RESEARCH ARTICLE

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

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Abstract

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

Introduction

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

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

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

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

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

Vegetative filaments of *Zygnema* lack constitutive desiccation tolerance (McLean & Pessoney, 1971), and the effect of field acclimation (hardening) or dormant stages production is therefore assumed. The formation of various resistant cell types has been frequently reported in *Zygnema*, namely zygospores, parthenospores, and akinetes (Kadlubowska, 1984; Poulíčková *et al.*, 2007; Stancheva *et al.*, 2012); their role in stress resistance is not yet fully understood. *Zygnema* commonly forms green algal akinetes as described earlier – developed from vegetative cells, with thick cell walls and accumulated storage products. Akinetes were described as individual cells that form from stationary-phase cells in starved cultures (McLean & Pessoney, 1971). *Zygnema* akinete production can occur in naturally desiccated sites, and akinetes were found that could survive experimental desiccation in a *Zygnema* sp. from Texas (McLean & Pessoney, 1971) and *Zygnema stellinum* from Belarus (Genkel & Pronina, 1979). Nevertheless, it must be noted here that, in *Zygnema*, the term 'akinete' is ambiguous because it also refers to a special type of cell in the life cycle of *Zygnema*. Such akinetes are rectangular cells with cell walls colored and structured in the same way as in zygospores, but developing asexually within vegetative cells. They represent a highly specialized, morphologically distinct, species-specific cell type (Kadlubowska, 1984; Stancheva *et al.*, 2012).

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

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

other stresses and assessed photosynthetic activity by chlorophyll fluorescence.

Materials and methods

Algal material

The *Zygnema* samples were collected in the area surrounding Billefjorden in central Svalbard in August of 2011, and the experiments were conducted at field station Petuniahytta (Elster & Rachlewicz, 2012). Six natural populations of *Zygnema* were sampled. The populations represented three natural desiccation states: (1) 'wet' biomass floating in liquid water of seepage pools (populations 1 and 2) that had not experienced dehydration since the spring, (2) 'moist' biomass on soil surface that probably had experienced recent dehydration or rehydration, but which was apparently fully hydrated at the time of collection due to capillary water between filaments (population 3), and (3) 'dry' biomass on dry soil surface forming paper-like crusts, visibly dried out (populations 4–6). Prior to all measurements and experiments, biomass from all sampling sites was placed into stream water and kept overnight (8 h) in shade conditions at a photosynthetic photon flux density (PPFD) of 15–30 μmol m^{-2} s^{-1}.

Experimental design

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

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

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

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

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

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

Chlorophyll *a* fluorescence

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

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

Light and epifluorescence microscopy

The morphology of the cells and the occurrence of plasmolysis were observed in an Olympus BX53 microscope equipped with a 100 W ultrahigh-pressure mercury lamp. Both bright field and epifluorescence observations were performed. Chloroplasts were visualized by means of chlorophyll autofluorescence, and the cell viability was estimated before the experiments and after a 24-h recovery in water with the application of the SYTOX Green dye (SYTOX® Green nucleic acid stain, Molecular Probes). This dye penetrates into cells via damaged membranes and binds to DNA. Thus, cells with damaged membranes emit a bright green fluorescence. The membrane integrity as a proxy for cell viability is widely used in algae and other microorganisms (de los Ríos *et al.*, 2004; Knowles & Castenholz, 2008; Tashyreva *et al.*, 2013). For the visualization of SYTOX Green dye fluorescence, a U-FBWA cube of 460–495/510–550 nm was used. The samples were treated with 3 μM SYTOX solution for 10 min prior to counting. The concentration and incubation time were assessed by trial tests on cultured material following a method described by Tashyreva *et al.* (2013). The number of viable and dead cells was counted in three randomly chosen fields of view in each of three independent samples of biomass (> 500 cells).

