 Wm^{-2} UV-A and 0.89 Wm^{-2} UV-B (Stamenkovic and Hanelt 2014). Arctic *Zygnema* sp. were even exposed to gamma radiation (Choi et al. 2015), which resulted in drastic changes of photosynthesis related proteins; however, the potential for repair was shown by upregulation of proteins related to DNA repair, quinone oxigoreductase, cytoskeleton and cell wall biogenesis (Choi et al. 2015).

The aim of the present study was to expose *Zygnema* spp. of (A) different culture age, i.e. young vegetative cells and mature pre-akinetes to realistic simulated UV scenario in a sun simulation chamber. We hypothesize that older pre-akinetes could tolerate UV stress better. This hypothesis was mainly driven by the observations that pre-akinetes showed a generally better stress tolerance e.g. to desiccation stress (e.g. Pichrtová et al. 2014) or freezing during winter (Pichrtová et al. 2016). A recent transcriptomic study in *Zygnema cricumcarinatum* (*Zygnema* sp. S) upon desiccation stress revealed that about 1,200 transcripts were up- or down regulated in young vegetative cells, while in pre-akinetes only 400 transcripts were regulated (Rippin et al. 2017). This was attributed to a hardening process, making less regulation necessary. The comparison between young vegetative cells and pre-akinetes concerning UV-tolerance was never studied in an experimental approach before, as previously either field collected samples of pre-akinete stage (Holzinger et al. 2009) or young cultured material of *Zygnema* sp. (Pichrtová et al. 2013, Prieto-Amador 2016) were investigated.

Moreover, in the present study *Zygnema* spp. from (B) different geographic origin like the arctic (*Zygnema* sp. B), Antarctic (*Zygnema* sp. C) and a temperate isolate (*Zygnema* sp. S) will be investigated. As the polar strains are exposed to different UV scenarios in their natural habitat, we hypothesize that they might show differences in tolerating the experimental UV exposure. The significance of different geographic distribution in the UV tolerance has been investigated in different *Cosmarium* sp. strains (Stamenkovic and Hanelt 2014). Untreated and UV exposed samples will be investigated for changes in primary pigments and phenolic compounds and a metabolomics approach will be applied, to describe if there are differences between the individual strains, the culture age and the UV exposures. The structural changes are investigated by light- and transmission electron microscopy.

Material and Methods

Algal strains

For the present study, three different *Zygnema* sp. strains with different geographical origin were used. A strain *Zygnema* sp. S (Culture collection Göttingen, SAG 2419, previously isolated from a sandbank of the river Saalach, Salzburg, Austria, at about 440 m a.s.l., Herburger et al. 2015), an arctic isolate from Svalbard *Zygnema* sp. B (Culture Collection of Autotrophic Organisms in Trebon, Czech Republic CCALA, www.butbn.cas.cz/ccala/index.php; isolated on Svalbard in 2010, accession number CCALA 976), and the Antarctic isolate *Zygnema* sp. C (CCALA 880), previously isolated from James Ross Island. The algae were cultivated on Bold's Basal Medium (BBM) solidified with 1.5% agar. The cultures were maintained either at continuous illumination or a light-dark cycle of 16:8 h at 15 °C at ~38 µmol photons m^{-2} s^{-1}. For the experiments either young cultures (1 month) or 6 months old cultures, consisting of well-developed pre-akinetes were used (Pichrtová et al. 2014).

Experimental UV simulation

For the UV treatments, the algae were placed in the sun simulator at the Helmholtz Center Munich to study the algae's response under a simulated natural photophysiolgical environment. In the sun simulator, a combination of four lamp types (metal halide lamps: Osram Powerstar HQI-TS 400W/D, quartz halogen lamps: Osram Haloline 500W, blue fluorescent tubes: Philips TL-D 36W/BLUE, and UV-B fluorescent tubes: Philips TL 40W/12) was used to obtain a natural balance of simulated global radiation throughout the UV to infrared spectrum. The short-wave cut-off was achieved by selected soda-lime and acrylic glass filters. A detailed description of the sun simulator facility is given by Döhring et al. (1996) and Thiel et al. (1996). The experimental period lasted for 74 h, the radiation period lasted for 16 h per day with 400 µmol m^{-2} s^{-1} PAR (400-700 nm) plus UV-A (315-400nm) – this mimics that natural situation where PAR is always combined with UV-A (designated as: PA); UV-B radiation (280-315 nm) was added 1 h after start of illumination and switched off 1 h before the dark phase leading to a total UV-B exposure of 14 h per day (designated as: PAB); The samples were harvested on the 4th day, 2 h after the onset of the UV-B exposure. The intensities of UV-A and UV-B radiation are shown in Table 1. The spectral composition during the experimental procedure is illustrated in Suppl. Fig. S1.

Chlorophyll Fluorescence

Effective quantum yield (ϕ_{PSII}) measurements were performed with a PAM 2500 (Walz, Germany) on PA and PAB exposed cells during the experiment 2 h after switching on the UV-B lamp as previously described (Pichrtová et al. 2014). For the measurements, the samples were removed from the exposure chamber for the shortest possible time (i.e. up to 5 minutes).

HPLC analysis of primary pigments and phenolics

HPLC analysis of primary pigments and phenolic compounds was performed with untreated samples (harvested prior to the experiment, 0) and with samples harvested

at the end of the PA or PAB exposure. Vegetative and pre-akinete cells of *Zygnema* sp. C and *Zygnema* sp. S were used in 3 replicates each. For *Zygnema* sp. B not enough biomass was available to perform these analyses.

Freeze-dried material was ground with glass beads using a laboratory mill (Tissuelyser II, Qiagen, Venlo, the Netherlands) at 30 Hz for 10 min, and extracted as described by Aigner et al. (2013) with minor modifications. The powder was suspended in 1 ml methyl-tertbutylether (MTBE, SigmaAldrich, St. Louis, USA) containing 0.1% butylated hydroxytoluene (BHT, SigmaAldrich, St. Louis, USA) to prevent oxidation of pigments. Then extract was vortexed and sonicated for 15 min at 0°C and the supernatant was removed whereby the ground material was again resuspended in 1.5 ml MTBE to guarantee quantitative extraction. Both MTBE extracts were combined and then 2 ml of 20% methanol (v/v; Roth, Karlsruhe, Germany) was added to the material, shaken at 4°C before the samples were frozen overnight at -20°C. This extract was then centrifuged (1000 g, 5 min) at 4°C to support phase separation of the lipophilic supernatant (MTBE-phase) and the hydrophilic lower (methanol) phase. The upper and the lower phases were separated, evaporated to dryness in a SpeedVac (SPD111V, Thermo Fisher Scientific, Waltham, USA), and then re-suspended in 350 µl N,N-dimethylformamide (DMF, Scharlau, Sentmenat, Spain; depending on the species) and 350 µl 50% methanol (v/v; HPLC grade, Roth, Karlsruhe, Germany), respectively. The extracts were centrifuged (15 000 g, 45 min, 4°C) prior to injection into the HPLC.

Primary pigments were quantitatively analysed according to Remias et al. (2005) with minor modifications, on an Agilent Technologies 1100 system (Waldbronn, FRG), with a DAD-detector set at 440 nm for carotenoids and 662 nm for chlorophyll *a*. The column was a LiChroCART (C18, 100 × 4.6 mm, 5 µm, 120 A) column (Agilent, Waldbronn, FRG) at a flow rate of 1 ml min^{-1} using solvent A (acetonitril:methanol = 74:6) and solvent B (methanol:hexane= 5:1). The system was started at 0% solvent B for 4 min, followed by a gradient to 100% solvent B from 4 to 9 min, which was maintained for 9 min, followed by a 5 min post-run with 100% solvent A. All solvents were of HPLC gradient grade quality. Pigment calibration and quantification was undertaken for ß-carotene and zeaxanthin with standards from Carbon 14 Centralen, Hørsholm, DK, while chlorophyll *a* was obtained from Sigma-Aldrich. All experimental manipulations were carried out at dim light and low temperatures. The presence of phenolic pigments were analysed from the hydrophilic phase on the same system and separated using a Phenomenex Synergi Polar-RP column (150 x 3.0 mm, 4 µm, 80 A; Aschaffenburg, Germany) protected with a RP-18 guard cartridge (20 x 4 mm I.D.) of the same material was used at 25 °C with a flow rate of 0.3 mL min^{-1} and an injection volume of 25 µL.

Mobile phases: A: water + 0.5 % formic acid (v/v); B: methanol + 0.5 % formic acid (v/v). The binary linear solvent gradient was as follows: start 0 % B; 40 min: 100 % B; followed a 8 min post run with 100 % A. Whole absorbance spectra were recorded each second and DAD detection wavelengths were at 280 nm and 350 nm, respectively, after Aigner et al. (2013).

Metabolic profiling of Zygnema strains

Samples of vegetative and pre-akinete cells of *Zygnema* sp. B, *Zygnema* sp. C and *Zygnema* sp. S were taken before and after UV treatment in triplicates. Algal material was transferred

into NucleoSpin® Bead Tubes (Macherey-Nagel, Germany) and evaporated until dryness to calculate the dry weight. Cells were extracted with 500 µL 70% methanol (Chromasolv™, Sigma-Aldrich, Germany) in 30% purified water (v/v) using a Precellys® Homogenizer (Bertin Technologies, France) at around 4 °C and 5.000 rpm (3 times á 20 s). After centrifugation for 15 min at 4 °C and 14.000 rpm, supernatants were removed and stored at -80 °C for further analysis.

Metabolic analysis were performed using reversed phase ultrahigh performance liquid chromatography (UHPLC; Waters Acquity) coupled to a time-of-flight mass spectrometer (qToF-MS; Bruker Daltonik maXis) within positive ionization mode. The maXis qToF-MS provides a resolution of >50.000 at m/z 400 and a mass accuracy <2 ppm. All chemicals used were of LC-MS grade (Chromasolv™) provided by Sigma-Aldrich, Germany.

Mobile phases containing A: purified water with 0.1% formic acid (v/v) and B: acetonitrile with 0.1% formic acid (v/v) were applied for chromatographic separation on a Waters Acquity BEH C_{18} column (dimensions 100 mm x 2.1 mm ID, 1.7 µm particle size) at 40 °C. A 10 minutes gradient was processed from 0 to 1.12 min 0.5 % B, followed by a continuous increase of B until 99.5 % at 6.41 min and a stable highly non-polar plateau of 99.5% B until 10.01 min. Equilibration of stationary phase was ensured by a pre-run time set to 2 minutes with 0.5% B. Samples were stored at 4 °C during the measurements. 5 µL of each sample extract were injected at a flow rate of 0.4 mL min^{-1}. Mass spectra were acquired within a mass range of 100 – 1500 m/z at 2.0 Hz scan rate (for additional parameters see Suppl. Table S1).

Data were processed with Genedata Expressionist V10.5 (Genedata AG, Switzerland). To ensure quality of the spectra and reliability of measurements over time, a certified standard (ESI-L Low Concentration Tuning Mix, Agilent Technologies, Germany) was injected in the mass spectrometer at the beginning of each run. The resulting peak in each total ion chromatogram (TIC) was used to create a verified chromatogram grid over all data and the resulting exact masses were used for calibration of MS spectra. After blank subtraction remaining sample peaks were integrated and isotopic clusters were assigned automatically. Masses only present in one sample were not taken into account. Therefore, 617 molecular masses were determined within the sample set, which were further analysed statistically for their response to the UV treatments of the 3 investigated *Zygnema* strains.

Light- and transmission electron microscopy

Light microscopy was performed on 2.5% glutaraldehyde fixed cells (see below) at an Olympus BX5 microscope equipped with Olympus DP72 camera and QuickPhoto Camera 2.3 software. For transmission electron microscopy, specimens of *Zygnema* sp. B, *Zygnema* sp. C and *Zygnema* sp. S exposed to PA or PAB were fixed with a standard chemical fixation protocol according to Holzinger et al. (2009) with modifications. Briefly, cells were fixed in 2.5% glutaraldehyde at room temperature for 1.5 h, rinsed and postfixed in 1% OsO_4 at 4°C overnight, both fixatives were dissolved in 20 mM caccodylate buffer, pH 7. After dehydration in increasing ethanol steps, cells were embedded in modified Spurr's resin and heat polymerized. Ultrathin sections were counterstained with uranyl acetate and Reynold's lead citrate, and investigated in Zeiss LIBRA 120 transmission electron

microscopes at 80 kV. Images were captured with a TRS 2k SSCCD camera and further processed using Adobe Photoshop software (Adobe Systems Inc., San José, California, USA).

Statistical evaluation of the data

The evaluation of the data of the phenolic concentrations as well as the deepoxidation state was performed by a three-way ANOVA analysis, with three factors "strain", "UV treatment" and "culture age" considered as factors with fixed effects. Differences between individual UV treatments were tested by one-way ANOVA analyses followed by Tukey's post-hoc tests, separately for each strain and culture age. Relative values of the effective quantum yield corresponding with the recovery rate of the initial values measured at the end of the experiment were also tested by three-way ANOVA and additional two-way ANOVA analyses were performed for individual strains separately. For all analyses the significance value was set as $p<0.05$. The analyses were performed in Statistica 10 for Windows and PAST (Hammer et al. 2001). Summary of all results of statistical analyses can be found in Suppl. Table S2.

Statistical evaluation of metabolomics data was performed using Genedata Expressionist V10.5 (Genedata AG, Switzerland). Data were first normalized to the sample dry weight and categorized according to *Zygnema* strains, UV treatment, culture age and biological replicate. Applied N-Way ANOVA analyses including the factors strain type, culture age and UV treatment did not give significance values of $p<0.06$. Anyway, principal component analyses (PCAs) of covariances were performed based on relative contents, i.e. the peak area of a single peak in relation to the summed peaks in the spectra. Metabolite alignment was done using an adapted version of the MassTRIX webserver (Suhre and Schmitt-Kopplin 2008). The maximum error for annotated masses was set to 0.005 Da and the possible appearance of sodium and formic acid adducts was taken into account.

Results

Changes of effective quantum yield

The effective quantum yield (ϕ_{PSII}) was determined over the whole course of the 74 h experiment, measurements were taken 2 h after turning on the UV-B exposure. Relative changes compared to untreated samples prior to the experiment were observed (Fig. 1). The mean initial absolute values of ϕ_{PSII} were as follows: *Zygnema* sp. B – young vegetative cells: 0.55±0.012, pre-akinetes: 0.47±0.012; *Zygnema* sp. C – young vegetative cells: 0.61±0.02, pre-akinetes: 0.3±0.03, *Zygnema* sp. S – young vegetative cells: 0.7±0.012, pre-akinetes: 0.66±0.019. These values were set to 100 %. In all strains and most treatments, an initial depression of the effective quantum yield was observed (Fig. 1). In *Zygnema* sp. B, the initial value recovered during the experiment in young vegetative cells after both PA and PAB treatments (Fig. 1 a). In contrast, pre-akinete cells of *Zygnema* sp. B decreased to a much lower value (~ 60-70% of the initial value) and then remained stable throughout the experiment. In vegetative cells of *Zygnema* sp. C a similar tendency was found whereas the effective quantum yield of pre-akinetes did not recover during the 74 h duration of the experiment (Fig. 1 b). Finally, in *Zygnema* sp. S, the pre-akinetes reached 60-70% of their initial quantum yield on day 4, slightly higher values were measured for young vegetative cells (Fig. 1 c).

The recovery rate after 74 h was significantly higher in vegetative cells than in pre-akinetes ($p < 0.0001$, Suppl. Table S2; Fig. 1). UV treatment was not significant when analyzed by three-way ANOVA, showing that there is no general pattern in the effect of individual UV treatments on the recovery of the effective quantum yield. This is also supported by a significant interaction of strain and UV treatment ($p = 0.0014$, Suppl. Table S2) proving that the response differed among strains. Therefore, subsequent two-way ANOVA analyses were performed for each strain separately. In *Zygnema* sp. C PA treatments had significantly better recovery than PAB ($p = 0.0319$, Suppl. Table S2). In contrast, *Zygnema* sp. S showed better recovery in PAB treated samples ($p = 0.0058$, Suppl. Table S2).

Photosynthetic pigments and xanthophyll cycle pigments change upon UV treatment

From the total analysis of the primary pigments (Suppl. Fig. S2), we used the xanthophyll cycle pigments violaxanthin (V), zeaxanthin (Z) and antheraxanthin (A) (Suppl. Fig. S3) to determine the deepoxidation state (DEPS) = (A+Z)/(V+A+Z) of *Zygnema* sp. C and *Zygnema* sp. S. The effects of all tested factors and their interactions were proven significant when tested by three-way ANOVA indicating that the deepoxidation state of the cultures is influenced by UV treatment, but the response is different for each strain and culture age, too. In addition, we were able to determine significant differences between the untreated samples and the samples exposed to PA and PAB in all cases except for pre-akinetes of *Zygnema* sp. C (Fig. 2, Suppl. Table S2). However, no significant differences were found between the two different UV treatments, despite the mean values were higher in the PAB treatments in most cases (Fig. 2).

UV-absorbing phenolic compounds increase as a consequence of UV treatment

Both effects of culture age and UV treatment on the content of phenolics were shown to be significant when tested by three-way ANOVA (Table S2, Fig. 3). Both strains share the same pattern of the response to UV: In general, content of UV-absorbing compounds was higher in vegetative cells than in pre-akinetes ($p < 0.0001$) and there is a tendency of elevated mean values of phenolic content after PA and PAB treatment, however these changes were not statistically significant in *Zygnema* sp. C when analyzed separately by one-way ANOVA. In *Zygnema* sp. S phenolics increased significantly after PA and PAB treatment in vegetative cells and after PAB treatment in pre-akinetes when compared to untreated samples (Table S2, Fig. 3). This indicated that particularly in *Zygnema* sp. S, PA and PAB induced changes of UV-absorbing phenolic compounds, with retention times (RT) of 15.4 min, 24.8 min and 26.1 min (Suppl. Fig. S4). These peaks, while having their absorption maxima around 280 nm were also absorbing in the UV-A range. From further analysis all other phenolic substances (20 compounds) were excluded, which had only a single absorption maximum at 280 nm (e.g. the peak at RT 23.4 min, shown in Suppl. Fig. S4). These compounds are probably pre-cursors or intermediates but contribute only little in the biologically relevant waveband.

Light microscopy shows difference between vegetative cells and pre-akinetes

UV treatment had no visible effect on cellular morphology observed under light microscope (Fig. 4). Young cells of all investigated strains were highly vacuolated,

their chloroplasts had numerous lobes protruding towards the cell periphery and large nuclei were well visible in the central part of the cells (Fig. 4 a, b, e, f, i, j). Cytoplasm of the pre-akinetes appeared denser and contained numerous lipid bodies, chloroplast lobes were no more clearly discernible (Fig. 4 c, d, g, h, k, l).

Transmission electron microscopy shows only moderate changes upon addition of UV-B

In young vegetative cells of *Zygnema* sp. B, large accumulations of starch were found under PA exposure, indicating an active metabolism (Suppl. Fig. S5 a); the cells show a high degree of vacuolization and narrow chloroplast lobes (Fig. 5 a). Under PAB exposure an enhanced occurrence of electron dense bodies in the cell periphery was observed (Fig. 5 b; Suppl. Fig. S5 b). The cells still contained large starch accumulations at the pyrenoids (Fig. 5 c). Pre-akinetes of *Zygnema* sp. B contained large accumulations of lipid bodies, particularly in the cell periphery (Fig. 5 d), the occurrence of electron dense bodies was found in PA treated cells (Fig. 5 d, Suppl. Fig. S6 a) but slightly enhanced in PAB treated cells (Suppl. Fig. S6 b).

In *Zygnema* sp. C electron dense bodies were found in vegetative cells under PA treatment (Fig. 6 a), and were sometimes massive under PAB treatment (Fig. 6 b). This massive accumulation of electron dense bodies was not observed in all cells, but a general tendency of increasing occurrence of these structures under PAB treatment, when compared to PA in young cells of *Zygnema* sp. C was obvious (Suppl. Fig. S5 c-d). Pre-akinetes of *Zygnema* sp. C showed an accumulation of lipid bodies, starch grains, and electron dense bodies were abundant particularly in PAB treated cells (Fig. 6 c). The comparison between PA and PAB treated pre-akinetes however shows, that electron dense bodies are found in both (Suppl. Fig S6 c, d).

Zygnema sp. S had massive starch accumulations around the pyrenoids in young vegetative cells exposed to PA and PAB (Fig. 7 a,b). Around the nucleus dense accumulations of ER were observed in PA and PAB treated vegetative *Zygnema* sp. S cells (Fig. 7 a,b). The high degree of vacuolization of these vegetative cells is illustrated in Fig. 7 b and Suppl. Fig. S5 e. The occurrence of electron dense bodies was found in both, PA and PAB treated cells (Suppl. Fig. S5 e,f). Electron dense bodies were found in pre-akinete cells of PAB treated cells (Fig. 7 c) but they were also observed in PA treated cells (Suppl. Fig. S6 e). These cells contained numerous starch grains and lipid bodies (Fig. 7 c). The pyrenoids were surrounded by starch grains, and the arrangement of thylakoid membranes had a wrinkled appearance (Fig. 7 d).

Metabolomic analysis

The UHPLC-qToF-MS analyses revealed a total of 617 molecular masses in the whole set of differentially treated *Zygnema* strains. Masses were statistically evaluated for correlations according to UV treatments, culture ages and strain types. N-Way ANOVA analyses with significance values of $p<0.06$ defined the data set as non-significant but indicated a connection of applied factors. PCAs were performed to reproduce this indicated trend of metabolomics data. The results depicted no distinctions, when all samples were compared. Hence, data were divided in subsets of single *Zygnema* strains and vegetative cells and pre-akinetes, respectively. Correlations thus obtained gave again no separation of various UV

treatments, but showed clear trend of *Zygnema* strains of vegetative cells or pre-akinetes (Fig. 8 a-b).

384 molecular masses, being responsible for the separation of *Zygnema* sp. in PCAs, were extracted and aligned with chemical databases, i.e. Kyoto Encyclopedia of Genes and Genomes (KEGG), Human Metabolome Database (HMDB), LipidMaps, MetaCyc, KNApSAcK and PubChem, which yielded around 200 assigned features. Most of these metabolites were classified as alkaloids, steroids, terpenoids, pyrroles and phospholipids. Fig. 8 a depicted the number of metabolites within selected chemical classes related to *Zygnema* sp. B, C and S, respectively.

Metabolite compositions in vegetative cells of *Zygnema* sp. B and C were very similar, whereas fewer amounts of metabolites from selected chemical classes were detected in *Zygnema* sp. S (Fig. 8 a). Compared with pre-akinetes (Fig. 8 b) high amounts of phospholipid species were found in vegetative cells. The *Zygnema* sp. S pre-akinetes separated from the arctic and Antarctic strains according to higher amounts of alkaloids, polyketides and pyrroles, which indicated ongoing metabolite production in pre-akinetes.

Discussion

The present study investigates the effects of realistically simulated photosynthetic active radiation (PAR 400 μmol photons $m^{-2}s^{-1}$) in combination with UV-A (PA) or an enhanced UV-B (PAB) scenario in three Zygnema strains from different geographic regions (arctic, Antarctic and temperate), which was not performed so far. From each strain young vegetative cultures and pre-akinetes were investigated. Three-way ANAOVA analysis revealed significant differences for the effect of culture age in all physiological parameters tested. Due to their active metabolism, young cells could adjust to the experimental conditions much better by an increased production of protective substances. The effect of strain was significant in analysis of effective quantum yield (ϕ_{PSII}) and deepoxidation state (DEPS) of xanthophyll cycle pigments. Additionally, the metabolomics approach allowed a clear separation between the investigated strains, when young and vegetative cells were analyzed separately, however could not detect effects of the UV treatments.

Photophysiology suggests good adaptation to experimental UV simulation

Young vegetative cells of all investigated strains recovered their initial values of the effective quantum yield (ϕ_{PSII}) much better than pre-akinete cells during the course of the experiment. In *Zygnema* sp. C. the initial values of ϕ_{PSII} recovered significantly better in PA treated cells; this effect was reversed in *Zygnema* sp. S where the PAB treated cells showed a better performance. A similar observation was made by Stamenkovic and Hanelt (2014), where an ameliorating effect of UVB at 21 °C was observed in the tropical *Cosmarium beatum*, concluded from higher rates of recovery of maximum quantum yield after moderate UVB treatment. It has to be emphasized, that we compared the initial values of samples that were taken directly from the standard culture conditions (0 under low PAR of approx. ~38 μmol photons m^{-2} s^{-1}), with the sun simulator incubated samples that were exposed to PA or PAB, both at PAR of 400 μmol photons $m^{-2}s^{-1}$. We can conclude that the applied UV treatments did

not drastically change the photophysiological properties of PS II, indicating a still active physiological performance.

In contrast, negative effects on the F_V/F_M as well as ϕ_{PSII} were detected upon short-term treatment (6 h) with 1.4 Wm^{-2} UV-B in young cultures of an Antarctic *Zygnema* sp. isolate (Prieto-Amador 2016). The observations by Pichrtová et al. (2013), also showed a significant decrease of F_V/F_M, at least in two strains after experimental UV exposure suggesting that an initial effect on the photosynthetic apparatus cannot be denied.

In vegetative cells of both, the Antarctic *Zygnema* sp. C as well as the temperate *Zygnema* sp. S a statistically significant elevation of the deepoxidation state of the xanthophyll cycle pigments was found under PA and PAB exposure when compared to untreated controls (0 samples). There was however, no significant difference between PA and PAB, suggesting that the addition of UV-B was not driving the change. This corroborates earlier findings in *Zygnema* sp., where the UV treatment did not provoke an increase in the deepoxidation state of the xanthophyll cycle pigments in *Zygnema* sp. E and sp. G, while an increase in the deepoxidation state was found in *Zygnema* sp. B (Pichrtová et al. 2013). *Zygnema* sp. G showed a significantly higher deepoxidation state when compared to the other two investigated strains, *Zygnema* sp. B and sp. E (Pichrtová et al. 2013). Recently, the xanthophyll cycle turnover was perturbed in an arctic *Zygnema* sp. by the use of dithiotreitol (DTT), an inhibitor of the violaxanthin deepoxidation (Kakkou et al. 2016). This resulted in a slight increase in chlorophyll fluorescence in the time interval 0 to 0.2 s (J and I chlorophyll fluorescence levels), indicating the importance of the natural rapid conversion of violaxantin into zeaxanthin. In *Cosmarium* sp. xanthophyll cycle pigments were corresponding to that of high-light adapted plants and algae (Stamenkovic et al. 2014a). There was however an exception of an arctic isolate (*Cosmarium crenatum* var. *boldtianum*) that showed an incomplete violaxanthin cycle leading to the accumulation of antheraxanthin during high light stress (Stamenkovic et al. 2014a). In the present study we could also observe reduced values of DEPS in pre-akinetes of the Antarctic strain *Zygnema* C when compared to young cells or the temperate strain. This goes nicely along with the drastically reduced ϕ_{PSII} acclimation capacities (~ 20%-40% of the initial value) in pre-akinetes of *Zygnema* C.

Changes in phenolic compounds

Changes in UV-AB absorbing phenolic compounds as a consequence of UV treatments were found in both investigated strains, but only in *Zygnema* sp. S the effect of UV was significant. This is in good accordance with previous findings, where at predominantly UV-A treatment an increase of similar phenolic compounds was observed in arctic and Antarctic strains of *Zygnema* (Pichrtová et al. 2013).

The HPLC method used in the present study was slightly different form the previously used one (Pichrtová et al. 2013), however, all the major phenolic peaks were found, with similar absorption characteristics. According to the spectral characteristics, for analysis of phenolic compounds, we considered only peaks with absorption in the UV-A and UV-B range. In young cells of temperate *Zygnema* sp. S, a significant increase in UV absorbing phenolic compounds was observed in the PA and PAB exposed samples, in pre-akinetes only in PAB exposed samples, when compared to untreated samples ($p<0.05$). The significant increase in young cells might be explained by their generally higher metabolic activity. In *Zygnema* sp. C, untreated

young vegetative cells had already high values of phenolic compounds when compared to pre-akinetes, suggesting a constitutive protection mechanism already available under standard culture conditions. The observation that pre-akinetes contained less phenolics when compared to young vegetative material, might be explained by that fact that most of the cell volume is filled by lipids (Pichrtová et al. 2016), while the detected phenolics are water soluble. These observations do not support the hypothesis that pre-akinetes are better protected against UV irradiation. In the here investigated *Zygnema* strains, no visible coloration deriving from phenolic derivatives were observed in the light micrographs. However, a detailed chemical characterization of these compounds in *Zygnema* sp. is still missing.

Metabolomics allowed separation between strains

Metabolic analysis could not detect an influence of the UV treatments on *Zygnema* sp. strains. The results confirmed that substantial peculiarities of vegetative cells and pre-akinetes dominate metabolic differentiation. A detailed view on the metabolites detected in vegetative cells and pre-akinetes, respectively, showed a distinct separation of *Zygnema* sp. strains and indicated changes in their activity at both stages of cultivation. Vegetative cells of strains from polar origin (*Zygnema* sp. B and *Zygnema* sp. C) were found to be more similar in their metabolite composition (e.g. alkaloids, terpenoids, steroids, pyrroles and phospholipids) than the temperate strain *Zygnema* sp. S. Several of these metabolite classes were found in *Zygnema* sp. S only in the pre-akinete stage, suggesting that they synthesize these compounds later. This observation is interesting and could possibly point to a geographic attribution, where the temperate strain has more time in season to synthesize certain compounds. These observations, however, have to be investigated in more detail in future studies.

Structural alterations due to UV treatment

The light microscopic observations showed clear differences between young and pre-akinete cells, but no changes could be attributed to the respective UV treatment.

There are indications for stress protection observed by the ultrastructural investigations in the present study, i.e. (1) electron dense bodies in the cytoplasm and (2) cubic membranes in the chloroplast. The most prominent structures that have been attributed to UV protection were the electron dense bodies (Holzinger et al. 2009, Pichrtová et al. 2013). These structures were already described as 'inclusions' in beginning akinetes (McLean and Pessoney 1971), and they have been found in field samples of an arctic strain (Holzinger et al. 2009). It was speculated that these bodies with a diameter of 400-600 nm contain phenolics (Pichrtová et al. 2013). Here we show that they can be found basically in all treatments, but there is a tendency of accumulation of these electron-dense bodies in PAB treated cells, which was illustrated e.g. in *Zygnema* sp. C (Fig. 6 b), where in some cells massive accumulations were found in young cells. This observation would nicely go along with the increase of phenolic compounds in young vegetative cells of *Zygnema* sp. C as detected by the HPLC approach. However, we still cannot provide evidence for the chemical nature of these compartments, only that they are highly reactive with osmium tetroxide leading to the electron dense appearance.

Cubic membranes, as illustrated in *Zygnema* sp. S to occur upon PAB treatment (Fig. 7d) have been reported previously in *Zygnema* (e.g. McLean and Pessoney 1970, Zhan et al. 2017). These cubic membranes are attributed to a stress defense reaction, as they usually occur after high light exposure (Zhan et al. 2017). However, in the studies by McLean and Pessoney (1970) and Zhan et al. (2017) roughly the same light intensities were used. Recently, cubic membranes have been considered as an antioxidant defense system (Deng et al. 2015). They were also observed in the desmid *Cosmairum* after elevated temperature treatment (Stamencovic et al. 2014b).

In general, the ultrastructure of all investigated *Zygnema* strains showed an intact appearance in both, PA and PAB treated cells, confirming earlier results (Holzinger et al. 2009, Pichrtová et al. 2013). The massive occurrence of lipid bodies in pre-akinete cells was reported repeatedly (McLean and Pessoney 1971, Pichrtová et al. 2014, 2016), and is also found in the present study. These lipid bodies are formed during prolonged cultivation and were never observed in young vegetative cells (e.g. Bakker and Lokhorst 1987, Pichrtová et al. 2013). Lipid bodies are together with starch accumulations ideal for energy storage, but not involved in UV tolerance.

Conclusion

Against our hypothesis that pre-akinetes could tolerate UV radiation better, the results indicated that particularly young vegetative *Zygnema* sp. cells are well protected and able to acclimate to an increased PAB scenario. This can be concluded from the significantly better recovery rate of the ϕ_{PSII} values during the 74 h experiment. The young vegetative cells had higher initial ϕ_{PSII} values than the pre-akinetes, which was previously reported (Pichrtová et al. 2014). These observations are supported by the significantly higher amount of UV-absorbing phenolic compounds in young vegetative cells. In young *Zygnema* sp. S, PA and PAB treatment induced a significant increase of phenolic compounds, when compared to untreated cells. Moreover, the deepoxidation state of the xanthophyll cycle pigments increased significantly upon PA and PAB treatments, suggesting a good light protection in general. This was also supported by ultrastructural observations of protecting structures like electron dense bodies and cubic membranes in the chloroplast.

The investigated strains were nicely separated by the metabolomics approach (the metabolites of arctic and the Antarctic strain were more similar), and had differences in physiological performance (Antarctic strain significantly lower ϕPSII values after PAB, the temperate strain recovered better under PAB). A link of these observations to the geographic distribution of the strains is possible but difficult, as we investigated only one strain per region.

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Figures

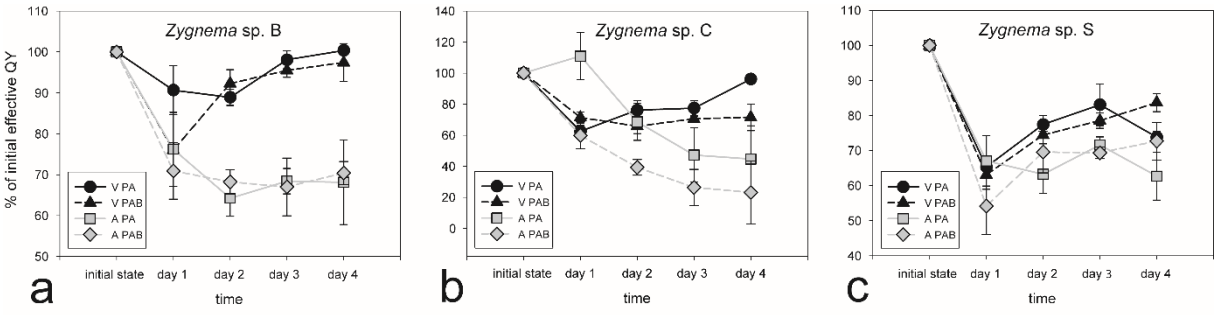

Fig. 1 Changes in effective quantum yield (ϕPSII) during the experiment. Values relative to the initial values before the UV exposure are shown (mean ± SD, n = 3). a *Zygnema* sp. B, b *Zygnema* sp. C, c *Zygnema* sp. S. Black circles: V PA – young vegetative cells, PAR-UV-A (PA) treatment; Black triangles: V PAB – young vegetative cells, PAR+UV-A+UV-B (PAB); Grey squares: A PA – pre-akinetes, PA; Grey rhomb: A PAB – pre-akinetes, PAB.

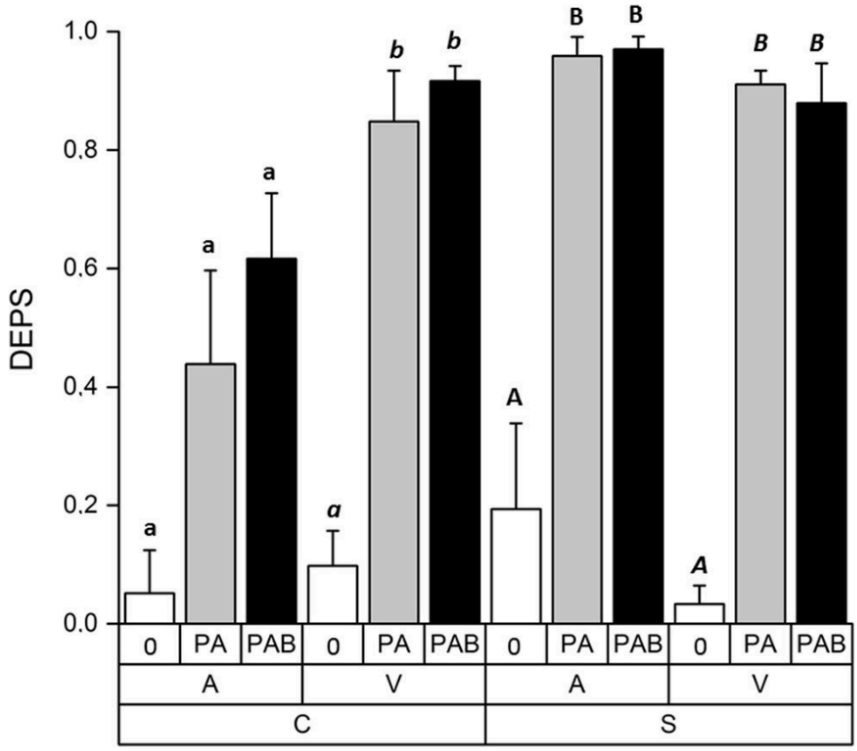

Fig. 2 Deepoxidation state - ratio of xanthophyll cycle pigments antheraxanthin, zeaxanthin and violaxanthin of *Zygnema* sp. C and *Zygnema* sp. S, (A) pre-akinetes and (V) vegetative cells either exposed to control condition (0) or PAR+UV-A (PA) or PAR+UV-A+UV-B (PAB). Statistical differences among individual UV treatments (One way ANOVA, Tukey's test) are marked with lower case letters (*Zygnema* sp. C, pre-akinetes), lower case letters in italics (*Zygnema* sp. C, vegetative cells), upper case letters (*Zygnema* sp. S, pre-akinetes) or upper case letters in italics (*Zygnema* sp. S, vegetative cells).

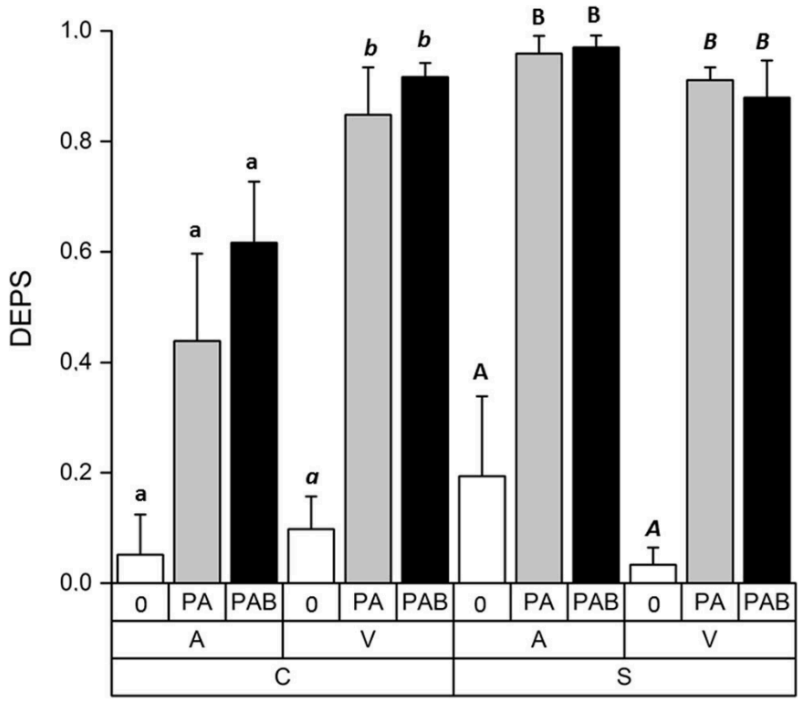

Fig. 3 UV absorbing phenolic compounds, illustrated as peak areas in *Zygnema* sp. C (C, left) and *Zygnema* sp. S (S, right). Pre-akinetes (A) are shown at the left side, vegetative cells (V) at the right side. The different treatments are indicated as follows (0) untreated control, PAR+UV-A (PA), PAR+UV-A+UV-B (PAB). Statistical differences among individual UV treatments (One way ANOVA, Tukey's test) are marked with lower case letters (*Zygnema* sp. C, pre-akinetes), lower case letters in italics (*Zygnema* sp. C, vegetative cells), upper case letters (*Zygnema* sp. S, pre-akinetes) or upper case letters in italics (*Zygnema* sp. S, vegetative cells).

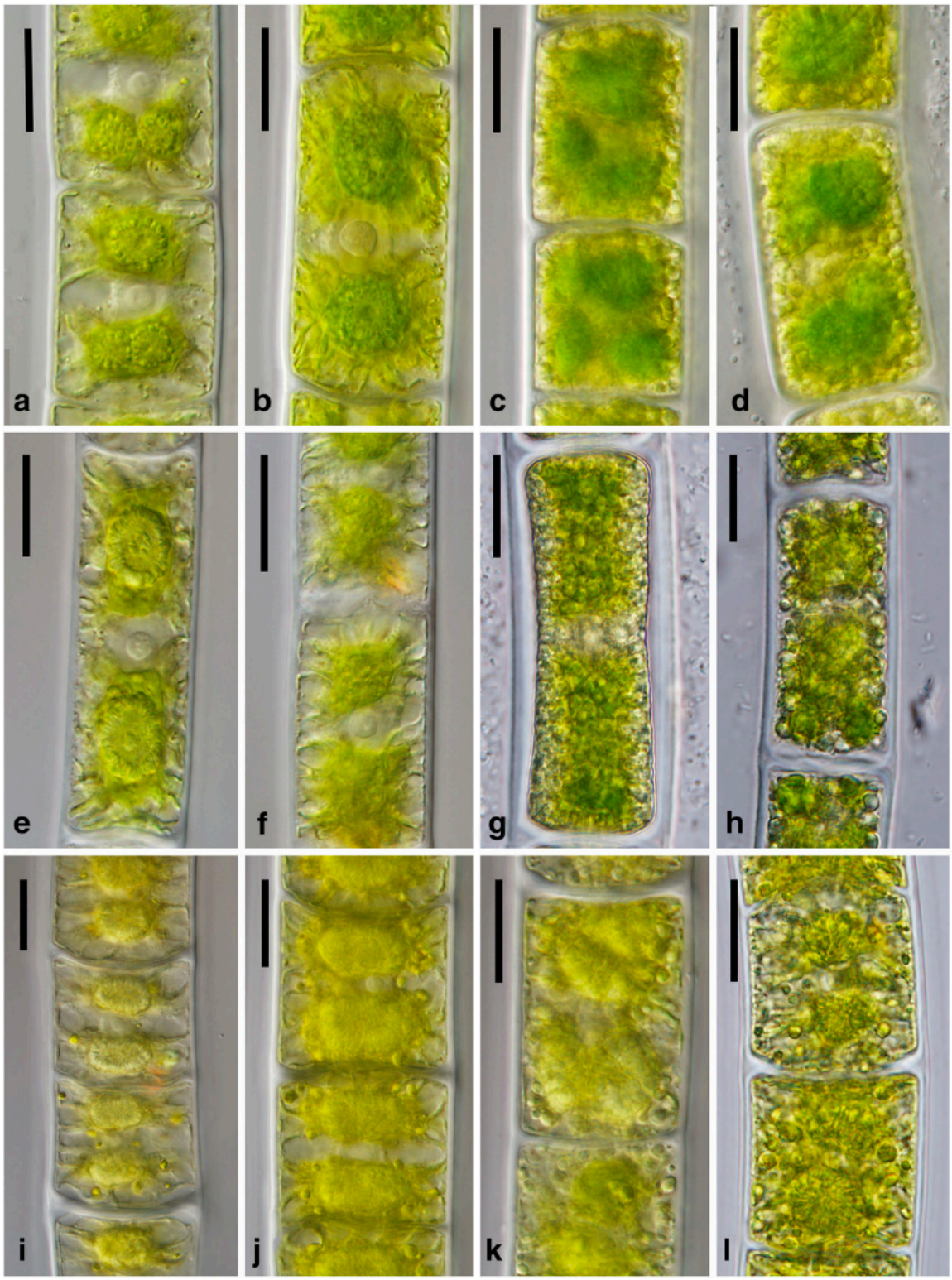

Fig. 4 Light micrographs of *Zygnema* cells after exposure to the experimental treatment. *Zygnema* sp. B (a – d); a young cells after PAR+UV A (PA), b young cells after PAR+UV-A+UV-B (PAB), c pre-akinetes after PA, d pre-akinetes after PAB. Zygnema sp. C (e – h); e – young cells after PA, f young cells after PAB, g pre-akinetes after PA, h pre-akinetes after PAB. Zygnema sp. S (i – l); i young cells after PA, j young cells after PAB, k pre-akinetes after PA, l pre-akinetes after PAB. Scale bars 20 μm.

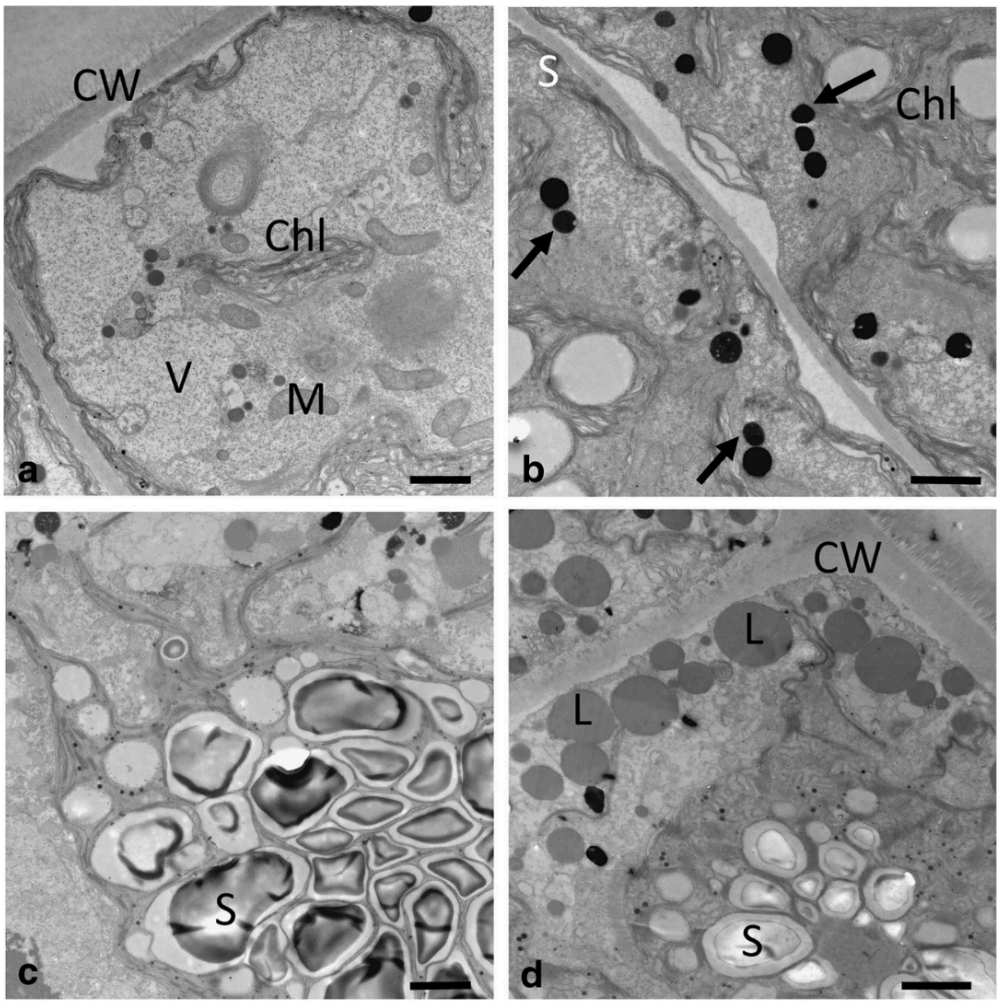

Fig. 5 Transmission electron micrographs of *Zygnema* sp. B young vegetative cells (a-c) and pre-akinete cell (d), exposed to (a, d) PAR+UV A (PA) or (b, c) PAR+UV-A+UV-B (PAB). a overview of young cell showing extensive vacuolization, and narrow chloroplast lobes, reaching towards the cell periphery, b electron dense bodies (arrows) are found in the cell periphery, c massive starch accumulations around the pyrenoids, d typical appearance of pre-akinete cells with massive lipid bodies in the cell periphery, the chloroplast shows starch accumulations and electron dense bodies are found. Abbreviations CW cell wall, L lipid body, M mitochondrion, S starch, V vacuole. Bars 2 μm