Data evaluation and presentation

The initial values of fluorescence parameters were tested by general linear model (GLM) two-way factorial analysis of variance (ANOVA) to determine whether there were any significant differences between populations and also between samples of the same population used for the separate experiments. The two factors tested – 'population' and 'experiment' were regarded as fixed effects. The differences in fluorescence parameters between the initial state and after 24 h incubation in individual sorbitol concentrations were tested by separate paired *t*-tests. Finally, GLM repeated-measures ANOVA was used to compare changes in parameters in time (in 2 M sorbitol and after 4 and 24 h of recovery). Factor levels were compared with Tukey's HSD *post hoc* tests. We also performed principal component analysis (PCA) to visualize correlations between the measured parameters and studied populations. Populations that had not plasmolyzed even in 750 mM sorbitol were arbitrarily given the value 1000 as the point of plasmolysis for PCA calculation purposes. No data transformations were performed, and the parameters ('species' data) were standardized and centered to make them comparable. ANOVA was performed in STATISTICA 10 for Windows and PCA in CANOCO for Windows, version 4.5 (Ter Braak & Šmilauer, 2002;). SIGMAPLOT 9.01, Adobe PHOTOSHOP 7.0, and Microsoft Office POWER POINT 2007 were used for the graphical elaboration of the results.

Results

Morphology and physiological performance in field-collected *Zygnema*

All investigated populations, including those in the natural desiccated state, were viable after collection in the field. Only a small proportion of dead cells were observed in some populations by means of light microscopy or epifluorescence (Fig. 1). In the light microscope, cells of all populations appeared similar (Fig. 2a–f). Their dense (brownish) cytoplasmic content was rich in storage material, so their typical star-shaped chloroplasts were not clearly discernible. Thick mucilaginous sheaths were

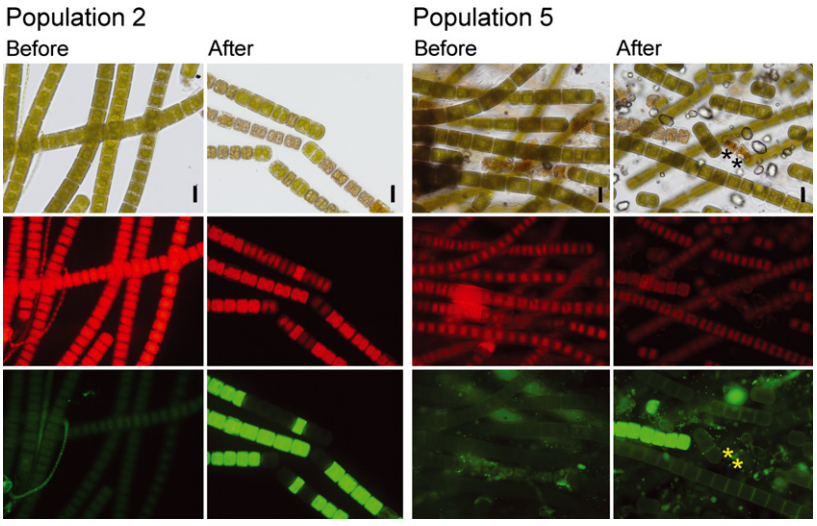

Fig. 1. Comparison between light microscope (top row), chlorophyll autofluorescence (middle row), and epifluorescence (bottom row) images of populations no. 2 (collected wet) and no. 5 (collected dry) in the initial state before incubation in 2 M sorbitol and after a 24-h recovery in water. The epifluorescence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