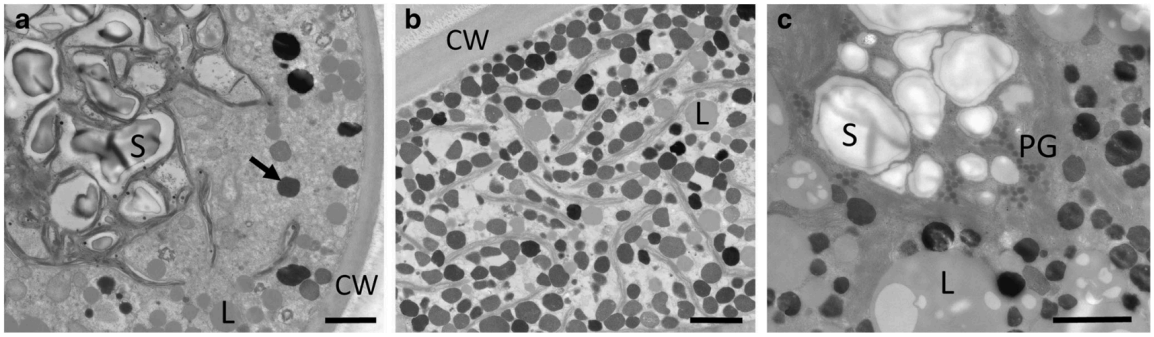

Fig. 6 Transmission electron micrographs of *Zygnema* sp. C vegetative cells (a, b) and pre-akinetes (c) exposed to (a) PAR+UV-A (PA) or (b-c) PAR+UV-A+UV-B (PAB). a numerous starch grains around the pyrenoid, several electron dense bodies (arrows) and lipid bodies, b cortical section with dense accumulation of electron dense bodies and lipid bodies, c chloroplast with starch grains and plastoglobules, electron dense bodies (arrows) and large lipid bodies. Abbreviations: CW cell wall, L lipid body, PG plastoglobules, S starch. Bars 2 μm

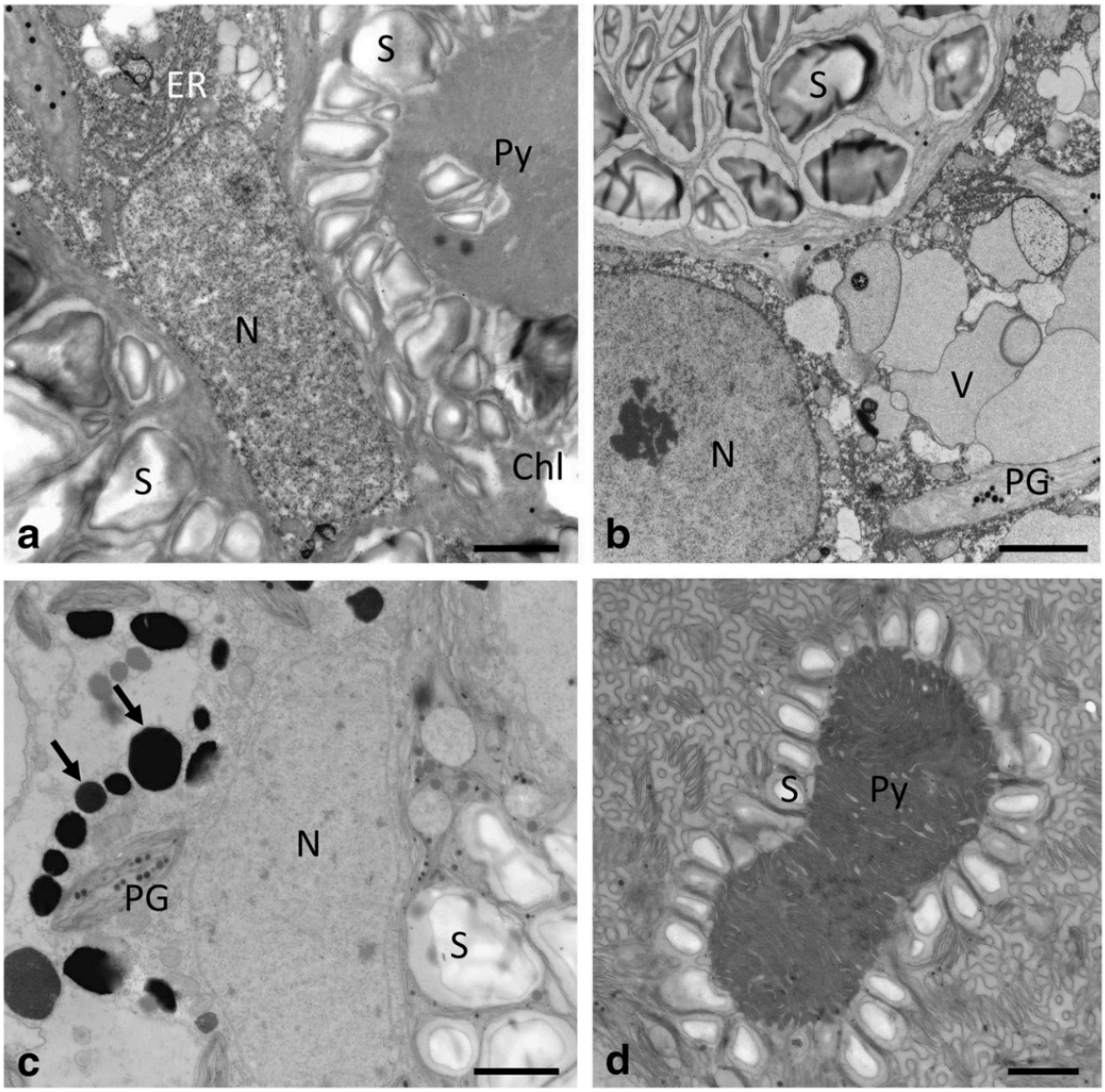

Fig. 7 Transmission electron micrographs of *Zygnema* sp. S vegetative cells (a, b) and pre-akinetes (c, d). Cells were either exposed to (a) PAR+UV-A (PA) or (b-d) to PAR+UV-A+UV-B (PAB). a central nucleus surrounded by two chloroplasts with prominent pyrenoids, surrounded by numerous starch grains, ER close to the nucleus; b nucleus with starch filled chloroplast and individual vacuoles, chloroplast lobes contain plastoglobules, c central area with nucleus, starch grains in the chloroplast and electron dense bodies (arrows), numerous plastoglobules, d pyrenoid surrounded by a single layer of starch grains, thylakoid membranes arranged in a cubic structure. Abbreviations: Chl chloroplast, ER endoplasmatic reticulum, N nuceleus, PG plastoglobules, Py pyrenoid, S starch. Bars 2 μm

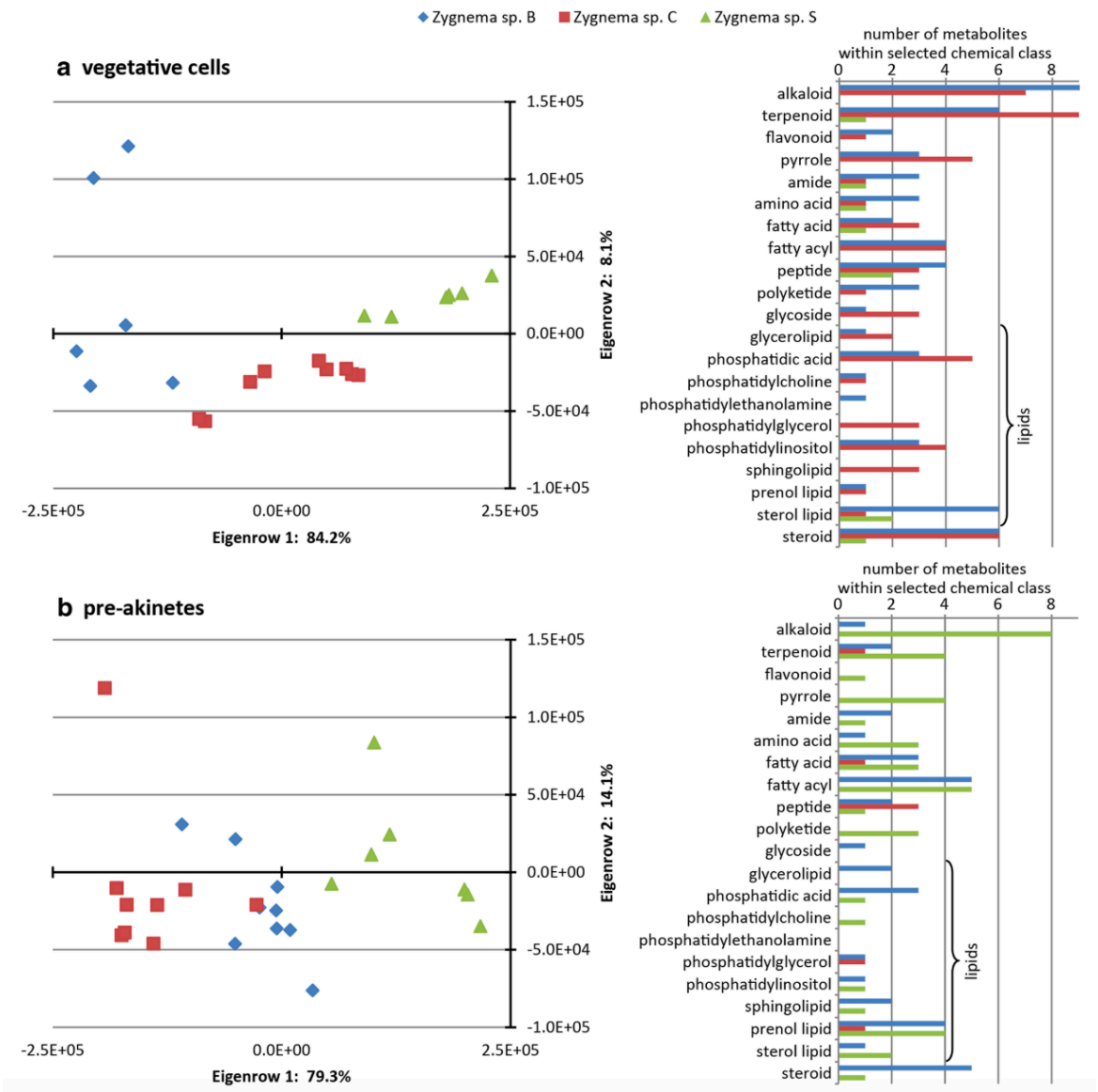

Fig. 8 PCA analysis of metabolomic data of (a) young vegetative cells and (b) pre-akinetes. Selected chemical classes driving the separation of *Zygnema* sp. strains within vegetative cells (a) and pre-akinetes (b) are listed on the right side. The different *Zygnema* strains are indicated by colors: blue: *Zygnema* sp. B, red: *Zygnema* sp. C, green *Zygnema* s

Paper III

The conjugating green alga *Zygnema* sp. (Zygnematophyceae) from the Arctic shows high frost tolerance in mature cells (pre-akinetes)

Protoplasma

The conjugating green alga *Zygnema* sp. (Zygnematophyceae) from the Arctic shows high frost tolerance in mature cells (pre-akinetes)

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Short title: Frost resistance by *Zygnema* sp.

Abstract

Green algae of the genus *Zygnema* form extensive mats and produce large amounts of biomass in shallow freshwater habitats. Environmental stresses including freezing may perturb these mats, which usually have only annual character. To estimate the limits of survival at subzero temperatures, freezing resistance of young *Zygnema* sp. (strain MP2011Skan) cells and pre-akinetes was investigated. Young, two-weeks-old cultures were exposed to temperatures of 0°C to -14°C at 2-K steps; whereas eight-months-old cultures were frozen from -10°C to - 70°C at 10-K intervals. Cell viability after freezing was determined by 0.1% Auramine O vital fluorescence staining and measurements of the effective quantum yield of photosystem II (Φ_{PSII}). At -8°C, the young vegetative cells were unable to recover from severe frost damage. But temperatures even slightly below zero (-2°C) negatively affected the cells' physiology. Single pre-akinetes could survive even at -70°C, but their LT_{50} value was -26.2°C. Severe freezing cytorrhysis was observed via cryo-microscopy at -10°C, a temperature found to be lethal for young cells. The ultrastructure of young cells appeared unchanged at -2°C, but severe damage to biomembranes and formation of small foamy vacuoles was observed at -10°C. Pre-akinetes did not show ultrastructural changes at -20°C; however, vacuolization increased, and gas bubbles appeared at -70°C. Our results demonstrate that formation of pre-akinetes increases freezing resistance. This adaptation is crucial for surviving the harsh temperature conditions prevailing in the High Arctic in winter and a key feature in seasonal dynamics of *Zygnema* sp.

Keywords: Auramine O, chlorophyll fluorescence, freezing, ice, live cell staining, ultrastructure

Introduction

Polar hydro-terrestrial ecosystems are characterized by changing and harsh environmental conditions. Freezing and desiccation are the primary environmental stresses in such habitats, although osmotic stress, high UV irradiation, nutrient deficiency, and seasonal changes in irradiance can also negatively affect the organisms living there. Algae and cyanobacteria are the main primary producers in polar ecosystems. They have to cope with long winters and even during growth periods they may be exposed to frequent freeze-thaw cycles (Davey 1989; Thomas et al. 2008). During freezing and thawing, their cells are exposed to a radically changing environment, which leads to cryoinjuries (damages caused by frost). Depending on the cooling rate, two different damaging mechanisms can occur: freezing cytorrhysis (collapse of a cell wall due to the loss of water though osmosis) caused by considerable cell dehydration, or mechanical disruption of membranes (Acker and McGann 2003).

Terrestrial algae inhabiting this harsh environment had to adapt to these stresses. One of the most widespread adaptation strategies is represented by specialized, stress-resistant life cycle stages, for example oospores of *Chara* sp. (Proctor 1967), aplanospores of *Coleochaete* (Delwiche et al. 1989) and many other green algae (revised in Leliaert et al. 2012), whereby reduced rates of physiological activity are complemented by additional protective mechanisms, i.e., changes in membrane composition or production of special protective substances (sumarized in Elster 1999). Nevertheless, many algae from harsh environments survive in a vegetative state, for example *Micrasterias denticulata* Brébisson ex Ralfs (Meindl et al. 1989), other examples are summarized in Sheath et al. 1996.

Zygnema is a common alga in polar hydro-terrestrial environments. As other filamentous representatives of the order Zygnematales (Zygnematophyceae), it forms extensive mats during the spring and summer season. Such mats were reported and studied mainly in localities fed by slow meltwater streams in the High Arctic (Holzinger et al. 2009, 2011; Pichrtová et al. 2014a, 2016b, 2018) and Antarctic regions (Hawes 1989, 1990). In the life cycle of *Zygnema,* different cell types can be distinguished. Highly resistant zygospores are formed by sexual process called conjugation, although they are very rarely observed in polar regions (Elster et al. 1997; Pichrtová et al. 2018). Commonly observed are other resistant stages; akinetes and aplanospores. They are formed by vegetative or asexual process (Stancheva et al. 2012). Young vegetative cells develop during the summer season into hardened mature cells termed pre-akinetes; these are characterized by smaller vacuoles, thicker cell walls, increased lipids and also their typical stellate chloroplasts are reduced (McLean and Pessoney 1971; Pichrtová et al. 2014a, b; Pichrtová et al. 2016a, b). The transition between young vegetative cells and pre-akinetes and *vice versa* is a continuous process (Herburger et al. 2015).

Numerous studies have focused on the ecophysiology and stress resistance of *Zygnema*. Pre-akinetes have been found to play a key role in the survival of these algae, as they are resistant to desiccation (Pickett-Heaps 1975; Pichrtová et al. 2014b; Herburger et al. 2015; Rippin et al. 2017), osmotic stress (Kaplan et al. 2013; Pichrtová et al. 2014a), and UV radiation; however, younger cells have a more flexible reaction to UV (Holzinger et al. 2009; Holzinger et al., 2018). Moreover, *Zygnema* is a representative genus commonly studied for its adaptation to terrestrial life, because Zygnematophyceaean algae are considered to be the closest relatives of land plants (Wodniok et al. 2011; Zhong et al. 2014; deVries et al. 2016; Lemieux et al. 2016;

deVries et al. 2018). Surprisingly, little is known about the role of pre-akinete formation in the overwintering strategy and freezing resistance of *Zygnema*. Living pre-akinetes embedded in ice were observed during the Arctic winter (Pichrtová et al. 2016b). Although these cells were capable of restoring their photosynthetic activity right after thawing, a large amount of dead algal biomass was found at the study site after winter (Pichrtová et al. 2016b). Hawes (1990) investigated freezing tolerance of both natural samples from Antarctica and cultures. He reported that all cells from the field resembled pre-akinetes. Repeated freeze-thaw cycles had no effect on photosynthetic rate. In addition, a small proportion of cells tolerated long-term (120 days) exposure to -20°C, suggesting their ability to survive long-term exposure to sub-zero temperatures during the Antarctic winter. However, no mechanism underlying this phenomenon has been proposed (Hawes 1990).

The present study focused on freezing resistance by an Arctic *Zygnema* sp. The formation of pre-akinetes was previously observed in the Arctic at the end of the growing season (Pichrtová et al. 2014a) and cells could overwinter in this state (Pichrtová et al. 2016b). Based on field observations, we aimed to estimate the limits of survival of young cells and pre-akinetes. Three experiments with cultures of different ages were performed. Hardened pre-akinetes were hypothesized to resist much lower freezing temperatures than young vegetative cells. Additionally, young cells were exposed to a freeze-thaw cycle to test if repeated freezing could harm them more. Changes in photosynthetic activity before and after freezing experiments were measured by the effective quantum yield of photosystem II (Φ_{PSII}). Frost injuries were observed via light and transmission electron microscopy. Moreover, the freezing process of young vegetative cells was investigated by cryo-microscopy to evaluate changes in morphology during the formation of ice. The results are discussed in the context of stress resistance and the seasonal dynamics of this ecologically important alga.

Methods

Algal material

Experiments were conducted with the Arctic *Zygnema* sp. strain MP2011Skan (Pichrtová et al. 2018: Tab. 1 Locality 4, Skansbukta, culture isolated from a field sample in 2011) whose *rbc*L sequence is identical to that of *Zygnema* sp. B (CCALA 976) and GenBank accession numbers JX075101 (Kaplan et al. 2013) and LN611664 (Pichrtová et al. 2014b). Cultures were maintained in Bold's Basal Medium (BBM; Bischoff and Bold, 1963) solidified with 1.5% agar and incubated at a light/dark regime of 16/8 h at 20°C and ~33 μmol photons m^{-2} s^{-1}. Cultures at two different stages were used for the experiments: 1) young vegetative cells obtained two weeks after inoculation and 2) eight-months-old pre-akinetes. The occurrence of pre-akinetes and the general condition of cultures was examined microscopically (Axiovert 200 M, Carl Zeiss AG, Jena, Germany) before the experiments.

Experimental freezing

Freezing experiments were carried out in commercial chest freezers (GT 2102, Liebherr, Lienz, Austria). The temperature inside the freezing compartment was controlled as described by Kuprian et al. (2016). Three different cooling experiments were performed. In the first experiment (1), young cultures were exposed to eight experimental temperatures

from 0°C to -14°C for 10 h, with a cooling and warming rate of 4 K h^{-1}. In the second experiment (2) the cells were frozen in two consecutive freezing cycles, both with exposure time of 8 h. Three experimental temperatures were investigated: -4°C, -6°C, and -8°C with cooling and warming rates of 4 K h^{-1}. Between cycles, samples were kept in the cultivation chamber at +20°C and ~33 µmol photons m^{-2} s^{-1}. The third experiment (3) was performed with mature cells (pre-akinetes) at seven experimental temperatures from -10°C to -70°C, exposure time of 8 h, and cooling and warming rate of 4 K h^{-1}. Controls without experimental treatment were kept in the cultivation chamber as described above. Detailed parameters of freezing cycles are given in Online resource 1.

A comparable amount of fresh biomass (expressed as average ± standard deviation) was used for the experiments: 0.003 ± 0.0008 g for young cells and 0.007 ± 0.0013 g for pre-akinetes (analytic balance, Sartorius, Göttingen, Germany). The experiment was performed in three independent biological replicates for each treatment. Cells were transferred onto glass microfiber filters GF/C Ø 47 mm (Whatman, UK), which were placed on filter paper Ø 90 mm (Whatman, UK) in a plastic Petri dish. The space between the microfiber filter with algal biomass and the wall of the Petri dish was covered with a tablespoon of crushed ice. To prevent artificial supercooling of algal cells, ice nucleation was triggered around -1.5°C by applying 400 µL of ice nucleation active bacterial suspension (*Pseudomonas syringae* van Hall) on the ice-covered part of the filter paper. This method is well established for higher plants (e.g., Hacker & Neuner 2007; Kuprian et al. 2016), but has been used here for the first time on algae. The algae were never mixed with the bacterial suspension, but the latter was applied on ice in the vicinity of the algal biomass. Direct application to filaments of *Zygnema* sp. caused cell death within a few hours during the recovery period. Additionally, thermocouples were mounted in the proximity of cells to monitor the temperature during experimental freezing cycles. The filter paper was removed right after freezing, and only the glass microfiber filter with algae was left in the Petri dish sealed with parafilm. Samples were placed under continuous illumination (~50 µmol photons m^{-2} s^{-1}) at around +19°C and rewetted with 200 µL water 6 h and 22 h after treatment to prevent additional desiccation stress.

Small aliquots of cultures containing pre-akinetes were frozen to -50°C and -70°C and, after the experiments, were transferred to Petri dishes with BBM media (Bischoff and Bold 1963) solidified with agar, and incubated at a light/dark regime of 16/8 h at +20°C and ~33 µmol photons m^{-2} s^{-1} for three weeks. These cells were microscopically examined (Zeiss Axiovert 200 M) for the presence of living cells after two and four weeks of cultivation.

Measurement of the effective quantum yield

The effective quantum yield of photochemical energy conversion in PSII (Φ_{PSII}) 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. The first measurement was taken before experiments, after placing the filters with the algal material into plastic Petri dishes. Subsequent measurements were performed 1 h, 3 h, 6 h, and 24 h after the end of freezing experiments.

Vital staining and light microscopy

Algal samples were examined by light or fluorescence microscopy (Zeiss Axiovert 200 M) using a Zeiss Axiocam HRc camera and a Zeiss filter set 09 (for fluorescence microscopy). A small amount of biomass was stained with 0.1% Auramine O (Sigma Aldrich, Steinheim, Germany) to estimate the viability of the cells directly after thawing. Auramine O stains the endomembrane system of metabolically active cells in bright yellow-greenish color (Harris and Gates 1984; Hawes and Davey 1989; Hawes 1990). Staining was performed in 1.5-mL Eppendorf tubes in 1 mL of staining solution for 10 min in darkness. At least twenty images were taken with approximately 600 cells per experimental temperature. The fluorescent cells were counted in ImageJ with the Cell counter plugin (Rasband 2016). At least 500 active/non-active cells were counted for every experimental temperature and control.

Transmission electron microscopy

Transmission electron microscopy was performed with young cells after a single freezing experiment at -2°C and -10°C, and with old cultures (pre-akinetes) after freezing to -20°C and -70°C, plus the respective untreated controls. Chemical fixation of filaments was as described by Holzinger et al. (2009) with some modifications. Briefly, samples were fixed in 2.5% glutaraldehyde in 25 mM sodium cacodylate buffer (pH 6.8) for 1.5 h, post-fixed with 1% OsO_4 at 4°C for 12 h, rinsed and dehydrated in increasing ethanol concentrations, transferred *via* propylene oxide, and embedded in modified Spurr's embedding resin (Science Services, Munich, Germany). Ultrathin sections were prepared with an Ultra-microtome (Leica Microsystems GmbH, Wetzlar, Germany), counterstained with 2% uranyl acetate and Reynold's lead citrate, and investigated on a Zeiss LIBRA 120 transmission electron microscope (Carl Zeiss AG, Oberkochen, Germany) at 80 kV. Images were taken with a TRS 2 k SSCCD camera and processed with Adobe Photoshop 7.0 software (Adobe Systems Inc., San Jose, CA, USA).

Cryo-microscopy

Cryo-microscopical observation was performed on a light microscope (DM1000, Leica Microsystems GmbH) equipped with a cooled slide holder and Leica EC3 camera. Cooling was performed by a custom-built cryo-stage filled with ethanol, which was pumped through the slide holder. The temperature on the sample slide was monitored by thermocouples. Cryo-stage and holder were controlled by custom-designed 'Cryostage' software developed by Dr. Othmar Buchner (University of Innsbruck, Austria). Only two-weeks-old cells were investigated with this system. The cells were frozen to -10°C and images were taken at minute-intervals during freezing until the filament was completely embedded in solid ice. Images were acquired with Leica LAS EZ software and further processed in Adobe Photoshop 7.0.

Freezing resistance

Lethal temperature at 50% frost damage (LT_{50}), a measure of freezing resistance, was determined in two ways. In the first, Auramine O staining was used to count the number of viable cells. The LT_{50} value indicated the temperature at which 50% of cells did not show

any metabolic activity 1 h after the end of the freezing treatment. In the second, LT_{50} was calculated from Φ_{PSII}. All Φ_{PSII} values were expressed as a percentage of the initial value. The resulting value represented the freezing temperature whereby chlorophyll fluorescence was at 50% of the maximum 24 h after the experiment. LT_{50} was calculated using Boltzmann's function in Origin 2017 software (Origin Lab Corporation, Northampton, MA, USA).

Statistical analyses

The effect of experimental temperature and time of recovery on Φ_{PSII} values was evaluated using two-way ANOVA with repeated measurements in each of the three experiments. Differences between Φ_{PSII} values before and 24 h after treatment were tested with a two-sample *t*-value test. For all analyses, the significance value was set as $p<0.05$. All tests were carried out in the PAST statistical program (Hammer et al. 2001).

Results

Physiological performance after freezing

Mean Φ_{PSII} values (± standard deviation) before the experiment were higher in young vegetative cells (0.60 ± 0.02) than in mature pre-akinetes (0.38 ± 0.09). For both types of cells, the initial Φ_{PSII} values dropped significantly after experimental freezing (two-sample *t*-value test, Table 1). In general, a lower freezing temperature caused a more pronounced decline in Φ_{PSII} during the recovery period (Fig. 1). Notably, Φ_{PSII} decreased considerably between -6°C and -8°C in both experiments with young cells (Fig. 1a–b). However, the effect of experimental temperature on Φ_{PSII} was statistically significant ($p<0.05$) only in the single freezing experiment (Table 2). Freezing temperature also significantly affected the Φ_{PSII} value of pre-akinetes (Table 2).

Estimation of viability

Staining with Auramine O was used to estimate the number of viable cells after freezing (Fig. 3a–d). Viable cells with a yellow-greenish fluorescence (Fig. 3a) were clearly distinguishable from dead cells exhibiting only red chloroplast autofluorescence (Fig. 3b–d; asterisks). Staining intensity was the same in young cells and pre-akinetes. Moreover, filaments surrounded by mucilage were more metabolically active than separately laying ones. Bacteria in mucilage envelopes were stained green and marked the amount of mucilage surrounding the cells. Pre-akinetes had much more mucilage around them than young vegetative cells (Fig. 3d).

Pre-akinetes frozen to -70°C and -50°C were additionally transferred to BBM agar medium. Even though there was no detectable Φ_{PSII} in the specimens after freezing, 10–20% of cells survived both treatments, as they were able to recover after four weeks. However, most of the biomass was dead.

LT_{50}

LT_{50} values calculated from Φ_{PSII} were -5.9°C for the single freezing experiment and -6.5°C for the double freezing experiment (Table 3). No photosynthetic activity could be detected below -10°C. Pre-akinetes were more resistant to freezing temperatures than young cells. Their LT_{50} value obtained by Φ_{PSII} was determined to be -26.2°C.
Based on the numbers of viable cells stained with Auramine O, LT_{50} values were -8.6°C for the single freezing experiment and -5°C for the double freezing experiment (Table 3). A large difference in the number of viable cells was observed between these two experiments. After freezing to -8°C, 71.5% of cells remained active in the single freezing experiment (Fig. 4), but only 16.5% did so after the double freezing experiment. The LT_{50} value was -26.1°C for pre-akinetes (Table 3).

Light microscopy investigations

Young control cells contained two stellate chloroplasts and many small perennial vacuoles. In contrast, pre-akinetes had small chloroplasts, lipid bodies, and a thicker cell wall. The morphology of young cells changed profoundly at experimental temperatures below -10°C, but remained unchanged compared to controls at temperatures slightly below 0°C (Fig. 2a–c). More than 80% of young cells were damaged at -10°C; their protoplast was shrunken and detached from the cell wall, and it appeared darker than in untreated controls, sometimes with a yellowish tinge. The number of damaged cells increased as the experimental temperature decreased for both cell types. Pre-akinetes (Fig. 2d–f) exhibited similar damage as young cells, with a shrunken and darker protoplast (Fig. 2f). In pre-akinetes, cells with shrunken protoplasts started to appear in samples frozen to -20°C and their number increased further as the temperature became even lower. Still, most cells frozen down to -20°C maintained a normal morphology (Fig. 2e). Moreover, a clear tendency to form vacuoles in the protoplasts was observed after freezing cycles at temperatures below -30°C. The vacuoles were localized in the apical part of the cells (Fig. 2f). We also observed air bubbles forming inside thawed cells previously frozen to temperatures below -30°C. The number of cells with vacuoles and gas bubbles increased further at even lower temperatures.

Cryo-microscopy

Morphological changes to the cells were observed by cryo-microscopy during the freezing process (Fig. 5). The shape of the cells gradually changed from normal to clavate, especially in the terminal cell of the filament (Fig. 5a–d). All the cells in the filament were thicker at the same apical part, and parallel bends appeared on squeezed cell walls (Fig. 5d; arrow). Cells recovered to their initial shape in a few seconds during thawing. When ice around the filament became softer, their cell walls returned to their original shape immediately. However, the protoplast formed a sphere in the middle of the cell and gained its normal shape only later on. Damage to the cells was visible after thawing; the protoplast was detached from the cell wall in several places (Fig. 5e; arrow) and the structure of the chloroplast was changed (Fig. 5e).

Changes of the ultrastructure

Young vegetative cells of *Zygnema* sp. showed a high degree of vacuolization (Fig. 6a). The two stellate chloroplasts contained pyrenoids with starch grains and were surrounded by a thin layer of cytoplasm (Fig. 6a). The center of large pyrenoids had a typical multilayer gyroid cubic membrane organization of the thylakoid membranes (Fig. 6b). The lamellar structure of thylakoid membranes was observed in chloroplasts (Fig. 6c). By contrast, pre-akinetes had thicker cell walls and little vacuolization (Fig. 6d). The size of the pyrenoids was reduced in comparison to young vegetative cells, and they also lacked the multilayer gyroid cubic membrane organization of the thylakoids. The thylakoid membranes in the small chloroplasts of pre-akinetes had a lamellar structure (Fig. 6e), whereas the cell periphery was full of lipid and electron-dense bodies (Fig. 6f).

Young vegetative *Zygnema* sp. cells exposed to -2°C displayed minor modifications of the ultrastructure (Fig. 7a–c) when compared to control cells. Golgi bodies and endoplasmic reticulum remained intact (Fig. 7b). There were minor changes in the structure of the pyrenoid, organization of thylakoid membranes was altered, and no multilayer cubic membrane organization was observed (Fig. 7c). In contrast, exposure to -10°C (Fig. 7d–f) led to drastic ultrastructural changes in young vegetative cells (Fig. 7d–f). Protoplasts were full of small vacuoles, and the structure looked "foamy" particularly in the electron-dense compartments (Fig. 7d–e). The structure of the pyrenoid was damaged, and there was no sign of multilayer cubic membrane organization. The electron-dense thylakoid membranes in the pyrenoids displayed a lamellar structure (Fig. 7f).

Pre-akinetes exposed to -20°C showed only minor changes in ultrastructure (Fig. 8a–c) when compared to control cells. The only observed difference was in the size of lipid bodies, most of which were drastically bigger than in controls (Fig. 8b). The structure of chloroplasts and the nucleus was the same as in untreated cells (Fig. 8a, Fig. 6d–f). As in the case of young cells, exposure to lower freezing temperatures had a more profound effect on pre-akinetes' ultrastructure and their morphology changed substantially when exposed to -70°C (Fig. 8d–f). Overall, protoplasts contained large and multiple vacuoles (Fig. 8d), chloroplast ultrastructure was severely altered, the cytoplasm was full of vacuoles and electron-dense material (Fig. 8e), and lipid bodies accumulated at the periphery of the cell (Fig. 8f).

Discussion

In this work, we investigated the limits of survival to frost by *Zygnema* sp. from the High Arctic region. We compared the freezing resistance of young vegetative cells and pre-akinetes. The latter are not specialized cells, but they gradually develop from young vegetative cells that stop dividing (Herburger et al. 2015). We report a substantial difference in the ability to deal with freezing temperatures between these two developmental stages. Young cells died at about -6°C to -10°C, whereas a small proportion of pre-akinetes could survive even down to -70°C. This corroborates our hypothesis, because pre-akinetes are much more tolerant to desiccation than young vegetative cells (Pichrtová et al. 2014b).

Limits to survival of young vegetative cells and pre-akinetes

Young vegetative cells were able to resist freezing temperatures, but frost drastically changed the ability to recover their photosynthetic activity. Except for cells at 0°C and controls, all other cell samples failed to restore initial Φ_{PSII} values (Fig. 1). Thus, a temperatures even slightly below zero (-2°C) negatively affected the cells' physiology. At - 8°C, the cells were unable to recover from severe frost damage as no Φ_{PSII} could be detected after 24 h of recovery. Hawes (1990) investigated freezing tolerance of *Zygnema* sp. cultures in exponential phase (i.e. young vegetative cells). They retained 92% viability after freezing to -15°C for 60s, also showing a certain level of frost tolerance even in young cells. Comparable, the LT_{50} value of vegetative cells of *Tribonema bombycinum* (C. Agardh) Derbès & Solier after 5 day incubation was -3°C (Nagao et al. 1999). In addition, Nagao et al. (2008) found that 15% of young *Klebsormidium* cells survived freezing at -10°C.

In contrary to our findings, 12 tested strains of *Klebsormidium* survived experimental freezing to -40°C retaining 80% viability (Elster et al. 2008). However, the age of cultures used in the experiment was not given in the study. Similarly, all tested strains of Antarctic wetland green algae of the genera *Chlorella*, *Chlorosarcina*, *Pseudococcomyxa,* and *Klebsormidium* survived all freezing treatments (-4°C, -40°C, -100°C and -196°C), even though the viability was reduced in comparison to control (on average 56% at -40°C); (Šabacká & Elster 2006).

It is difficult to estimate the exact temperature limiting survival. Here, cell viability was estimated by two different methods; indeed, this is the first study that uses staining and measures of chlorophyll fluorescence of PSII together for this purpose. The LT_{50} value calculated based on the number of young viable cells (-8.6°C) was lower than the one calculated based on Φ_{PSII} (-5.9°C). However, these two values are not comparable. The first LT_{50} value indicated the temperature at which 50% of cells did not show any metabolic activity. The second LT_{50} represented the freezing temperature whereby chlorophyll fluorescence of the living cells was at 50% of the maximum 24 h after the experiment. It should be noted that staining was performed immediately after the end of the experiment to capture the cells' status right after thawing. The decline in Φ_{PSII} observed after 24 h of recovery indicated that the cells' photosynthetic machinery was severely damaged and, indeed, cells died soon afterwards. Frost has many damaging effects, including changes to the ultrastructure of cell membranes, loss or fusion of membrane bilayers, and organelle disruption (Mazur 2004). Thylakoids are profoundly affected by frost (Garber and Steponkus 1976) and the cells' survival depends on their ability to recover from the damage. Therefore, we believe that the LT_{50} value determined from Φ_{PSII} reflects the physiological status of the cells more realistically than Auramine O staining.

In contrast to young vegetative cells, pre-akinetes showed astonishingly high freezing resistance. The LT_{50} value determined from a number of viable cells was -26.1°C and we counted 28.6% of cells to be alive after freezing to -70°C. Whereas none of the frozen samples was able to fully recover Φ_{PSII}, a few viable cells were nevertheless found in a culture previously exposed to -70°C. This indicates that a small proportion of pre-akinetes survives even when its fluorescence cannot be detected. The present findings confirm the results of Hawes et al. (1990), who tested long-term exposure of *Zygnema* sp. to -20°C. The small proportion of cells they found alive after long-term exposure to frost were most likely resistant pre-akinetes. Similar freezing resistance was found with akinetes of *Tribonema bombycinum* (C. Agardh) Derbès

& Solier. Cells incubated under nutrient-limiting conditions formed resistant akinetes capable of surviving below -30°C (Nagao et al. 1999). Interestingly, both *Tribonema* (Xanthophyceae) and *Zygnema* sp. share the same life strategy even though they are phylogenetically unrelated; *Zygnema* sp. belongs to Archeplastida and *Tribonema* sp. to Sar (Burki et al. 2016; Brown et al. 2018).

We also investigated the effect of repeated freezing on young cells. Elster and Komárek (2003) showed that freezing down to -4°C was common during the polar summer periods and even at that temperature the liquid content of cells was not frozen, so that species could survive without frost injuries. As shown by Hawes (1990), field collected *Zygnema* sp. maintained its photosynthetic capacity without any cryoinjury during repeated overnight exposures to -4°C. In our experiments, repeated freezing to -4°C and -6°C had only a minor effect on Φ_{PSII} values. However, Φ_{PSII} dropped markedly after 24 h of recovery, as in the case of single freezing. In addition, exposure to -8°C resulted in 71.5% viable cells in the single freezing experiment but only 16.4 % in the double freezing experiment. Thus, the results clearly show that repeated freezing can harm young vegetative cells more than a single freezing event. In the case of this experiment, we had low statistical support regarding the effect of temperature on Φ_{PSII}, possibly due to fewer tested levels of this factor.

Frost injuries

The most prominent differences between young vegetative cells and pre-akinetes included the size of vacuoles, chloroplast shape, and the massive occurrence of lipid bodies in pre-akinetes (Pickett-Heaps 1975; Pichrtová et al. 2014b; Pichrtová et al. 2016a). Frost injuries incurred during the experiments represented classical examples of freezing cytorrhysis, with deformed cell walls and retracted protoplasts (Pearce 2001; Beck et al. 2007; Buchner and Neuner 2010). They were similar to the plasmolyzed pre-akinete cells observed by Pichrtová et al. (2014a). However, damages derived from strong mechanical stress during freezing experiments were more severe than those observed in only plasmolyzed cells. As shown by cryo-microscopy, cells lost water during freezing cytorrhysis, but the cell walls remained flexible. Cells were squeezed completely during continuous freezing to -10°C. However, unlike protoplasts, cell walls remained unharmed, and cells restored their shape.

Morris and McGrath (1981) observed the formation of gas bubbles during fast thawing of *Spirogyra grevilleana* (Hassall) Kützing filaments. The solubility of gasses increases during cooling and is predicted to double between 0°C and -20°C (Hobbs 2010). Accordingly, when liquid water is removed to form ice, gasses remain concentrated in the residual solution. Small gas bubbles may become trapped between ice crystals when intracellular ice is formed. During thawing, they fuse before returning onto solution in the protoplast. Authors have not observed any remaining gas bubbles during slow warming (less than 0.5 K min^{-1}). However, they applied much more moderate freezing temperatures than those used in the present study with pre-akinetes.

The ultrastructure of young cells and pre-akinetes of *Zygnema* sp. corroborated earlier studies (e.g., Kaplan et al. 2013; Pichrtová et al. 2013). The effect of freezing in young cells was clearly visible, as judged by the appearance of small foamy vacuoles. Freezing injuries affected mainly vacuoles and the structures of chloroplasts in young cells, whereas whole protoplast content seemed to be harmed by mechanical damage (Fig. 7d–e). Electron-dense

bodies were present in young cells and particularly in pre-akinetes (Fig. 8a, d). They tended to fuse in pre-akinetes after exposure to -70°C and had an irregular shape (Fig. 8d–f). Lipid bodies, too, showed the same agglutination behavior; however, this could be an effect of their physical properties rather than a stress response. Liquid water is removed from the cells during freezing, which means that other compounds get closer to each other and may merge.

Mechanisms of freezing protection

During the formation of pre-akinetes, vacuoles are reduced, and lipids accumulate (Herburger et al. 2015; Pichrtová et al. 2016a). Accumulation of storage compounds represents a strategy for dealing with environmental stresses for many algae (Morison and Sheath 1985; Meindl et al. 1989; Karsten & Holzinger 2012; Herburger et al. 2016). They serve as a source of energy and carbon when optimal conditions are restored.

Freezing resistance is also triggered by the accumulation of organic osmolytes which are produced to prevent water loss and maintain homeostasis during osmotic stress caused by desiccation, excessive salinity, or freezing. *Klebsormidium flaccidum* (Kützing) P.C.Silva, K.R.Mattox & W.H.Blackwell increases the content of sugars and other compounds during cold acclimation (Nagao et al. 2008). *Zygnema* collected from the Antarctic was found to contain 9.9 ± 0.8% soluble sugars in ash-free dry weight; the sugars were identified as sucrose, with traces of glucose, fructose, and mannitol (Hawes 1990). However, this concentration seems to be too low to depress the freezing point of the protoplast (Hawes 1990). Nevertheless, the field samples used in the study of Hawes (1990) lost photosynthesis and showed high level of membrane disruption already at -8°C, simalrly as our young vegetative cells. Recently, production of organic osmolytes (sucrose) in *Zygnema* sp. upon desiccation stress has been analzed by a transcriptomic approach, and several crucial pathways have been found (Rippin et al. 2017).

In addition, prior work has documented the importance of hardening for stress resistance by *Zygnema* pre-akinetes. Naturally slowly desiccated pre-akinetes are resistant to severe osmotic stress (Pichrtová et al. 2014a) and pre-akinetes grown under mild desiccation stress are resistant to very fast desiccation (Pichrtová et al. 2014b). In contrast, our results showed that pre-akinetes induced by nutrient starvation were highly resistant to freezing. Indeed, additional frost hardening might even strengthen their resistance. Similarly, in another streptophytic alga, *Klebsormidium flaccidum* (Kützing) P.C.Silva, K.R.Mattox & W.H.Blackwell, survival rates after freezing increased by 70% after exposure to 2°C for seven days (Nagao et al. 2008).

Dynamics of Zygnema mats and influence of frost in early spring

The current findings confirmed the hypothesized increased freezing resistance of pre-akinetes. However, the experiment was set to investigate rather short-term freezing (10 h), whereas polar winters last many months. Longer exposure to frost was previously found to lead to a gradual decrease in viability of *Zygnema* sp. cells at -3°C, whereas viability was lost rapidly at -20°C (Hawes 1990). Frozen pre-akinetes are metabolically active right after thawing and they start to divide immediately (Pichrtová et al. 2016b), transforming back to young

vegetative cells and losing their freezing resistance. In addition, repeated freezing turned out to be more harmful to cells than single freezing. If frost or freeze-thaw cycles occur unpredictedly during spring, young vegetative cells are severely damaged or die, which is probably the main cause of the perennial character of the mats (Pichrtová et al. 2016b).

On Svalbard, ground surface temperatures on a study site covered by snow dropped to -30°C several times during winter and the temperature oscillated around zero during spring and autumn indicating the occurrence of freeze-thaw cycles (Láska et al. 2012). Nevertheless, accumulation of snow and ice offers very effective thermal insulation (Hawes 1989). While the air temperature of the Antarctic study sites fell to -25°C, the temperature under thick ice (30–50 cm) was only -4°C (Hawes 1989). Young vegetative cells could potentially survive the winter underneath snow and ice. So far, though, they have not been observed during the winter season.

Conclusion

The production of pre-akinetes is a crucial strategy for overwintering in *Zygnema* sp. In accordance with our hypothesis, pre-akinetes were able to resist extreme temperatures substantially below the freezing point, whereas young vegetative cells turned out to be more susceptible to unexpected frost events. Development of young vegetative cells in the beginning of the spring season is crucial for rapid growth during the short summer season and the future of a population. Nevertheless, young dividing cells can be easily harmed by frost, which could endanger the whole population. Further research, e.g., long-term freezing or winter field observations are required to fully understand stress resistance by this alga.

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Tables and figures

Young cells—single freezing			
N, 24			
Before freezing		24 h after freezing	
Mean	0.60562	Mean	0.19687
Median	0.602	Median	0.125
t			9.036
p (same)			0.000000004995
Young cells—double freezing			
N, 9			
Before freezing		24 h after freezing	
Mean	0.56044	Mean	0.29633
Median	0.561	Median	0.392
t			4.181
p (same)			0.003077
Pre-akinetes			
N, 21			
Before freezing		24 h after freezing	
Mean	0.38195	Mean	0.089667
Median	0.352	Median	0
t			8.012
p (same)			0.000000114

Values were compared using paired *t* tests. Each of the three experiments was tested separetly.

Table 1 The values of ΦPSII in 1h during recovery period were compared with values before freezing treatment using paired t-tests.

Effect	Sumsqrs	df	Meansqr	*F*	*p*
Young cells—single freezing					
Recovery time	0.05187	3	0.01729	0.007971	0.9989
Temperature	6.813	8	0.8517	6.506	0.0007648
Interaction	0.2327	24	0.009694	− 0.02993	1
Young cells—double freezing					
Recovery time	0.05217	3	0.01739	0.009226	0.9986
Temperature	1.453	3	0.4843	3.71	0.08061
Interaction	0.05039	9	0.005599	− 0.008379	1
Pre-akinetes					
Recovery time	0.03498	3	0.01166	0.03643	0.9898
Temperature	1.261	7	0.1801	11.53	0.00007394
Interaction	0.08617	21	0.004103	− 0.1038	1

Tested Φ_{PSII} values are from four dependent measurements during the recovery time (1 h, 3 h, 6 h, and 24 h). Significant interactions between the factors were not encountered.

Table 2 Summary of the results of two-way ANOVA with repeated measurements (variable: ΦPSII values; factors: recovery time, temperature).

	Young cells—single freezing	Young cells—double freezing	Pre-akinetes
LT_{50} values for ΦPSII	− 5.9°C	− 6.5°C	− 26.2°C
LT_{50} values for viable cells	− 8.6°C	− 5°C	− 26.1°C

Numbers were determined from Boltzmann's function fitted to the number of viable cells after experimental freezing and stained with Auramine O or to Φ_{PSII} values. Effective quantum yield values were recalculated as a percentage of initial values.

Table 3 LT50 values of *Zygnema* sp. (strain MP2011Skan).