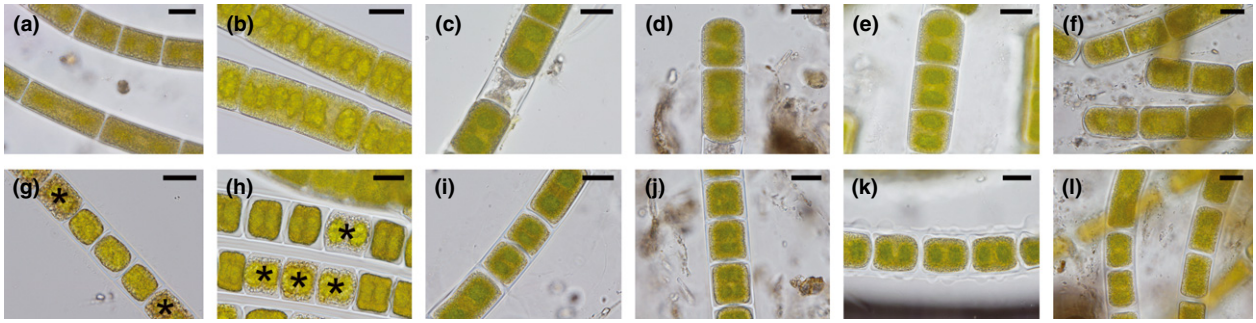

Fig. 2. Light microscope images showing *Zygnema* cells in their natural state in water (a–f) and plasmolyzed cells in 2 M sorbitol (g–l). Population 1: a, g; 2: b, h; 3: c, i; 4: d, j; 5: e, k; 6: f, l. Cells irreparably damaged are marked by asterisks. Scale bars: 20 μm.

observed around filaments in moist and dry samples (Fig. 2c–f). The filaments tended to break into short fragments in populations 5 and 6. Typical vegetative cells of *Zygnema* sp. with clearly visible stellate chloroplasts were observed only rarely.

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

Occurrence of plasmolysis

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

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

Osmotic stress and recovery

Plasmolysis occurred in all populations after a 4-h incubation in 2 M sorbitol ($\Psi_\pi = -5.63$ MPa; Fig. 2g–l). The

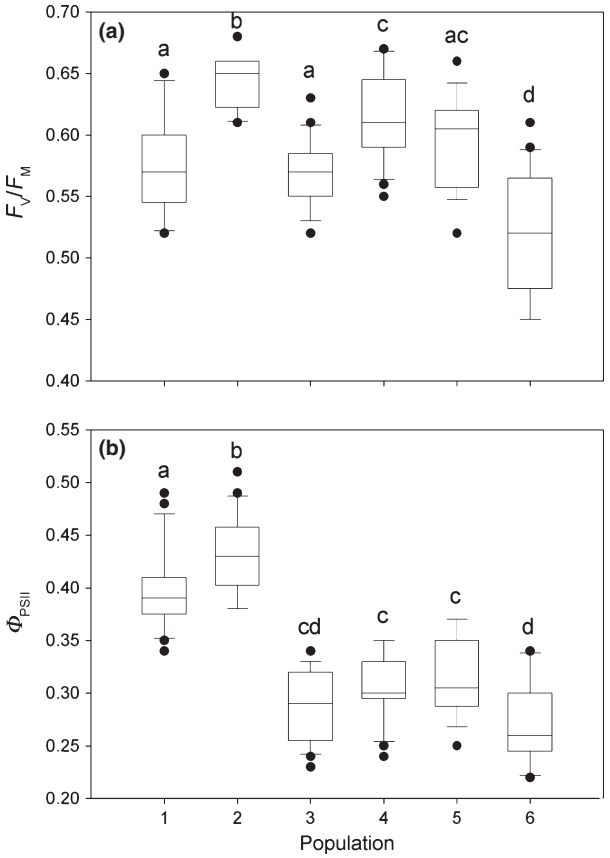

Fig. 3. Initial values of chlorophyll fluorescence parameters measured prior to the experiments on all samples from individual populations ($n = 21$). The samples were kept in water for 8 h after collection in the field; (a) maximum quantum yield of PS II (F_V/F_M), (b) steady-state quantum yield of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

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

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

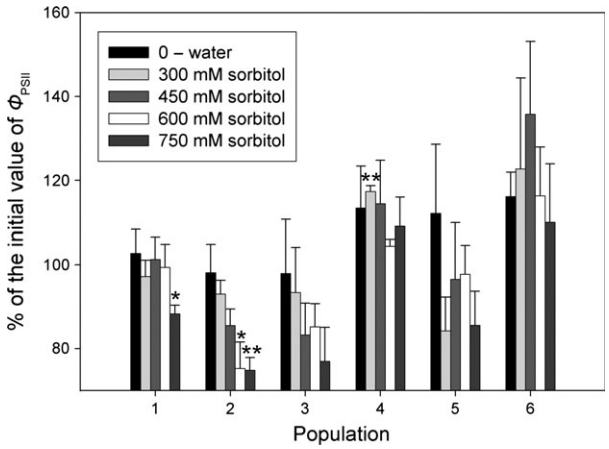

Fig. 4. Relative values (compared with the initial state) of steady-state quantum yield of PSII in the light (Φ_{PSII}) after 24-h incubation in water and four sorbitol concentrations (means + SD). The mean absolute measured values at the beginning of the experiment were as follows: population 1 (Φ_{PSII}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