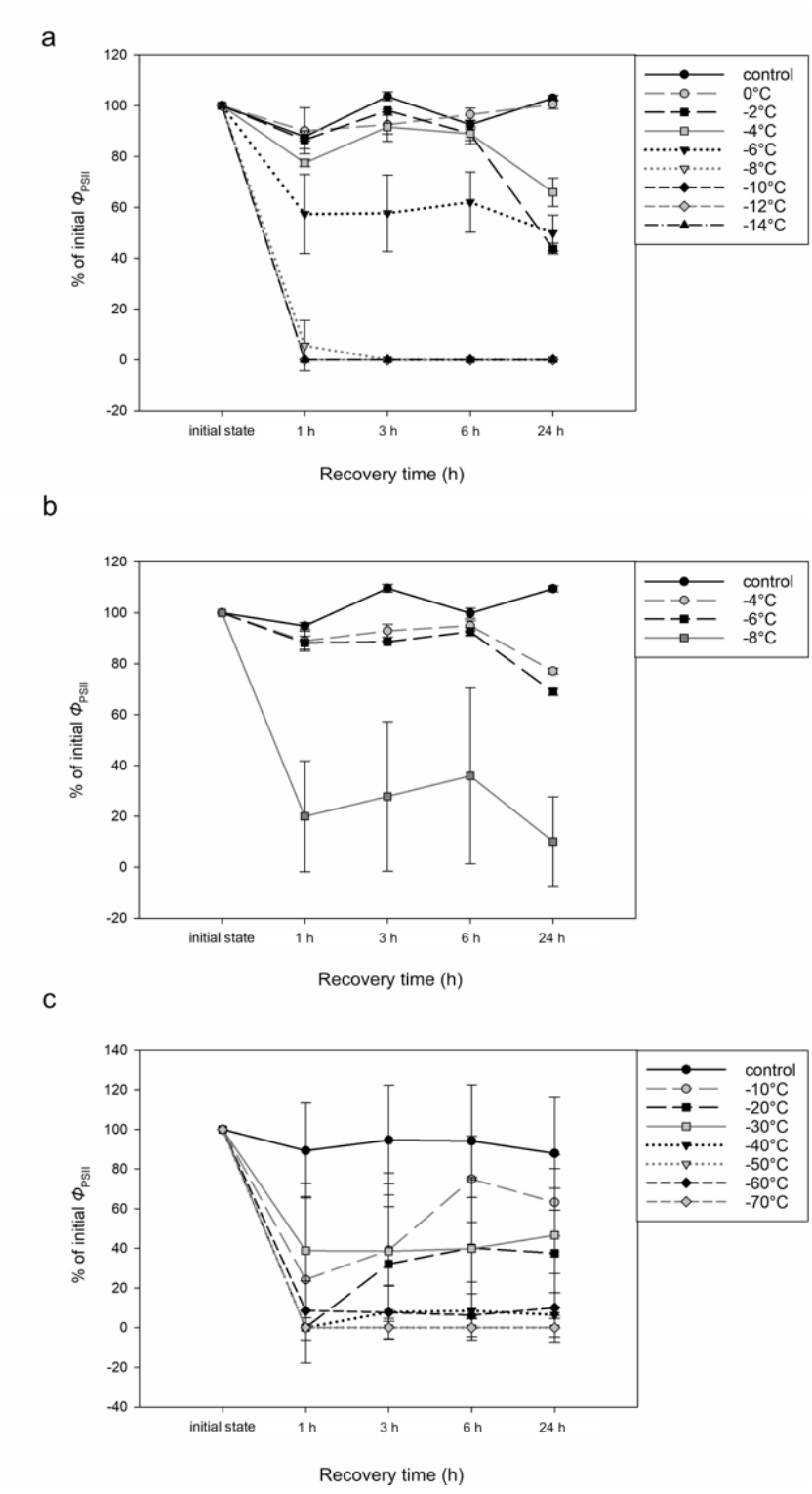


Fig. 1 Changes in steady-state quantum yield of PSII in the light (ΦPSII) of *Zygnema* sp. (strain MP2011Skan) measured before the experiments and during the recovery period. Values relative to the initial values before the experiment are shown (mean ± standard deviation): a, young cell, single freezing cycle; b, young cells, double freezing cycle; c, pre-akinetes

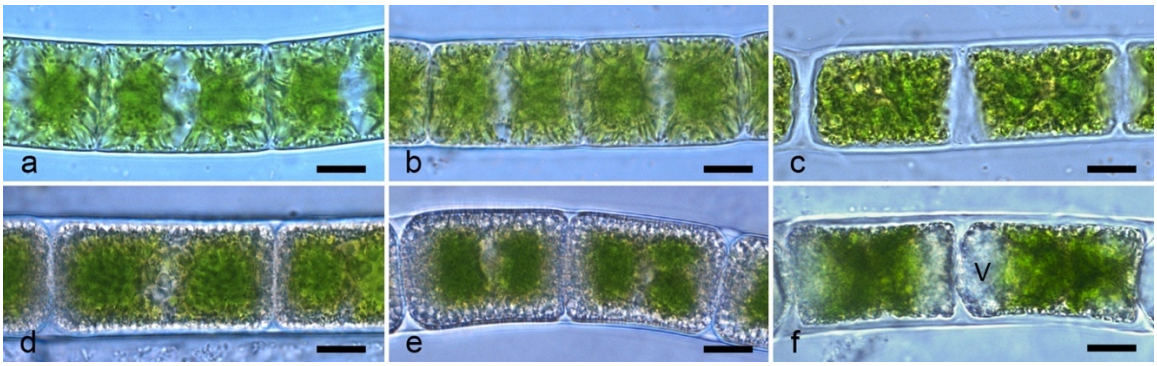


Fig. 2 Light micrographs of *Zygnema* sp. (strain MP2011Skan) before and after experimental freezing: a–c, young cultures (a, control; b, -2°C; c, -10°C); d–f, pre-akinetes (d, control; e, -20°C; f, -70°C, V vacuole). Scale bars 10 μm

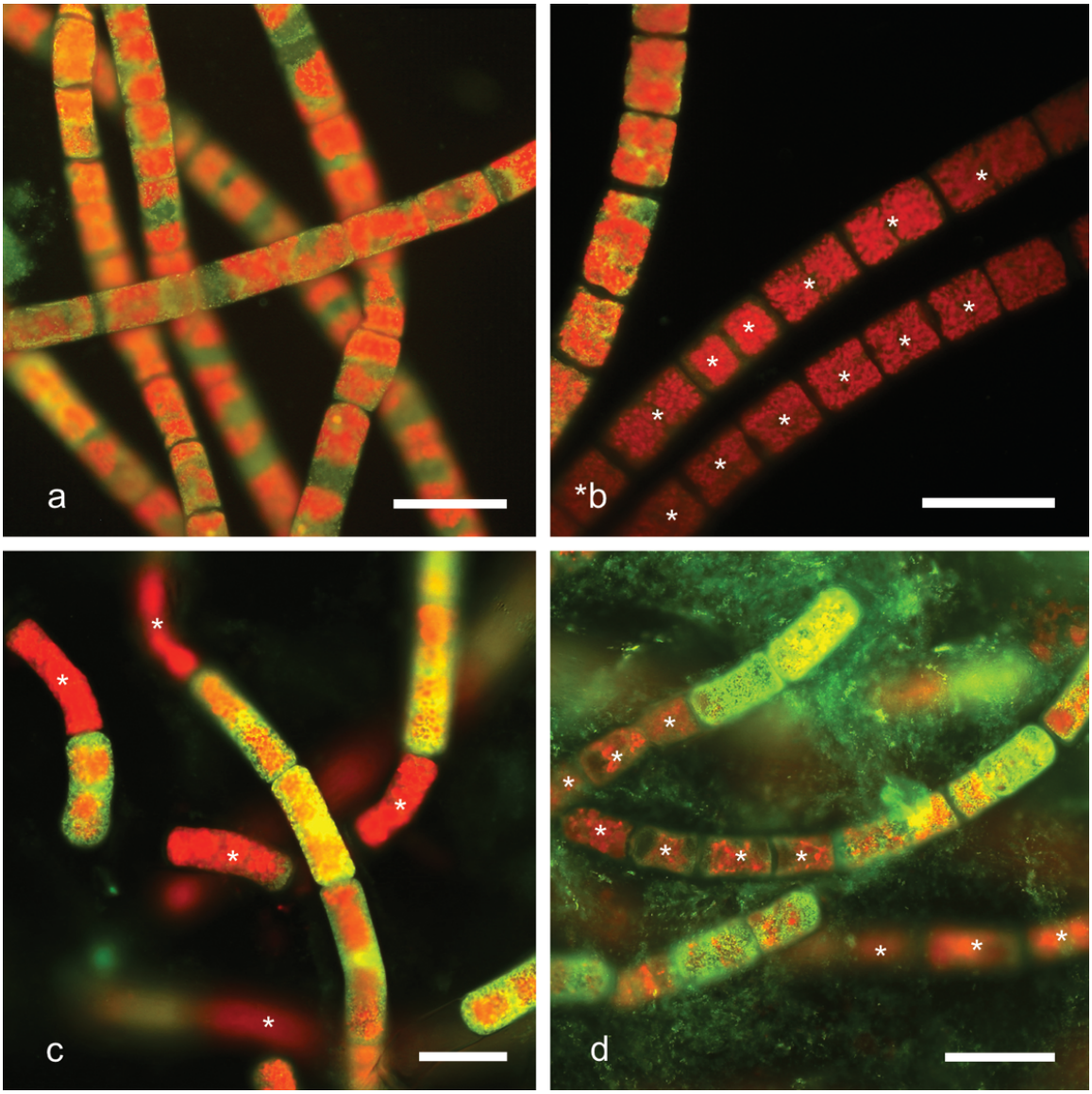


Fig. 3 Microphotographs of *Zygnema* sp. (strain MP2011Skan) stained with 0.1% Auramine O. Dead cells are marked with asterisks. a–b, young cultures (a, -2°C; b, -10°C); c–d, pre-akinetes (c, -20°C; d, -70°C). Scale bars, 40 μm

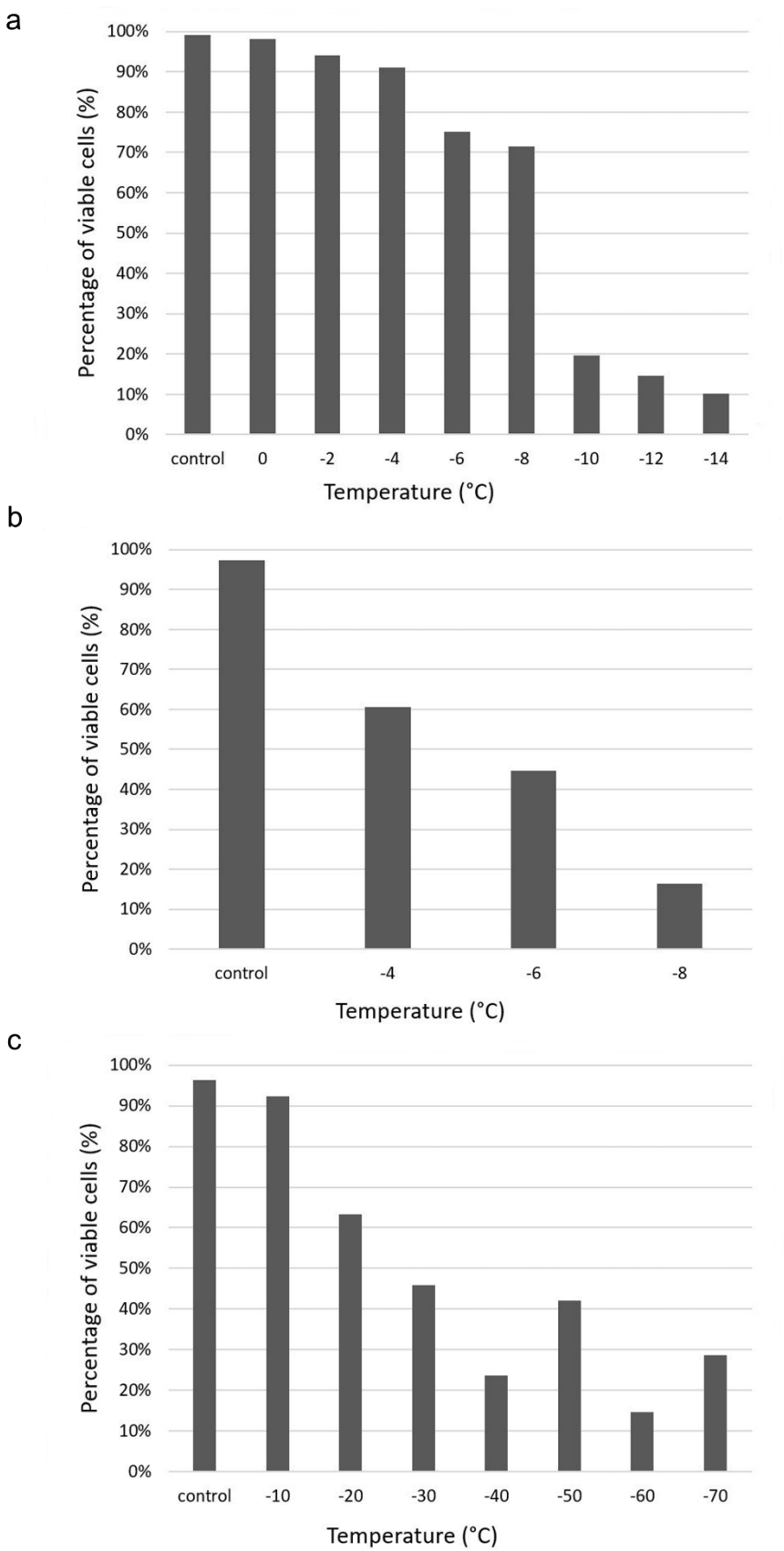


Fig. 4 Percentage of viable cells 1 h after freezing experiments. Cells were stained with Auramine O to detect viability

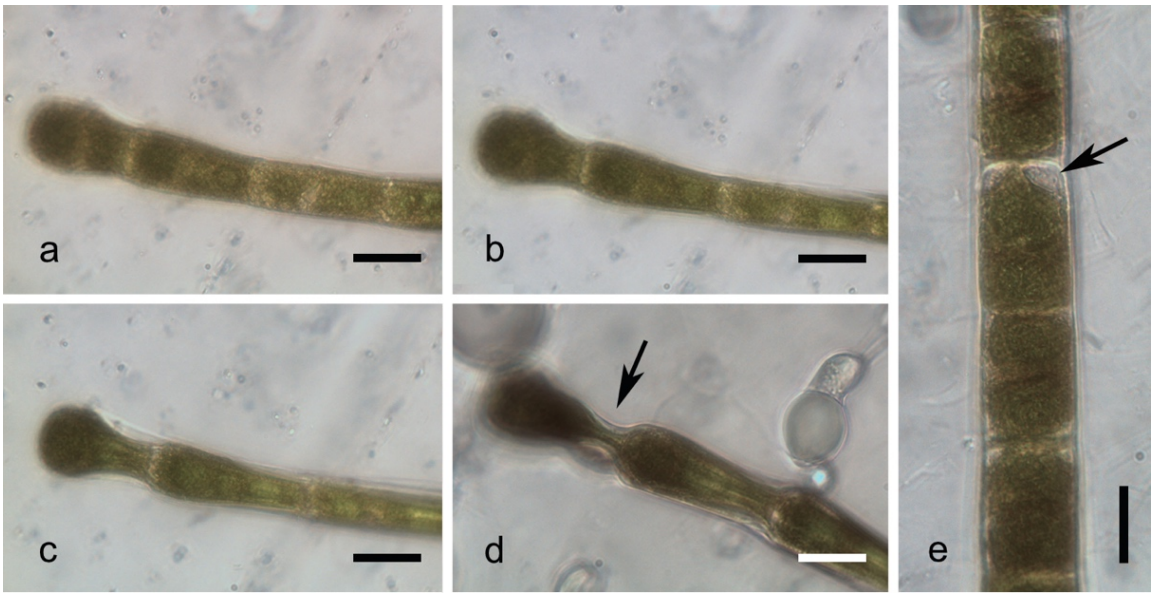


Fig. 5 Microphotographs of young vegetative filaments of *Zygnema* sp. (strain MP2011Skan) taken during the process of freezing (10 min at -10°C) by a light microscope equipped with a cooled holder. a – d, filament in the process of freezing showing the clavate shape of the cell (arrow); e, thawed filament, showing a damaged cell with broken plasma membrane (arrow). Scale bar, 20 μm

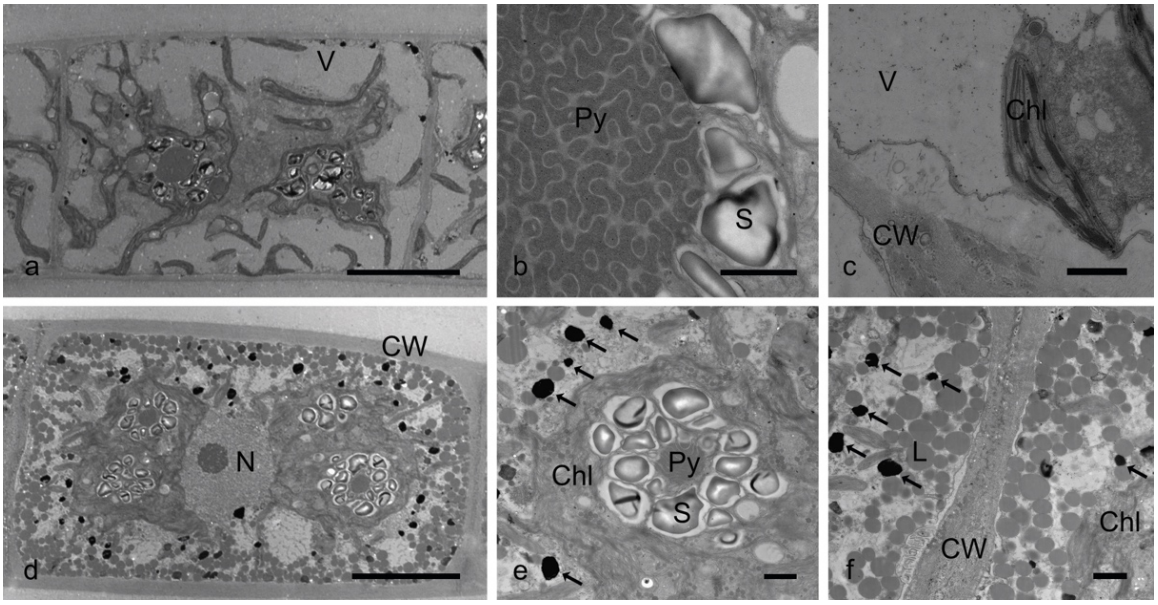


Fig. 6 Transmission electron micrographs of *Zygnema* sp. (strain MP2011Skan) control cells. a–c, young vegetative cell (a, whole cell with vacuoles; b, pyrenoid with starch grains; c, chloroplast, cell wall, and vacuole). d–f, pre-akinetes (d, whole cell with nucleus); e, chloroplast with reduced pyrenoid, electron-dense bodies (arrows); f, lipid bodies, thickened cell walls, electron-dense bodies (arrows). Chl chloroplast, CW cell wall, L lipid body, N nucleus, Py pyrenoid, S starch, V vacuole. Scale bars, 10 μm (a, d); 1 μm (b, c, e, f)

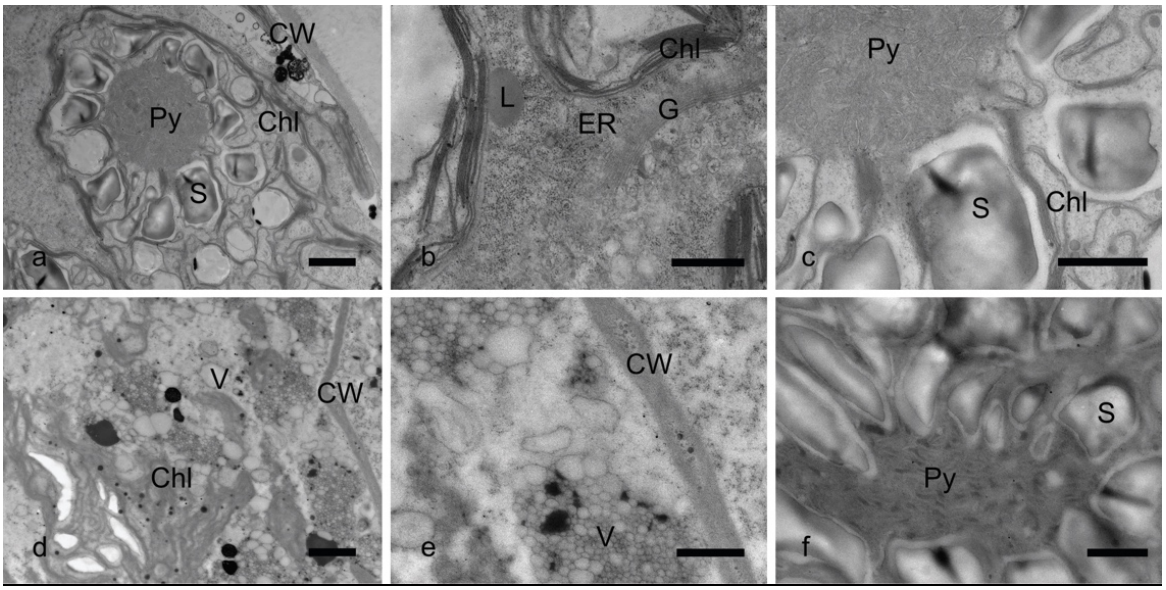


Fig. 7 Details of transmission electron micrographs of *Zygnema* sp. (strain MP2011Skan) young vegetative cells after freezing to different temperatures. a–c, -2°C (a, chloroplast with pyrenoid and starch grains; b, detail of thylakoids and Golgi apparatus; c, detail of the pyrenoid). d–f, -10°C (d, cell with destroyed protoplast; e, detail of the foamy structure in the cell; f, detail of the pyrenoid). Chl chloroplast, CW cell wall, ER endoplasmic reticulum, G Golgi body, L lipid body, Py pyrenoid, S starch, V vacuole. Scale bars, 20 μm (a, d); 1 μm (b, c, e, f)

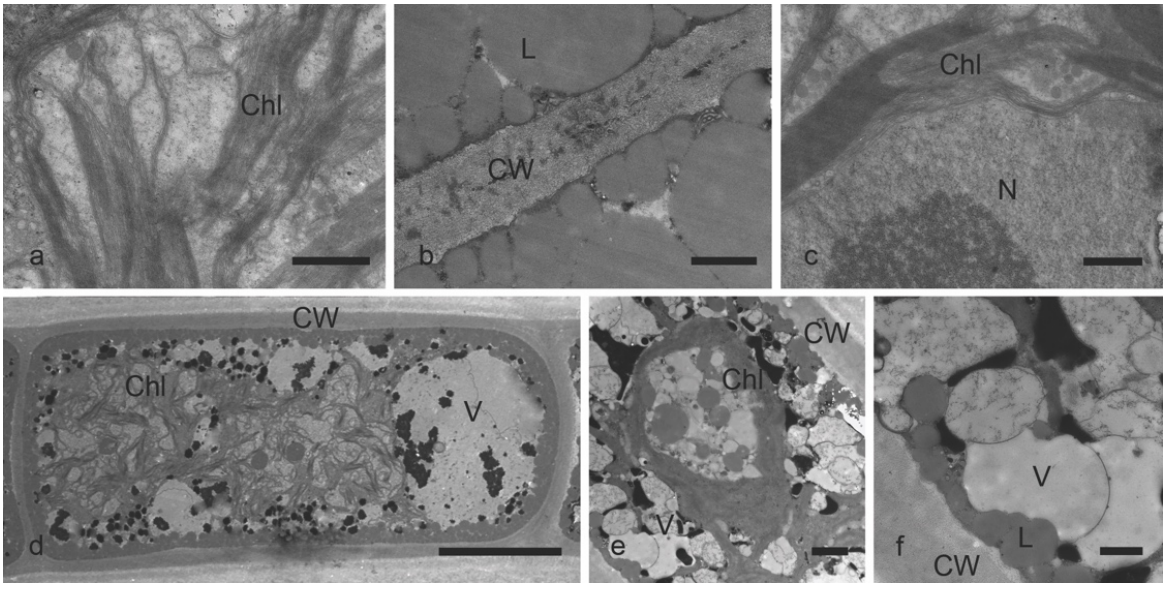
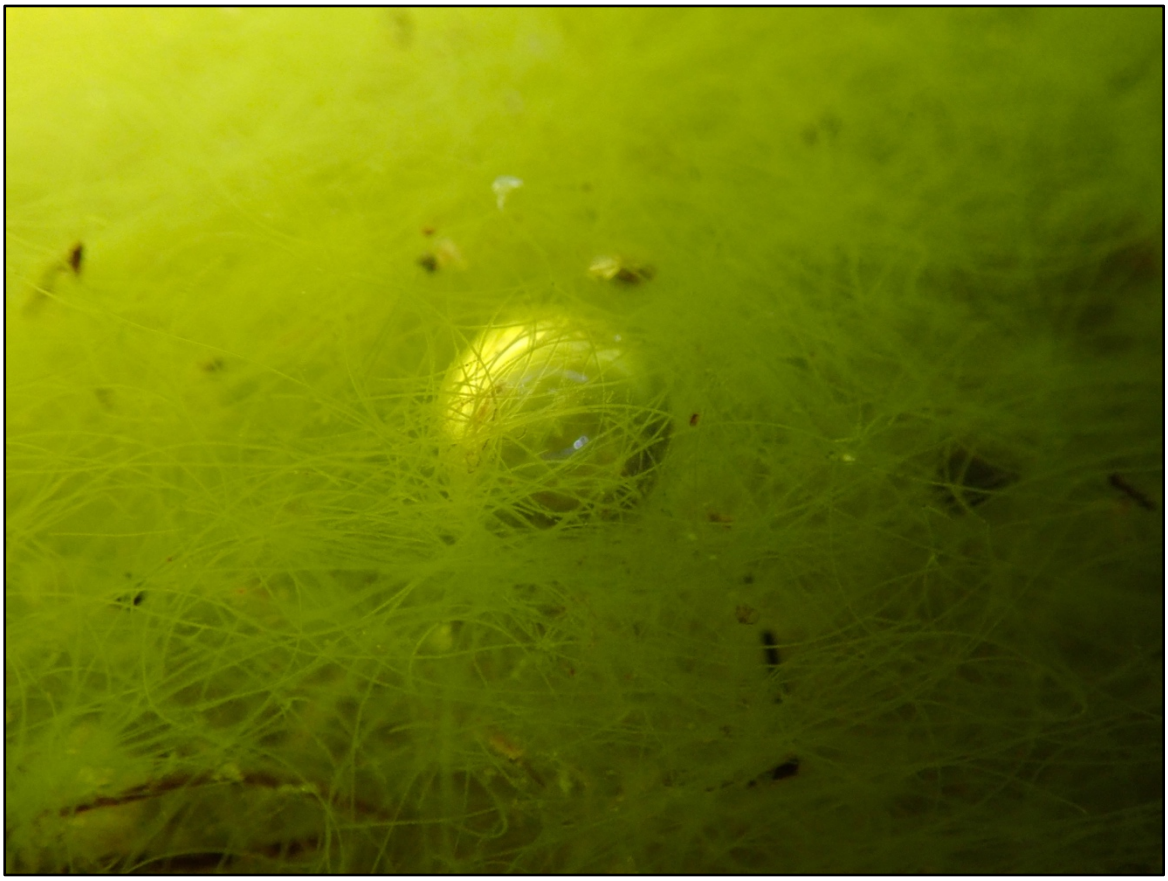


Fig. 8 Details of transmission electron micrographs of *Zygnema* sp. (strain MP2011Skan) pre-akinetes after freezing to different temperatures. a–c, -20°C (a, detail of chloroplast and unchanged thylakoids; b, large lipid globules and thick cell walls; c, nucleus). d–f, -70°C (d, cell with damaged protoplast, large vacuole on the right side, and electron-dense bodies marked by arrows; e, chloroplast with destroyed structure and electron-dense bodies marked by arrows; f, detail of vacuoles in the protoplast, lipid bodies, and electron-dense bodies marked by arrows). Chl chloroplast, CW cell wall, L lipid body, N nucleus, V vacuole. Scale bars, 20 μm (d); 1 μm (a, b, c, e, f)

Paper IV

Seasonal dynamics of *Zygnema* (Zygnematophyceae) mats from the Austrian Alps

Microbial Ecology



Seasonal dynamics of *Zygnema* (Zygnematophyceae) mats from the Austrian Alps

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Abstract

Filamentous green algae of the genus *Zygnema* are an essential part of hydro-terrestrial ecosystems. Despite several studies on their resistance to natural stresses, little is known about the composition of their assemblages and the changes they undergo over time. Two sites at altitudes above 2200 m a.s.l. in the Austrian Alps were selected for a two-year observation period and sampled five times. Molecular phylogenetic analysis of the 152 isolated strains of *Zygnema* sp. was performed based on the *rbcL* and *trnG* sequences. Seven genotypes were found at these sites during the samplings, but their proportion varied throughout the seasons. The site with a more stable water regime also had a more stable representation of genotypes, in contrast to the site with fluctuating water availability. The mats formed resistant pre-akinetes at the end of the season with reduced photosynthetic activity. Contrary to expectations, the mats were not exposed to extremely cold temperatures in winter due to snow cover. Some genotypes have been previously observed at this site, indicating that the population composition is stable. This work highlights the importance of resistant pre-akinetes in surviving winter conditions, the ability of algae to re-establish mats, and the need to address the hidden diversity of the genus *Zygnema*.

Key words: overwintering, cryptic diversity, chlorophyll fluorescence, freezing, hidden diversity

Introduction

Periodical changes in environmental conditions during the year are accompanied by weather changes (fluctuations in water availability, high or low temperatures, changing illumination levels, etc.), which often consist of periods of unfavorable conditions. These are particularly challenging for living organisms that must be adapted to surviving in varying environments. Pronounced seasonal differences and long periods of subzero temperatures are characteristic of both polar and high-altitude temperate regions [1]. Long winters act as the main stressor, resulting in a short growing season, which leads to a decline in higher plant vegetation. Thus, tundra ecosystems are dominated by non-vascular plants, including algae, which often form large-scale macroscopic mats [2–5]. Changing environment leads to the development of complex life cycles with different seasonal stages, which can ensure their long-term survival. In addition, patterns of changes in abundances of numerous algal genera have been documented [6].

Wet places in polar and high alpine tundra habitats are dominated by mucilaginous algal mats, often formed by filamentous conjugating green algae (Zygnematophyceae, Streptophyta). They have a simple morphology of unbranched filaments, and the group is characterized by a unique type of sexual reproduction called conjugation [7]. Conjugation results in a dormant zygospore, whose morphology is useful in traditional species determination [8, 9]. Moreover, conjugating algae are considered the closest relatives to land plants (Embryophyta); therefore, elucidating their stress tolerance and life strategies is important from an evolutionary point of view [10, 11].

Many studies have recently investigated the stress resistance of Zygnematophycean algae in the vegetative state [12, 13]. Zygnematophycean algae showed resistance to desiccation [14–16], osmotic stress [17, 18], ultraviolet (UV) irradiation [19–21] and freezing [22–24]. However, stress tolerance is often a characteristic of a single cell types. For example, there is a difference between the stress tolerance of young cells and hardened pre-akinetes of *Zygnema* sp. [14] or the green and purple morphs of *Zygogonium ericetorum* [21]. In contrast, unspecialized vegetative cells of *Zygnema* sp. showed high resistance to UV irradiation [25]. They are also sensitive to high temperatures [26]. In the genus *Serritaenia*, the UV resistance of vegetative cells was mediated by their pigment-rich mucilage envelopes [27]. *Spirogyra* sp. is known for its resistance in the vegetative stage, and it has been repeatedly found in Antarctic ponds, while no spore formation has been observed [28].

Despite the stress resistance of vegetative cells, field observations in different climatic conditions confirmed the seasonality of the mats formed by Zygnematophycean algae; characterized by new, fast-growing biomass developing every year [5, 28]. Seasonality was observed in several Zygnematophyceae, particularly *Spirogyra*, *Zygnema* and *Mougeotia* [28–31]. The mass growth of Zygnematophycean algae begins in spring, and their biomass volume usually peaks between May and September in temperate regions [29, 32]. Algal mats in the Arctic and Antarctic also showed higher abundances in spring and summer; however, this period is noticeably shorter there [5, 28]. The formation of lipid-rich pre-akinetes at the end of the growth season enabled survival through winter freezing [5, 33]. Mats of zygnematophycean algae usually grow quickly at the beginning of the growth season. *Spirogyra* can triplicate its biomass within only three days [31]. Spring coverage of the total water surface by floating algal mats can reach 70%–80% under suitable conditions [29].

Reproduction usually occurs during the spring and summer. In the Netherlands, [29], sexual reproduction of *Spirogyra* species was observed from April to August. The abundance of algal communities usually fluctuates throughout the year. The decline in conjugating-algal communities may be caused by summer drought [34] or anoxic conditions under ice [28].

Several studies have investigated the genetic diversity of *Zygnema* in Svalbard [14, 35], New Zealand [36], California [8], and Czechia [9]. As mentioned above, characteristics associated with sexual reproduction are used in species delimitation; however, only sterile populations are often found in field conditions. Thus, the diversity within the genus *Zygnema* is most likely underestimated, and an understanding of the seasonal pattern of individual species (strains) is still lacking. A recent study in Svalbard [4] showed unexpectedly high diversity hidden within the algal mats; the mats were composed up to four different genotypes. Zygospores of *Zygnema* and *Zygnemopsis* were found during sampling, but their occurrence was rare.

In the present study, we focused on the diversity within the mats formed by *Zygnema* sp. in the Alps and its changes within two years and different seasons. We hypothesized that, like in the Arctic, zygospores would be very rare and hidden diversity will be detected. Moreover, we aimed to understand which stress conditions directly affect the annual character of the mats and whether genotypes tended to inhabit one place for a longer time. The results showing trends in long-term persistence are discussed in the context of this alga's environmental conditions and stress resistance.

Materials and Methods

Studied sites

The field study was performed in the Austrian Stubai Alps, near Kühtai village. This site has an alpine tundra characteristic with a cold temperate climate. Summer stretches from the end of June to September [37]. The average annual temperature is 0.9 °C, and annual rainfall is 1653 mm [37]. Two localities with a high abundance of algal mats, designated "Lake" (47.2273°N, 11.0152°E, 2354 m a.s.l.) and "Streamlet" (47.2223° N, 11.0283° E, 2243 m a.s.l.), were selected for the long-time observation study. "Lake" was the tributary area of a stream flowing into a study site (Fig. 1a). Water was available throughout the year, and the conditions were relatively stable. Algal mats were distributed in the shallow waters of the tributary area and submerged most of the time during the vegetative season (Fig. 1b). "Streamlet" was a wet meadow (Fig. 1c), which was fed by melting water from the mountains above through small, branched streamlet. Algal mats were distributed throughout the wet area in the depressions filled with retained water (Fig. 1d). Water levels fluctuated strongly, from almost complete desiccation to a large amount of available slow-streaming water. The mats were subjected to repeated desiccation and were exposed to direct sunlight for most of the year.

Sampling pattern

Both localities were sampled five times: August and November 2018, and July, August, and October 2019. Sampling covered the vegetative seasons in both years, mainly summer and autumn, when the largest biomass was expected. Sampling was performed at multiple locations throughout the mat to ensure that the entire mat was covered. We used

120 mL plastic containers for sampling to collect a sufficient volume of biomass (approx. 20 mL) to estimate the proportion of pre-akinetes. The water temperature in the two observed sites was measured during the study period (from 21/8/2018 to 16/8/2020) using a Minikin Tie datalogger (Minikin Tie, ESM, Brno, Czech Republic) at 1-h intervals during the study. The dataloggers were placed close to the algal mats and secured using a cord. Data from the data loggers were graphically displayed using Sigma plot 14.0. The pH and conductivity of the water were measured during the first sampling (WTW pH/Cond 340i). To correlate the presence of mats and cell types with the water level in the localities, we created a scale of estimated water levels from 1–5, where 1 means completely dry, and 5 means high amount of water during the spring snowmelt. Scales applied to localities 1 and 2 are not fully comparable because of the different water regimes in both localities.

Four additional localities around Kühtai were also sampled once to determine the total diversity of *Zygnema* sp. in the region and to enrich the dataset for phylogenetic analysis: Lake Gossenkollesse (47.2302°N, 11.0136°E, 2417 m a.s.l.), Pond 1 Kühtai (47.2206°N, 11.0235°E, 2260 m a.s.l.), Pond 2 Kühtai (47.2214°N, 11.0240°E, 2268 m a.s.l.), and Upper Lake Kühtai (47.2284°N, 11.0089°E, 2436 m a.s.l.).

Cultivation and origin of strains, light microscopy observations and measurements

The samples were collected and subsequently transferred to the laboratory in a cooled box. They were microscopically examined for *Zygnema* sp. filaments, and the young vegetative cells/pre-akinetes ratio in each locality was estimated. Using a single-filament isolation method, we obtained 152 monoclonal cultures that were cultivated on Bold's basal medium (Merck, Germany). Additionally, four strains from Kühtai were obtained from a previous sampling campaign in 2015 and used in this study. Cultures were incubated at 18–19 °C and with continuous illumination at 40 mmol m^{-2} s^{-1} using 18 W cool fluorescent tubes (Philips TLD 18W/33, Royal Philips Electronics). The strains were microscopically examined after three weeks of cultivation. Microphotographs of the strains representing all clades were obtained using a Leica DM2000 LED equipped with a camera, Leica ICC50W, with the LASX program (Leica Microsystems). Microphotographs were graphically adjusted using Affinity Photo (Serif Ltd.). Thirty cells for each genotype were randomly chosen for width and length measurements in ImageJ software (http://imagej.nih. gov/ij). To reveal possible significant differences between strains, measured cellular width and length were tested by one way analysis of variance (ANOVA) followed by Tukey's post hoc test in PAST 4.10 [48].

DNA isolation, PCR reaction, sequencing

Genomic DNA was extracted using the Instagene matrix (Bio-Rad), according to a previous protocol [38]. Two molecular markers were used for the phylogenetic analysis: plastid *rbc*L and *trn*G. The *rbc*L region was amplified using the primers RH1 and 1385R [39] or ZygF and ZygR [4]. Primers trnG-uuc-F-5′ and trnG-uuc-R-3′ [40] were used to amplify the *trnG* intron. The PCR reactions were carried out in 20 µL volumes: 13.9 µL of sterile Mili-Q water, 2 µL of $MgCl_2$ (25 µM), 2 µL of PCR Buffer 10× (Applied Biosystems), 0.4 µL of dNTP, 0.25 µL of each primer, 0.2 µL of AmpliTaq GOLD polymerase (5 U/µL), and 1 µL of template DNA (not quantified). PCR amplification of the *rbcL* region (temperatures and times for the *trnG* intron are given in brackets) was set to an initial denaturation at 95 °C

for 10 min (94 °C for 2 min), followed by 35 (40) cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min, elongation at 72 °C for 2 min (2,5 min), and a final extension at 72 °C for 10 min. The PCR products were purified using AMPure XP beads (Beckman Coulter, Inc.) and sequenced using an Applied Biosystems automated sequencer (ABI 3730xl) by Macrogen (Seoul, Korea). Sequencing reads were assembled and edited using the SeqAssem program [41]. The obtained unique sequences were concatenated using the MEGAX program [42], assigned with capital letters Z1–Z7, and submitted to GenBank under accession numbers ON338067, ON338068 and ON989204 to ON989211 for *the rbcL* region and ON980776 to ON980783 for the *trnG* region.

Sequence alignment and phylogenetic analysis

The concatenated phylogeny of the plastid *rbcL* gene and *trnG* intron was inferred to illustrate the phylogenetic position of the newly obtained *Zygnema* strains. The dataset of 152 newly isolated strains was enriched with 24 sequences collected from Svalbard and the Czech Republic [4] and sequences obtained from public databases. A total of 180 strains were analyzed. Sequences were aligned using MEGA X [42] and trimmed manually. The most appropriate substitution model was estimated for each data set using the Bayesian information criterion (BIC) with jModeltest 2.1.4 [43]: GTR+I for the first, JC for the second, and HKY+Γ for the third codon position of the *rbcL* dataset and HKY+G model for the *trnG* alignment. The phylogenetic tree was inferred by Bayesian inference (BI) using MrBayes version 3.2.6 [44]. Bootstrap analyses were performed using maximum likelihood (ML) criteria on GARLI version 0.951 [45]. The phylogenetic tree obtained was displayed in FigTree [46] and Mega X [42]. Finally, the displayed phylogenetic tree was graphically adjusted using Affinity Designer (Serif Ltd.).

Chlorophyll a fluorescence

The photosynthetic activity was assessed using chlorophyll fluorescence. Biomass samples for fluorescence measurements were harvested from Lake and Streamlet in August and October 2018 and transported to the laboratory. Prior to the measurement, the samples were dark-adapted for 30 min. The maximum quantum yield (F_V/F_M) and relative electron transport rate (rETR) curves were measured using a pulse-amplitude-modulated fluorometer (FluorCam, PSI, Czech Republic). The light response curves showed photoinhibition and were fitted according to Webb et al. [47]. The parameters rETRmax (μmol electrons m^{-2} s^{-1}), α (electrons $photon^{-1}$), and I_k (μmol) were calculated. The difference in the values of the calculated fluorescence parameters between seasons was tested using the t-test in PAST [48].

Results

Phylogenetic diversity of mats formed by Zygnema sp.

Phylogenetic analyses revealed seven *Zygnema* sp. genotypes in the Kühtai region (Fig. 2). These genotypes were distributed among other *Zygnema* strains in both major clades of the genus. Most strains isolated during sampling in Kühtai belonged to genotype Z1 (Fig. 2). This lineage mainly comprised isolates from the Lake (66 isolates), Streamlet (10), Lake Gossenkollesse (7), and two in ponds 1 and 2. Genotype Z2 was represented by fewer isolates

from the Lake (8), upper lake (2), and pond 2 (2). Lineages Z3 and Z5 comprised exclusively the isolates from Lake and Streamlet. Genotype Z4 comprised isolates from Streamlet (2) and upper lakes (2). Genotypes Z6 and Z7 belonged to Clade 2. Lineage Z6 comprised isolates from Lake (1) and Streamlet (16), together with the two isolates from 2015. Genotype Z7 comprised exclusively isolates from Lake (2) and Streamlet (4). The number of isolates found in all localities and their lineages are summarized in Table 1. Four isolates of *Zygnema* sp. found in 2015 by Martina Pichrtová clustered together with those identified during the observation period. Isolate Kühtai 8 was identical to other isolates of genotype Z1, and isolates Kühtai 5 and 7 belonged to genotype Z6. Isolate Kühtai 4 was not detected during the observation period.

Vegetative morphology

Isolated strains from seven Kühtai lineages shared a similar vegetative morphology typical of this genus (Fig. 3 a–g). The young vegetative cells had two stellate chloroplasts, and vacuoles occupied most of the remaining space in the protoplast. Pre-akinetes showed only imperceptible vacuolization, while the cells were full of storage lipids, and chloroplasts were reduced. We did not observe any conjugation or zygospore formation despite our sampling efforts.

Width of cells varied from 24.5 μm in Z5 (mean value, ±SD 0.72) to 26.6 μm in Z3 (mean value, ±SD 1.63). Cell lengths varied from 37.4 μm in Z6 (mean value, ±SD 9.81) to 52.5 μm in Z1 (mean value, ±SD 8.16). Widths and lengths of cells measured for each genotype are given in Supplementary Information 1. Cell length varied significantly among some strains, but cell width, on the contrary, was quite uniform. Only the difference between strains Z3 and Z5 was proven to be weakly significant ($p = 0.01061$). Complete results of statistical analyses are given in Supplementary Information 2.

Seasonal changes of Zygnema sp. mats and their persistency

The two sampling sites differed in diversity throughout the year (Fig. 4). Genotype Z1 prevailed in the Lake during all sampling periods (Fig. 4a). Additionally, the less abundant genotypes Z2, Z5, Z6, and Z7 inhabited the Lake. However, they occurred only in small volumes and were not present year-round. The Streamlet showed a higher diversity of *Zygnema* sp. In total, six different genotypes were identified during sampling on this site. The prevailing genotype differed among samplings on the Streamlet locality (Fig. 4b). Genotypes Z3 and Z6 prevailed during the four sampling periods when the algal mats were present. Sampling sites differed in the average number of genotypes found during the sampling; we found 1–3 genotypes in the Lake contrary to the 2–5 genotypes in the Streamlet. No algal mats were found at the sites during the two sampling events: in the Streamlet in November 2018 and the Lake in July 2019.

Pre-akinetes were found in both areas. Their proportion in mats fluctuated from 5%–95%, depending on the season and locality. The lowest number of pre-akinetes (5%–10%) were observed during the vegetative period in July and August. We observed massive production of pre-akinetes during the autumn, up to 95% of cells in the pre-akinete stage in October and November (Fig. 5).

Seasonal changes of the habitats

Changes in water availability at the two observed localities were recorded during each sampling. The sampling sites differed in their water regime. Water availability in the Lake fluctuated between levels 3 and 5 (Fig. 5), where 3 meant a stable water availability, while 5 referred to a high amount of water during spring snow melting. We observed stable conditions in the Lake, with no periods of drought, except in winter, characterized by frozen water.

In contrast, the water regime in the Streamlet differed fundamentally. The sampling site experienced significant changes in water availability (Fig. 5). Water levels fluctuated from levels 2 to 4, where 2 meant almost dry, while 5 meant a completely wet locality with a strong current of the streamlet and depressions filled with a large amount of retained water. Algal mats were exposed to desiccation events when water levels dropped, and the biomass was exposed to air. Water conductivity and pH measured during the first sampling was 0.04 mS cm^{-1} and 6.8 in the Streamlet and 0.06 mS cm^{-1} and 7.6 in the Lake, respectively.

The temperature of the water measured by the Minikin sensors and the average air temperature in Kühtai are shown in Fig. 6. The lowest and highest recorded water temperature at the Lake and Streamlet were –0.2 °C (21.11.2018) and 20.7 °C (25.07.2019), and –0.04 °C (26.11.2018) and 21.3 °C (31.08.2019), respectively. The average winter temperature of the water in the Lake and Streamlet was 0.31 °C and 0.9 °C in 2018 and 2019 (from November to April), respectively. The average winter temperature of the air in Kühtai measured from November to April was –5.3 °C (data collected from 1999 to 2019) [37]. The average daily minimum and maximum air temperature was –13.7 °C in January and 15.4 °C in August, respectively [37].

Photosynthetic activity during the year

Both localities showed similar declines in photosynthetic activity throughout the season. The maximum quantum yield reached significantly lower values at the end of the season than that in summer at both localities, although the actual values differed only slightly in the Lake (Table 2). A clear difference in photosynthetic performance was revealed by the rETR curves (Fig. 7, Table. 2). At both sampling sites, autumn samples showed clearly different kinetics from that of summer samples: mean rETR max values decreased significantly in both the Lake and Streamlet. The decrease in α and I_k values was significant only for the Streamlet. All parameters and t-test results are summarized in Table 2.

Discussion

In this study, mats of *Zygnema* sp. growing in the alpine tundra habitat were thoroughly investigated for the first time. We found eight different genotypes within the study area, seven during this investigation, and one additional unpublished genotype collected in 2015. In both studied localities, pre-akinetes formed at the end of the vegetative season. This adaptation helps the population overcome an unpleasant winter period.

Diversity of Zygnema sp. in the studied region

The diversity within the mats of *Zygnema* sp. growing in the alpine tundra habitat was unexpectedly high. Seven genotypes, assessed by *rbcL* and *trnG* sequences, grew in the Kühtai streams. Another unique *rbcL* genotype was reported in the same study area in 2015 and added

to our dataset. None of the 152 isolated strains exhibited any morphological characteristics related to sexual reproduction, restricting species determination. Therefore, we used the term "genotype" instead of "species." Genotypes growing in Kühtai were distributed in clades 1 and 2 (Fig. 2). Although the DNA sequences of most of the genotypes were not identical to any *Zygnema* strains from public databases, they should not be considered endemic to the region because the diversity of this genus has not been satisfactorily investigated yet, and recent molecular diversity studies revealed previously unknown genotypes [4, 8]. Moreover, there is no working species concept based on which species can be reliably distinguished. Traditional taxonomy based on morphological characters associated with sexual reproduction is not applicable to most natural samples because of the rarity of sexual reproduction. Furthermore, the conjugation of *Zygnema* has not yet been successfully induced. In contrast to our previous study from the Arctic, the Alpine strains did not show considerable variation in cell width [4]. In the current study, only a small area was investigated, while the sampling sites in the Arctic covered a larger region with a high variation of abiotic conditions [4] which might be reflected by morphological differences. Variations in cell size can also be explained by differences in genome size. However, only little is known about genome size variation in *Zygnema* [49] and its correlation with *Zygnema* phylogeny and morphology is currently under investigation.

Spatial and temporal dynamics of Zygnema sp. diversity

The mats were usually formed by several *Zygnema* sp. genotypes growing together (Fig. 4). A similar phenomenon of up to four genotypes in one mat was previously observed in the tundra habitats of Spitzbergen, which are very similar to high alpine habitats [4]. In the genus *Spirogyra*, up to three genotypes have been observed to grow together in one location [50, 51]. Sympatric occurrence has also been described for other microalgae such as desmids [52] or diatoms [53].

Seasonal variation in the proportion of genotypes differed among the two localities. The total diversity was higher at the Streamlet than that at the Lake, and the Lake showed higher stability in genotype occurrence than the Streamlet (Fig. 4). This difference cannot be explained based on climatic conditions, which are identical over such small areas. However, both localities differed in the fluctuation of water availability. The algae do not have enough time to grow to large volume of biomass in the Streamlet because of the fluctuation in water level, resulting in the mats being constantly renewed from a small number of cells. This is incongruence with the intermediate disturbance hypothesis, a general ecological rule describing the positive effects of a certain level of disturbance on species diversity [54, 55]. Nevertheless, the rediscovery of two identical genotypes that occurred at the site Streamlet 4 years prior to this research indicates that the species pool is stable at this site for a longer period.

Moreover, the Streamlet sampling site showed greater diversity compared to the Lake, and had several distinct microhabitats, which may have contributed to the persistence of this diversity, as different genotypes might have different microhabitat preferences. For example, this phenomenon has been observed in the diversity of Arctic *Prasiola* sp., whose occurrence was correlated with natural conditions [56]. Ryšánek et al. [57] suggested that different lineages of *Klebsormidium* are adapted to the substrate on which they originally occur, independent of their evolutionary distance, and closely related lineages differ

ecophysiologically to the same extent as unrelated clades. Hainz et al. [58] observed larger vegetative *Spirogyra* sp. filaments in nutrient-rich waters, whereas morphotypes with narrower cells occurred in nutrient-poor water.

Seasonal changes in morphology and physiology

No sexually formed zygospores were observed at these sites. However, their presence cannot be completely ruled out given that we did not study soil samples. Nevertheless, at both sites, pre-akinete formation occurred at the end of the growing season (Fig. 5). Pre-akinetes are mature lipid-rich vegetative cells with thick cell walls and distinct mucilage layers [16, 18]. Pre-akinete formation is generally associated with nutrient deficiency [14], as metabolism shifts from the formation of nitrogen-rich proteins toward nitrogen-free sugars and lipids [59]. In addition, acclimation via slow desiccation promotes stress tolerance in pre-akinetes [14]. Pre-akinetes are resistant to many stresses, including drought [14] and frost [22, 24], and their growth ensures the population's survival under adverse conditions.

Seasonal production of pre-akinetes has been observed several times in algae of the genus *Zygnema* and seems to be independent of climatic conditions, as has been observed at both the Arctic [5] and Texas [60]. The decrease in temperature in autumn (Fig. 6) and the associated slowing of metabolic processes may lead to cell starvation, promoting this process. A decline in physiological activity was also observed in their photophysiological response. Pre-akinetes always reach a lower maximal electron transport rate ($rETR_{max}$) than young vegetative cells [61]. Nevertheless, the decline in F_V/F_M, alpha, and I_K was not significant in the Lake despite pre-akinete formation. This was due to water availability. Similarly, pre-akinetes were formed in all types of localities in the Arctic, but populations that had experienced natural drought were photosynthetically less active and stress-tolerant [18].

At both sites, periods of macroscopic disappearance of *the Zygnema* sp. populations were observed. In the Streamlet, no biomass was present in the autumn of 2018, despite there being sufficient water at the site (Fig. 5). The algal population was visibly present in noticeably smaller numbers at the following observation in July 2019, and gradually increased over the year (Fig. 4). This could be due to the previous drying of the site in the summer or a strong current in the stream that carried biomass away [34]. In the Lake, the biomass disappeared in July 2019 but rejuvenated quickly (Fig. 5). This rapid recovery is due to resilient pre-akinetes. Their presence was observed at 5%–10%, even during the period when young vegetative cells were predominant (Fig. 5). This small proportion of mats is constantly prepared for unfavorable conditions, even during the vegetative season, which substantially increases the chances of long-term survival [5, 24].

How Zygnema sp. survives winters

Because the observed sites were used as ski slopes during winter, it was impossible to carry out sampling under the snow. However, considering the small amount of biomass present at the sites in spring (Fig. 4), it can be assumed that most of the cells in the mat do not survive winter likely due to freezing stress. Therefore, we hypothesized that winter temperatures in Kühtai are too low for the survival of most *Zygnema* sp. cells. However, this hypothesis could not be proven, as snow cover effectively insulated the soil surface

and protected it from subzero temperatures (Fig. 6). Similarly, Hawes [3] reported that the air temperature of the Antarctic study sites fell to –25 °C, while the temperature under thick ice (30–50 cm) was only –4 °C. Steiner et al. [62] reported winter survival of *Micrasterias denticulata* (Zygnematophyceae) in unfrozen peat bog pools despite air temperature minima went down to −17.3 °C. Pichrtová et al. [5] also showed survival of *Zygnema* sp. under ice.

Moreover, recent laboratory experiments showed that *Zygnema* sp. vegetative cells can survive at –8 °C, and pre-akinetes at even lower temperatures [22]. Therefore, we can assume that even direct exposure to air temperature at our sampling sites would not be lethal to the entire population of *Zygnema.* Adaptation of vegetative cells to winter is crucial and has been observed not only in the genus *Zygnema* but also in other algae such as *Spirogyra* sp. [28], *Klebsormidium* sp. [63, 64], the genus *Tribonema* [65], and diatoms [66]. Adaptation to frost is related to the ability of cells to acclimatize to it, which has been observed in the genus *Klebsormidium* [63]. *Klebsormidium crenulatum* showed reinforcement of cells by additional wall layers during freezing stress [64]. Resistance to frost is universally present among organisms [67], so it can be assumed that algae of the genus *Zygnema* have this ability, although this has not been studied yet.

In addition to low freezing conditions, other stress factors are also associated with winter. Hawes [28] observed a winter decline in *Spirogyra* population in a lake in Antarctica, resulting in the death of almost the entire population because of the lack of light and anoxic conditions. Although the winter conditions in the Alps are not extreme, there is undoubtedly a lack of light owing to shorter days and snow cover. However, low or undetectable respiration at low temperatures in Alpine *Zygnema* [61] and other Alpine or polar terrestrial green algae [68, 69] indicated good adaptation against potential cellular carbon loss during long winter periods in the dark under snow cover.

Conclusion

This study provides the first insight into the seasonal dynamics of mats formed by *Zygnema* sp. in the Alps, based on phylogenetic classification. Algae of this genus exhibit distinctive seasonal dynamics. The largest stands were observed during the summer growing season, followed by gradual dieback and the formation of pre-akinetes. During autumn, virtually the entire population forms resistant pre-akinetes, which have reduced photosynthetic activity and can survive under adverse conditions. Annual mats are renewed each year because of the small number of cells that survive winter. Contrary to our expectations, the algae in this habitat were not exposed to extreme winter temperatures. However, snow cover that insulates the algae also prevents access to light throughout the winter, which is a probable reason for the survival of a small number of cells after winter. The individual mats consisted of up to five genotypes, which varied in their proportions during the observation period. Seasonal changes in genotype composition probably reflect only local site conditions, while diversity remains stable over a more extended period. Even after four years, the same genotypes were found at the observed sites. However, further work is needed to study the ecology and diversity of this alga, specifically for future taxonomic use.

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Tables and figures

Observation	Month of collection	Locality	Genotype	Number of isolates	Code in the phylogenetic tree	Coordinates	*rbcL* Genebank accession number	*trnG* Genebank accession number
Isolates found during the observation period								
1	August 2018	Lake (1)	Lineage Z1	2	K13L-1	47.2273°N, 11.0152°E	ON338067	ON980776
		Streamlet (2)	Lineage Z1	2	K15S-1	47.2223°N, 11.0283°E	ON338067	ON980776
		Streamlet (2)	Lineage Z3	1	K15S-3	47.2223°N, 11.0283°E	ON989204	ON980778
		Streamlet (2)	Lineage Z6	5	K15S-5	47.2223°N, 11.0283°E	ON989207	ON980781
		Lake Gossen-kollesse (3)	Lineage Z1	7	K12G-1	47.2302°N, 11.0136°E	ON338067	ON980776
		Pond 1 Kühtai (4)	Lineage Z1	1	K11P1-1	47.2206°N, 11.0235°E	ON338067	ON980776
		Pond 2 Kühtai (5)	Lineage Z1	1	K14P2-1	47.2214°N, 11.0240°E	ON338067	ON980776
		Pond 2 Kühtai (5)	Lineage Z2	2	K14P2-2	47.2214°N, 11.0240°E	ON338068	ON980777
2	November 2018	Lake (1)	Lineage Z1	27	K23L-1	47.2273°N, 11.0152°E	ON338067	ON980776
		Lake (1)	Lineage Z5	2	K23L-5	47.2273°N, 11.0152°E	ON989206	ON980780
3	July 2019	Streamlet (2)	Lineage Z3	3	K35S-3	47.2223°N, 11.0283°E	ON989204	ON980778
		Streamlet (2)	Lineage Z5	1	K35S-5	47.2223°N, 11.0283°E	ON989206	ON980780
4	August 2019	Lake (1)	Lineage Z1	12	K43L-1	47.2273°N, 11.0152°E	ON338067	ON980776
		Lake (1)	Lineage Z2	8	K43L-2	47.2273°N, 11.0152°E	ON338068	ON980777
		Lake (1)	Lineage Z7	7	K43L-7	47.2273°N, 11.0152°E	ON989208	ON980782
		Upper lake Küh-tai (6)	Lineage Z2	2	K42UL-2	47.2284°N, 11.0089°E	ON338068	ON980777
		Upper lake Kühtai (6)	Lineage Z4	8	K42UL-4	47.2284°N, 11.0089°E	ON989205	ON980779
		Streamlet (2)	Lineage Z1	6	K45S-1	47.2223°N, 11.0283°E	ON338067	ON980776
		Streamlet (2)	Lineage Z3	7	K45S-3	47.2223°N, 11.0283°E	ON989204	ON980778
		Streamlet (2)	Lineage Z4	3	K45S-4	47.2223°N, 11.0283°E	ON989205	ON980779
		Streamlet (2)	Lineage Z6	1	K45S-6	47.2223°N, 11.0283°E	ON989207	ON980781
		Streamlet (2)	Lineage Z7	1	K45S-7	47.2223°N,	ON989208	ON980782
5	October 2019	Lake (1)	Lineage Z1	25	K53L-1	47.2273°N, 11.0152°E	ON338067	ON980776
		Lake (1)	Lineage Z6	1	K53L-6	47.2273°N, 11.0152°E	ON989207	ON980781
		Streamlet (2)	Lineage Z1	2	K55S-1	47.2223°N, 11.0283°E	ON338067	ON980776
		Streamlet (2)	Lineage Z3	4	K55S-3	47.2223°N, 11.0283°E	ON989204	ON980778
		Streamlet (2)	Lineage Z6	8	K55S-6	47.2223°N, 11.0283°E	ON989207	ON980781
		Streamlet (2)	Lineage Z7	3	K55S-7	47.2223°N, 11.0283°E	ON989208	ON980782
Other isolates used in the study								
	2015		Kühtai 4	1	Kühtai-4 2015	N/A	ON989211	N/A
	2015		Lineage Z6	1	Kühtai-5 2015	N/A	ON989210	ON980783
	2015		Lineage Z6	1	Kühtai-7 2015	N/A	ON989210	ON980783
	2015		Lineage Z1	1	Kühtai-8 2015	N/A	ON989209	ON980778

Table 1 Complete list of new strains isolated from mats on localities during five samplings from 2018-2019.

Locality	Season	F_V/F_M	*p-value*	$rETR_{max}$	*p-value*	alpha	*p-value*	I_k	*p-value*
Lake	August	0.69 ± 0.03	0.0076	108.8 ± 8.1	< 0.0001	0.5 ± 0.03	0.052	274.2 ± 58	0.16
	October	0.64 ± 0.02		61.1 ± 8.1		0.4 ± 0.06		210.4 ± 66.8	
Streamlet	August	0.74 ± 0.01	0.0008	134.4 ± 12.9	< 0.0001	0.6 ± 0.01	0.0001	359.1 ± 40.8	< 0.0001
	October	0.61 ± 0.01		41.5 ± 6.9		0.4 ± 0.06		141.3 ± 28.5	

Table 2 Summary of the calculated chlorophyll a fluorescence parameters. All values are averages derived from 6 independent measurements (n = 6). The difference between August and October values was tested by T test, and p-values were given. Parameters of the light curves are described from the curves fitted according to Webb et al. [47].

Fig. 1 Localities in Kühtai where long-term observation was performed. a: Lake locality, algal mats were located in the tributary area of the Lake in a stream – marked with arrow; b: detail of the algal mat in the stream water on the Lake locality; c: Streamlet locality – stream on a wet meadow, algal mats were distributed through the wet area in the depressions in-ground and a branched streamlet – marked with arrow; d: detail of the algal mat in a depression filled with retained water on the Streamlet locality.

Fig. 2 Phylogenetic tree based on the Bayesian analysis of the concatenated *rbcL* and *trnG*^{UCC} intron sequences. Strains of *Zygnema* sp. collected during the long-term observation in Kühtai are shown in bold. Values at the nodes indicate statistical support estimated by MrBayes posterior node probability and maximum likelihood bootstrap. Number of isolates per locality and sampling is given in brackets. Full statistical support is marked with an asterisk. Branch lengths within the outgroup were shortened to improve visualization.

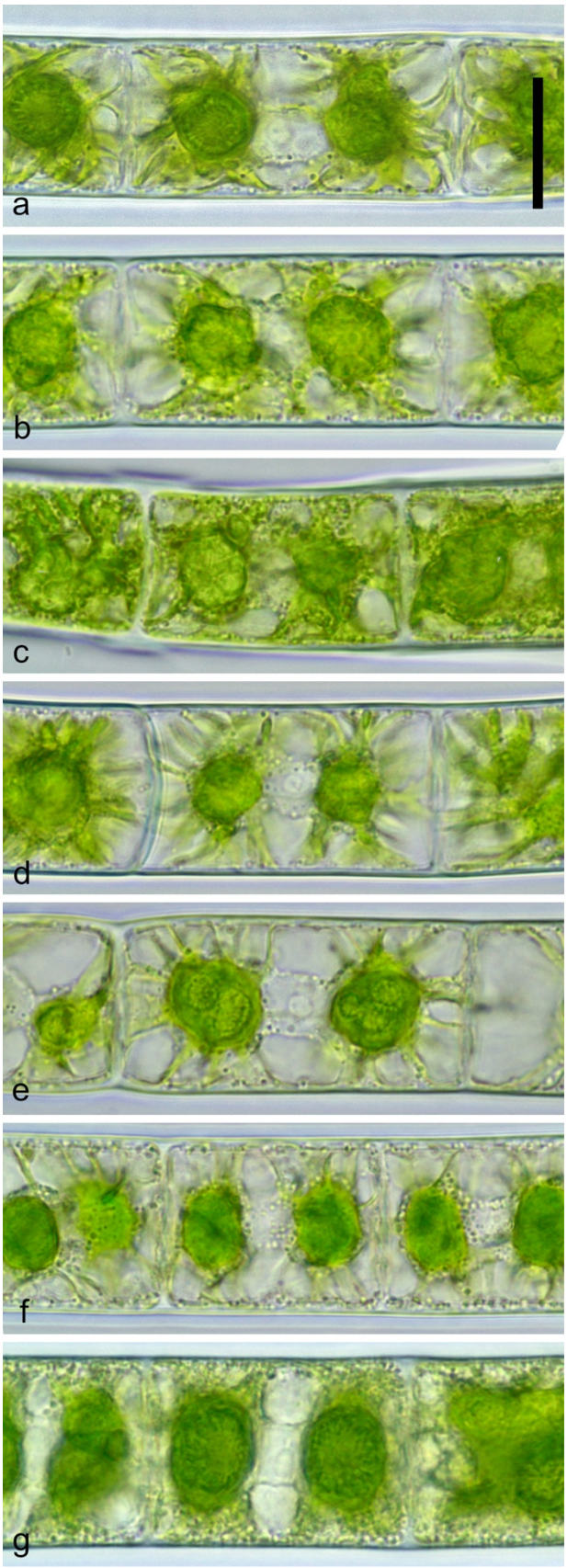

Fig. 3 Young vegetative cells of the genotypes found during the long-term observation. Each image represents a unique genotype from Kühtai lineages Z1 – Z7, genotype name is written in the brackets. a: lineage Z1 (K23L-1); b: lineage Z2 (K43L-2); c: lineage Z3 (K45S-3); d: lineage Z4 (K45S-4); e: lineage Z5 (K23L-5); f: lineage Z6 (K53L-6); g: lineage Z7 (K45S-7). Scale bar = 20 μm in all images.

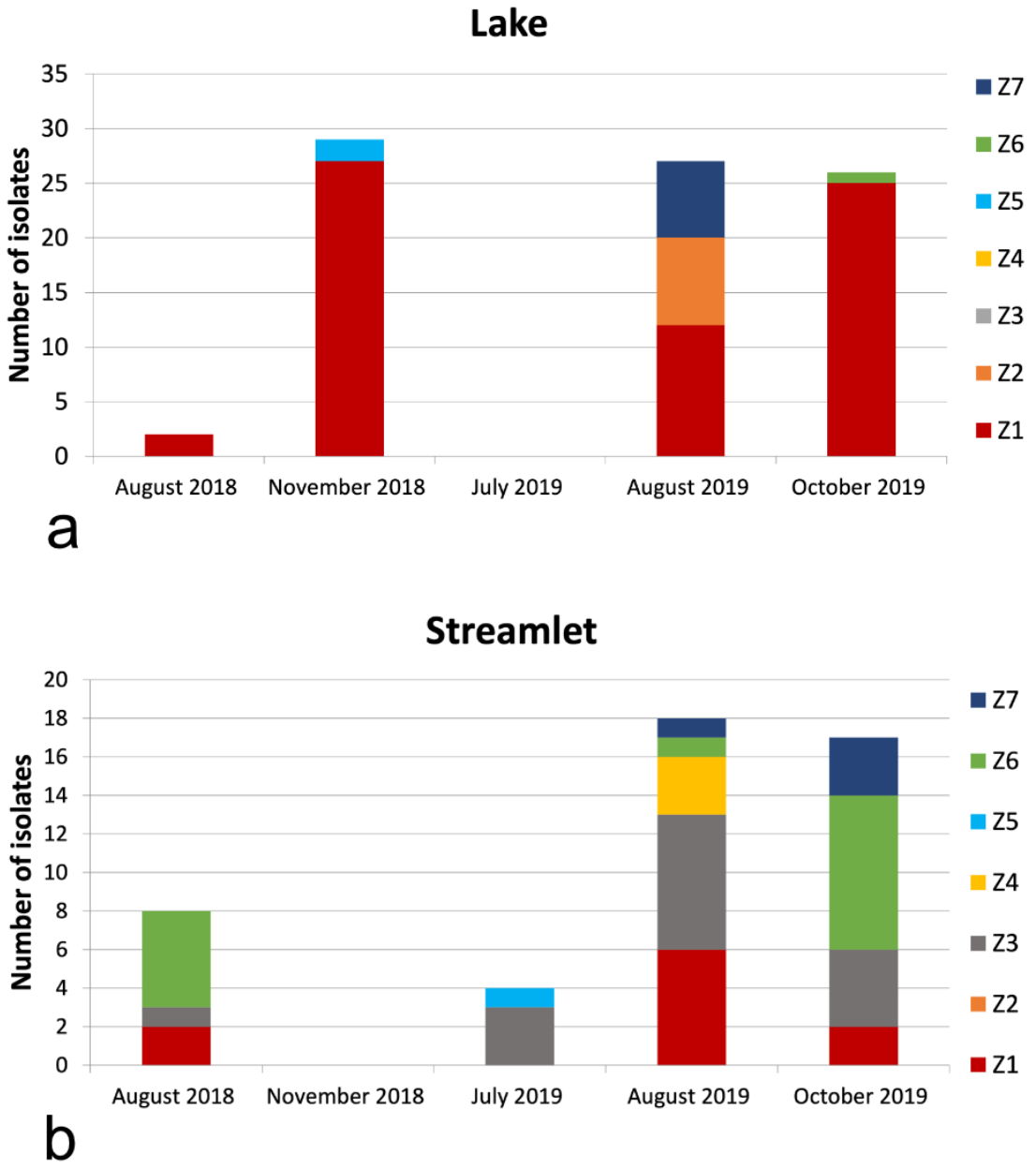

Fig. 4 Number of *Zygnema* sp. isolates of seven genotypes found during the five samplings in the two observed localities.

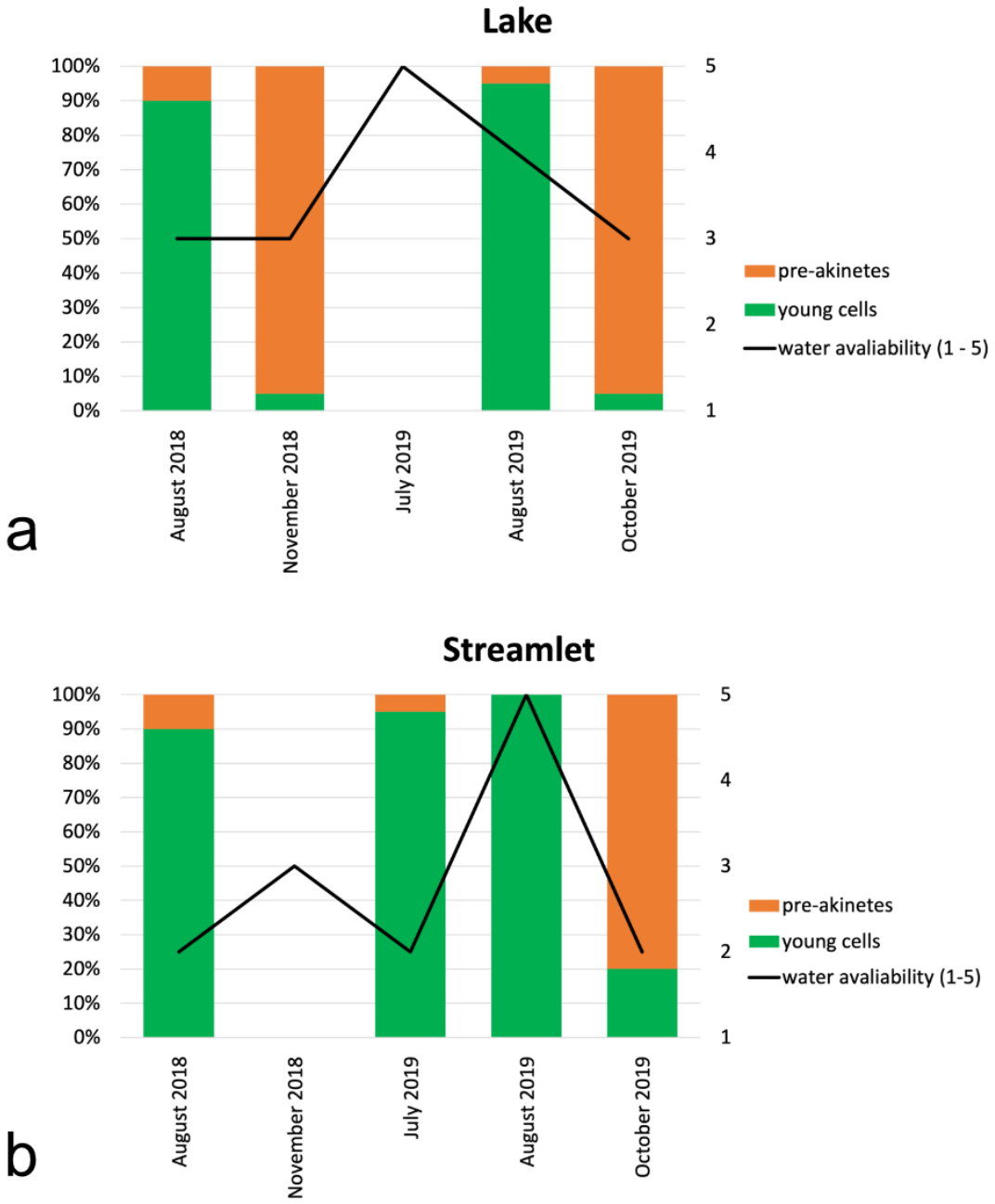

Fig. 5 Proportion of pre-akinetes vs. young vegetative cells and water availability during five samplings in two localities in Kühtai. Proportion of the cell types was estimated for 200 living cells in each sample.

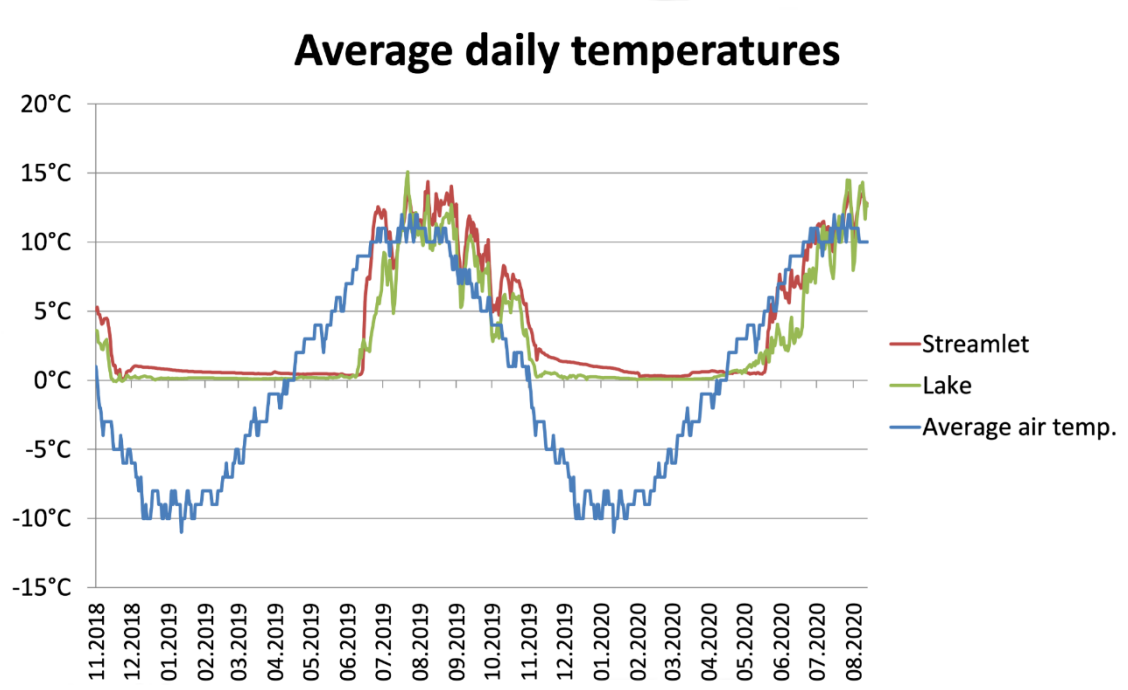

Fig. 6 Average daily temperature of water measured in-situ on the observed Lake and Streamlet localities during 11/2018 – 08/2020. The blue line shows average daily temperature of air in Kühtai.

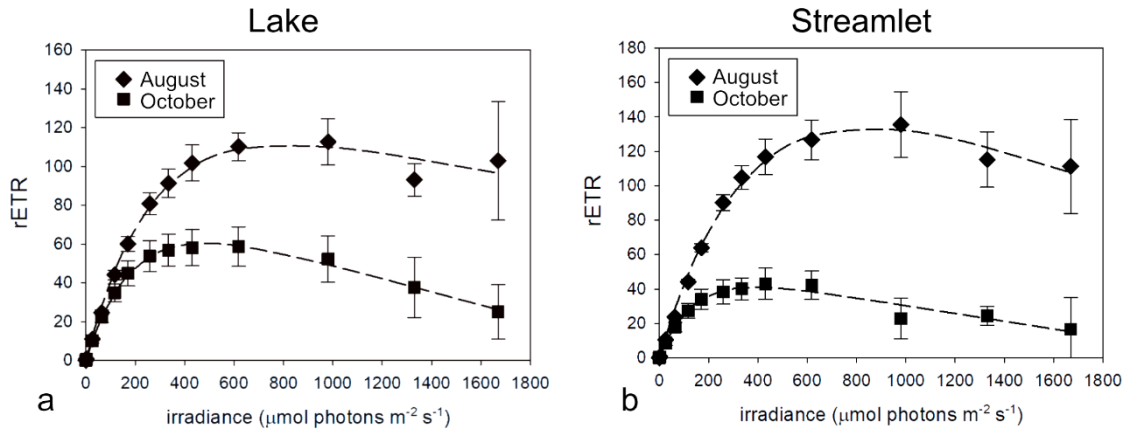

Fig. 7 Relative electron transport rate (rETR) curves of natural population of *Zygnema* sp. From the Lake and Streamlet localities. Samples for the measurements were collected during summer sampling (August 2019) and at the end of vegetative season (October 2019).

4 Key results and conclusions

Zygnema sp. is a typical inhabitant of hydro-terrestrial habitats and streams worldwide. Regardless of numerous studies on their stress resistance, complex information about their life strategy is missing. Although mats formed by *Zygnema* sp. can overwinter under ice, a massive loss of biomass was observed during the spring (Pichrtová et al., 2016b). Their ability to withstand frost is also known (Hawes, 1990); however, knowledge about the frost resistance of different cell types is lacking. Results of studies compiled in this thesis revealed trends in the life strategy of alga *Zygnema* sp. with an emphasis on annual comparison between the mats.

4.1 Diversity of mats formed by *Zygnema* sp.

The morphological and molecular diversity of *Zygnema* sp. has been already investigated before, mainly in Spitzbergen (Pichrtová et al., 2013; Pichrtová et al., 2014b), New Zealand (Novis, 2004), California (Stancheva et al., 2012), and Czechia (Poulíčková et al., 2007). Here, we investigated 156 strains isolated in the Alps and 143 Arctic strains using markers *rbc*L (Paper I, IV) and *trn*G (Paper IV) for detecting diversity. The molecular marker *rbc*L was used to make our results comparable with previous studies, while *trn*G was selected because of its proven reliability in species delimitation in Zygnematophycean algae, mainly in desmids (Nemjová et al., 2011; Neustupa et al., 2010, 2011, 2014). Overall, phylogenetic analyses revealed seven different genotypes in the alpine location of Kühtai (Paper IV) and 12 in Spitzbergen (Paper I). Although the diversity in Kühtai might appear lower, the studied area was considerably smaller than in Spitsbergen.

Species of *Zygnema* sp. cannot be determined based on vegetative morphological traits; characteristics connected with sexual reproduction are required for species delimitation (Stancheva et al., 2012). Microscopic investigation of strains used in this study did not lead to any species recognition, except for one strain from the Arctic that was found during the conjugation and identified as *Zygnema calosporum* cf. (Paper I). Confocal laser scanning microscopy revealed differences in the plastids of the strains from Spitsbergen. However, although plastid shape did not reflect the phylogenetic position, it provided sufficient information to distinguish the genera *Zygnema* and *Zygnemopsis* (Paper I). In contrast to the Arctic genotypes (Paper I), Alpine strains did not exhibit considerable variation in cell width (Paper IV). The investigated area in Alps was comparably considerably smaller and less variable than that in the Arctic, which might be reflected in the morphological differences.

The diversity within the mats of *Zygnema* sp. was found to be unexpectedly high. We demonstrated that mats are generally formed by different *Zygnema* sp. genotypes growing together (Paper I, IV). Genetic diversity within the mats was higher in the Alps; we found five and six genotypes, respectively, on our two sampling sites during a two-year observation period (Paper IV). A higher number of mats were investigated in Spitzbergen, where fourteen of 39 mats included greater than one genotype. One site with an area of 1 m^2 was subjected to detailed investigation and four genotypes were found (Paper I). Up to three genotypes were observed to grow together in genus *Spirogyra* (Chen et al., 2012; Stancheva et al., 2013). High

diversity was also discovered in the morphotypes forming *Spirogyra* mats in Lake Baikal (Volkova et al. 2018), where the mats comprised one to seven morphotypes. Unlike *Zygnema* sp., fertile filaments were recorded in 50% of sampling sites. However, one mat typically included only one fertile species, with the remaining sterile (Volkova et al. 2018).

The number of genotypes in a mat can vary from one to a mix of six genotypes, and their proportion varies tremendously (Paper IV). For example, we found only two different isolates from one mat in total 27 isolates, which indicates the possible risk of overlooking real diversity when only a little amount of biomass is investigated. However, most of the mats we investigated exhibited a balanced ratio of genotypes, with one of them generally prevailing in the mat. Although the diversity in the mats varied seasonally, it is evident that even after the disappearance of a few genotypes by the next sampling, it may have increased (Paper IV). In addition to the two sites selected for long-term observation, we also sampled another three localities in Kühtai once. Where we found the same genotypes (Z1, Z2, Z4). This indicates that the entire area shares the same diversity. Owing to the large number of small streams, algal dispersal is possible through this route. Dry algae are also likely to become airborne which was observed not only for genus *Zygnema* but several other genera for different groups of algae (Genitsaris et al., 2011). Further, as the entire area is used as pasture in summer, the algae may also spread by free-ranging cattle.

Most of the genotypes were not endemic to Spitzbergen (Paper I) or Kühtai (Paper IV), and the investigated genotypes were distributed within the two largest clades. Only two genotypes (R and G) were found exclusively on Spitzbergen. However, we cannot exclude the possibility of their occurrence in any other part of the world as the diversity of genus *Zygnema* has not been investigated to date in several areas. The most abundant genotype from Kühtai was also present in seven mats from Spitzbergen (22 isolates in total). Moreover, the other alpine genotypes were not exclusive to this area and their *rbc*L sequences can be found in public databases (Paper IV). *Zygnema* strains from California (Stancheva et al., 2012) and isolates form New Zeeland used in phylogenetical analysis in Paper I were distributed within both clades. *Zygnema* sp. tends to have a worldwide rather than endemic distribution according to our data. *Klebsormidium* species (Klebsormidiophyceae) from different lineages are known to be adapted to the substrate on which they originally occur, regardless of their evolutionary distance (Ryšánek et al., 2016). Furthermore, the occurrence of Arctic *Prasiola* is correlated to the conditions of the place of collection (Moniz et al., 2012). The most likely scenario for *Zygnema* sp. is that individual genotypes prefer specific microhabitats.

4.2 Changes in *Zygnema* sp. mats and their persistency

Seasonal changes in local diversity were investigated in both sampling areas; on Spitzbergen and in an alpine locality in Kühtai (Paper I, IV), with repeated sampling revealing the same trends. Four sampling sites on Spitzbergen were investigated repeatedly and retained the same genotypes (Paper I). Results from our long-term study focused on seasonal changes in mat composition in the Alps confirmed these observations (Paper IV). Two sampling sites in the alpine location of Kühtai were observed for two vegetative seasons (2018–2019). The pattern of seasonal dynamics was the same for both. However, these two sites differed in diversity during the year. Genotype Z1 prevailed on the site Lake and was present during all samplings. Although four less abundant genotypes (Z2, Z3, Z6, Z8) were

also observed, they occurred only in a small volume and were not present all year round. The second alpine sampling site differed in the seasonal changes of the diversity. Overall, we found six genotypes growing on the site Streamlet and none of them prevailed during our two-year-long observation. Over an average season, 3–4 genotypes were present at the Streamlet compared with two genotypes in the location Lake (Paper IV). We suggest two possible explanations for this difference. The first is that algae do not have sufficient time for heavy growth in the location Streamlet because of the unstable water regime and periods of drought. The Streamlet also diverged in the possible number of microhabitats, which could contribute to better survival of a higher number of genotypes. Volkova et al. (2018) also found seasonal changes in the *Spirogyra* sp. population in Lake Baikal. Morphotypes dominating in early summer were replaced by different ones later. Similar to Hawes (1988), our data revealed occasional disappearance of visible biomass during the year. While the *Spirogyra* mat was greatly reduced during the winter, we observed a noticeable loss of biomass in July (Lake) and November (Streamlet). We explain these fluctuations by sudden changes in conditions, mainly strong water flow and drying. Hawes observed that this decline during the winter for *Spirogyra* was caused by anoxia. We were unable to obtain samples during this period, as the site was on a ski slope. However, we hypothesize that significant biomass loss also occurred here during the winter, which was indicated by the absence of algae at the Lake site during snowmelt (Paper IV).

We also found two same genotypes even after three years of the first sampling on this site by Martina Pichrtová (Paper IV). Our results indicate that mats generally persist at one site. However, the abundances of genotypes and their occurrence in a season can vary depending on conditions. Although we did not observe conjugation and akinete formation during the sampling, pre-akinetes were produced massively in autumn and almost all cells were transformed (Paper IV). Pre-akinete formation is associated with starvation (Pichrtová et al., 2014b) as the cells start to produce nitrogen-free lipids and sugars instead of nitrogen-rich proteins (Vitova et al., 2015). Small proportion of pre-akinetes was present in the mat even in summer when nutrients were available (Paper IV).

This readiness of the mats for unexpected events was also observed in the climatic conditions of the Central European lowlands (Trumhová, unpublished observation). During a very warm summer, the pond, which contained a considerable mat of *Zygnema* sp. and other filamentous algae, dried up quickly (Fig. 8). The green algal growth turned into a completely dry black crust (Fig. 8 a–c). Even after several months of storage after collection, this crust resumed its growth under the right conditions (Fig. 8d). This small number of pre-akinetes is constantly prepared for unfavorable conditions and substantially increasing changes for long-term survival, as it was also observed in previous studies (Hawes, 1990; Pichrtová et al., 2016b).

Although seasonal dynamics were evident in the observed algae (Paper I, IV), classifying the investigated strains of *Zygnema* sp. into any of ecological groups according to Transeau (1916) is difficult. No sexual reproduction occurred in the observed algae; however, mats typically reached their highest biomass during summer and early autumn (Paper IV, personal observation). Because the mats comprised up to five genotypes that could not be distinguished macro- or microscopically from each other, determining which one occupied the largest biomass volume was not possible. However, we assume that *Zygnema* sp. probably

has a summer annual life strategy, or it can be a perennial alga, depending on environment and ecological preferences of distinct genotypes.

Fig. 8: Summer development of *Zygnema* sp. mat observed in a natural monument Na Plachtě in Hradec Králové (Czechia; a–d). a: mat during spring bloom in May; b: drying mat when water is unavailable; c: completely dry mat forming black crust; d: restoration of mat growth on the media (Trumhová, unpublished observation).

The investigated mats in this study were formed not only by different genotypes (Paper I, IV), but also by multiple genera growing together, as is has been observed before (Hillebrand, 1983; Simons & van Beem, 1990). Except *Zygnema* spp., we observed *Spirogyra* sp. and *Oedogonium* sp. growing at the same site together (Trumhová, unpublished observation). *Zygnemopsis* was also found in the same mats too, unlike *Zygnema* during conjugation (Paper I). The strategy of forming mats also provides protection from stresses, as it was demonstrated in mats of *Zygogonium ericetorum* (Aigner et al., 2013) and *Zygnema* sp. (Rippin et al., 2019).

4.3 Adaptation to UV damage in young cells of *Zygnema* sp.

Excessive UV irradiation is one of the seasonal stress factors connected mainly with the summer season. Therefore, understanding how it affects cells is extremely important. We tested the effect of realistically simulated UVR conditions in a sun-simulation chamber (Paper II). Three investigated *Zygnema* genotypes of different geographical origins (Arctic, Antarctic, and temperate) in two developmental stages (one-month-old young cells and six–month-old pre-akinetes) were used for the experiment. UVR treatments applied during the experiment did not drastically change the physiological properties of the cells. Against our hypothesis, young cells adjusted better to the experimental conditions in comparison with pre-akinetes. They recovered their Φ_{PSII} values better during the 74-h experiment. In young cells

of *Zygnema* sp., genotype S treatment induced a significant increase in phenolic compounds (Paper II). Their accumulation has already been recorded after exposure to high levels of UV radiation in *Zygnema* sp. (Pichrtová et al., 2013a). They also accumulate in cells of *Zygogonium ericetorum* (Aigner et al., 2013) and *Spirogyra* (Cannell & Alio, 1988). Moreover, *Zygnema* sp. and *Zygogonium ericetorum* are known for their mat protection ability using self-shading (Aigner et al., 2013; Rippin et al., 2019). A study on the effects of UV-B radiation on *Zygnema* originating in higher altitudes (1,880 m a.s.l.) revealed no damage to the photosynthetical apparatus (Germ et al., 2009). Further, an arctic strain of *Zygnema* exhibited no damage after UV treatment. However, Pichrtová et al. (2013) observed cell damage after UV treatment. Higher amount of phenolics in young cells can be explained by their solubility in water, as they contain large vacuoles that are strongly reduced in pre-akinetes (Pichrtová et al., 2016a).

Pigments of the deep oxidation state of the xanthophyll-cycle increased upon PA and PAB treatment (Paper II), which agrees with the previous findings in *Zygnema* (Pichrtová et al., 2013a). Protective structures such as electron-dense bodies or cubic membranes were observed in chloroplasts (Paper II). The metabolomics well separated the three strains and exhibited differences in physiological performance. However, the geographical separation of strains based on these observations must be interpreted carefully.

Excessive irradiation also leads to heat stress, which can be expected especially in summer. Previous studies have demonstrated the considerable resistance of *Zygnema* sp. to heat stress and its ability to achieve significant photosynthetic production up to 35 °C and 30 °C in isolates from the lowlands and the Alps, respectively (Herburger et al., 2015). *Zygnema* strains isolated from subpolar and polar habitats exhibited maximum photosynthetic oxygen production at lower temperatures of 20 °C and 15 °C (Permann et al., 2022c). The highest water temperature we recorded in the Alpine sampling spot was only 21.3 °C (Paper IV), indicating that the algae living there were not even close to their temperature limit. However, we must consider that each strain living there might have a different temperature optimum.

4.4 Frost resistance of *Zygnema* sp.

Understanding frost resistance was an important experimental part in this study because frost is the main seasonal stressor in both Alps and Arctic environments. Experiments performed on young vegetative cells (two weeks old) and hardened pre-akinetes revealed unexpected frost tolerance of *Zygnema* sp. strain from Spitzbergen (Paper III). Primarily, we reported a substantial difference between the two developmental stages. Although young vegetative cells could survive temperatures below 0 °C, the frost drastically altered their recovery abilities. Even temperatures slightly below zero affected the cell physiology. Cells could not recover at all in 24 h after freezing to -8 °C. On the contrary, pre-akinetes exhibited considerably higher resistance, and a small proportion of cells recovered after freezing to -70 °C. This observation confirmed the results of Hawes at al. (1990), who found a small proportion of cells alive after long-term exposure to -20 °C. Repeated freezing can also negatively affect cells. Repeated exposure to -8 °C resulted in only 16.4% viable cells compared with 71.5% after single freezing. Moreover, *Spirogyra* exhibited tolerance to low temperatures, even without forming any specialized cells. However, only a small proportion of the biomass survived anoxic conditions under the ice in Antarctic (Hawes, 1988). However, *Spirogyra* was

observed to grow even under ice in Baikal Lake in March when temperatures dropped below 4 °C (Volkova et al., 2018).

Other mat-forming algae also exhibit resistance to frost. An LT_{50} value of -3 °C was reported for the vegetative cells of *Tribonema bombycinum* (C. Agardh) Derbès & Solier after 5-day incubation (Nagao et al., 1999). In another study, 15% of *Klebsormidium* cells survived experimental freezing to -10 °C. In contrast, Elster et al. (2008) reported high frost resistance in 12 tested *Klebsormidium* strains; however, the age of cells was not mentioned. The akinetes of *Tribonema bombycinum* (C. Agardh) Derbès & Solier, cells analogous with *Zygnema* akinetes, can also tolerate very low temperatures (up to -30 °C). Both algae share similar life strategies; they form mats together occasionally (Hillebrand, 1983), even after being phylogenetically unrelated (*Tribonema* belongs to SAR; Burki et al., 2016).

Although *Zygnema* sp. is extremely resistant to frost according to our results (Paper III) and previous studies (Hawes, 1990), it may not be as significantly exposed to frost in nature as we would expect. During our observations of Alpine sites, we did not record temperatures less than -0.2 °C (Paper IV). However, the thermometers were submerged in water the entire time and the area is generally covered with insulating snow cover in winter. The average winter temperature (November to April) at this location is -5.3 °C and the lowest average daily temperature was -13.4 °C. According to our results, such temperatures would cause a significant problem for young vegetative cells if they remained exposed on the soil surface during winter (Paper III).

Algal freezing tolerance can be also improved by cold acclimation. Nagao et al. (2008) demonstrated this process in *Klebsormidium*. Acclimation is associated with an accumulation of polysaccharides, reduction of vacuoles, and enlargement of chloroplasts and starch grains. This phenomenon has not yet been tested in *Zygnema* sp. and thus offers scope for future studies.

4.5 Conclusions

Results presented in this thesis provide a new insight into the life strategy of conjugating green algae of the genus *Zygnema*. We demonstrated that diversity within mats is higher than that expected earlier and assumed based on microscopic observation (Paper I, IV). Genotypes of *Zygnema* sp. definitely tend to inhabit the same area for long periods. Although the longest observed period is four years, it could be considerably longer. However, species composition changes from one season to another, probably in response to local conditions, and one genotype can prevail (Paper IV). Formation of sexual zygospores might be unnecessary for long-time survival as we did not observe conjugation in two years during the observation. Pre-akinetes were formed before every winter and exhibited a typical decrease in maximum electron transport rate, in accordance with previous observation (Herburger et al., 2015). Their presence is a key to winter survival, as we experimentally proved with the strain from Spitzbergen whose survival limit reached even to -70 °C (Paper III). Overwintering of pre-akinetes in solid ice has been observed earlier (Pichrtová et al., 2016b). Young vegetative cells can develop from pre-akinetes in days. It is a strategy for the colonization of new habitats, and also leads to the formation of extensive mats. Because young cells are quite sensitive to frost and freeze-thaw cycles (Paper III), their development can be disturbed by frost in early spring. Zygospores have been considered long-term spores, while the formation of pre-

akinetes was believed to serve as a strategy for surviving short-term stresses, mainly osmotic stress and desiccation (Kaplan et al., 2013; Pichrtová et al., 2014a, 2014b). However, we demonstrated that they can play the same role as zygospores in conditions where conjugation is rare owing to environmental limitations (the Arctic, high mountains). Young vegetative cells proved to exhibit noticeably higher resistance to UV radiation, as they contain a greater number of protective phenolic compounds (Paper II). Even during the summer, a small proportion of cells in mats remains in the form of pre-akinetes (Paper IV). These cells are constantly prepared for the sudden arrival of unfavorable conditions. The collected data in this study can contribute to the general knowledge about diversity and life cycle of *Zygnema*. Nevertheless, the studies were not aimed to resolve taxonomical problems of this genus and further research in this topic, as well as in possible adaptations of individual species, is required.

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Appendix: Curriculum vitae

Mgr. Kateřina Trumhová

Education

Since 2016:	Doctoral study - Charles University, Faculty of Science, study branch: Botany (Supervisor: RNDr. Martina Pichrtová, Ph.D.)
2014–2016:	Master study - Charles University, Faculty of Science, study branch: Protistology (Supervisor: Mgr. Jan Šťastný, Ph.D.)
2011–2014:	Bachelor study - Charles University, Faculty of Science, study branch: Biology (Supervisor: Mgr. Jan Šťastný, Ph.D.)

Employment

7/2020 – presence	Forensic expert in biology - botany, non-human genetics, and serology (Institute of Criminalistics, Prague)
4/2018 – 6/2020:	Laboratory technician (Charles University in Prague, Faculty of Science, Department of Botany) – part time job
10/2016 – 12/2017:	Research assistant (GAČR 15-34645L) – part time job
6/2016 – 9/2016:	Laboratory technician (GAČR 15-34645L) – part time job

Projects

2023 – 2025:	ISTA TAČR VK01010069: Application of species composition analysis of diatoms (Bacillariophyceae) communities in forensic practice – member of the team
2016 – 2021:	VI20162020015 - GENETIKA IV. - Introduction of new methods of human, animal, and plant material identification in forensic practice – member of the team
2018 – 2020:	Charles University Science Foundation Grant (GAUK) 980518: Seasonal dynamics of the mats formed by conjugating algae of the genus *Zygnema* (Zygnematophyceae) – principal investigator
2015 – 2017:	Czech Science Foundation project (GAČR) 15-34645L: Colonization of land by conjugating green algae – member of the team

Fellowships

29.11. – 3.12. 2022	Department of Medicine, University of Barcelona, Spain
1. – 5. 8. 2022	Department of Forensic Science, BKA Mainz, Germany
13. – 26.11. 2017	Department of Botany, Innsbruck University, Austria (Research Group Cell Biology lead by Prof. Andreas Holzinger)
1.2. – 30.6. 2017	Department of Botany, Innsbruck University, Austria (Research Group Cell Biology lead by Prof. Andreas Holzinger) – Erasmus+ and Action program

Teaching

Botany of non-vascular plants – practical lessons

Phylogeny and morphology of non-vascular plants (for teachers) - practical lessons

Phycology II – practical lessons

Exercise in Protistology

Scientific papers and other publications

Trumhová K., Klimešová V., Pichrtová M. (2022) Seasonal Dynamics of *Zygnema* (Zygnematophyceae) Mats from the Austrian Alps. Microb Ecol. https://doi.org/10.1007/s00248-022-02105-6

de Araújo, C. B., de Mattos Bicudo, C. E., da Silva, T. G., Šťastný, J., **Trumhová, K.**, & Škaloud, P. (2022). Hidden generic diversity in desmids: description of *Pseudomicrasterias* gen. nov. (Desmidiaceae, Zygnematophyceae). Phycologia, 1-14.

Lefnar, Zagatová, **Trumhová**: CM VaVaI No 102 (2021) Determination of genetic markers of different *Cannabis* varieties with the potential to determine the origin.

Zagatová, Lefnar, **Trumhová**: CM VaVaI No 103 (2021) Species genotyping and predictive geographic assignment of unknown plant material.

Trumhová, K., Holzinger, A., Obwegeser, S., Neuner, G. & Pichrtová, M. (2019): The conjugating green alga *Zygnema* sp. (Zygnematophyceae) from the Arctic shows high frost tolerance in mature cells (pre-akinetes). – Protoplasma https://doi.org/10.1007/s00709-019-01404-z

Pichrtová, M., Holzinger, A., Kulichová, J., Ryšánek, D., Šoljaková, **T., Trumhová**, K. & Němcová, Y. (2018): Molecular and morphological diversity of *Zygnema* and *Zygnemopsis* (Zygnematophyceae, Streptophyta) from Svalbard (High Arctic). – European Journal of Phycology 53(4): 492-508. https://doi.org/10.1080/09670262.2018.1476920

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