plasma membrane retracted only partly from the cell wall in the dry samples, whereas in the wet samples, clear convex plasmolysis was observed. Furthermore, a high proportion of the cells in the wet samples were irreversibly damaged after the osmotic stress (Fig. 2g and h). We observed in the light microscope that some cells lost their integrity in 2 M sorbitol, and instead of plasmolyzing, their cell content was homogeneously spread out. These cells did not recover in water, and further observation proved that they were dead.

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

Overall similarity of the samples

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

Discussion

Morphology and physiological performance in field-collected *Zygnema*

Our results clearly support our hypothesis that naturally desiccated populations of *Zygnema* become hardened, possessing an osmotic mechanism of desiccation resistance that prevents plasmolysis. The natural populations were mostly formed by old, stationary-phase cells that are usually called akinetes in green algae (McLean & Pessoney, 1971; Coleman, 1983). In fact, they were the only special cell type recorded in our samples. They were mostly not disintegrated from filaments, in which case we call them pre-akinetes in accordance with McLean & Pessoney (1971). It must be emphasized that these resistant stationary-phase cells are markedly different from the true *Zygnema* akinetes with zygospore-like cell walls, which we have never observed in the Arctic.

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

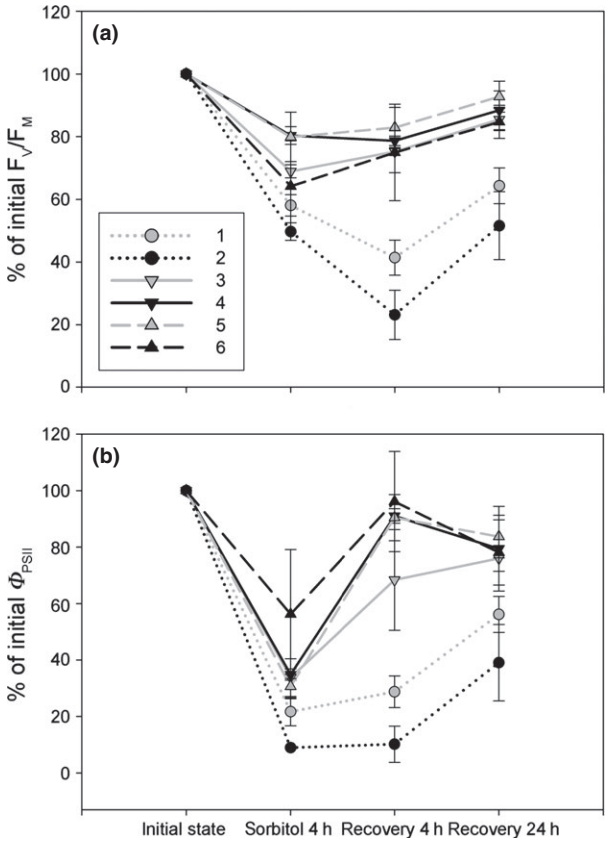

Fig. 5. Changes in chlorophyll fluorescence parameters during incubation of *Zygnema* samples in 2 M sorbitol and subsequent recovery. Values relative to the initial values before the experiment are shown (mean ± SD): (a) maximum quantum yield of PS II (F_V/F_M), (b) steady-state quantum yield of PS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

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

Osmotic stress and osmotic potential

Populations in various stages of desiccation differed in their reaction to the experimental osmotic stress treatments. The wet-collected populations (1 and 2) act similarly to laboratory-grown, nonhardened cultures of *Zygnema* without any stress-induced osmotic adjustment: Chlorophyll fluorescence was unaffected after 24 h in 400 mM sorbitol, but 800 mM sorbitol led to a significant reduction of F_V/F_M in cultivated material (Kaplan *et al.*, 2013). Moreover, the incipient plasmolysis occurred at −1.67 MPa (Kaplan *et al.*, 2013), a very similar value

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

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

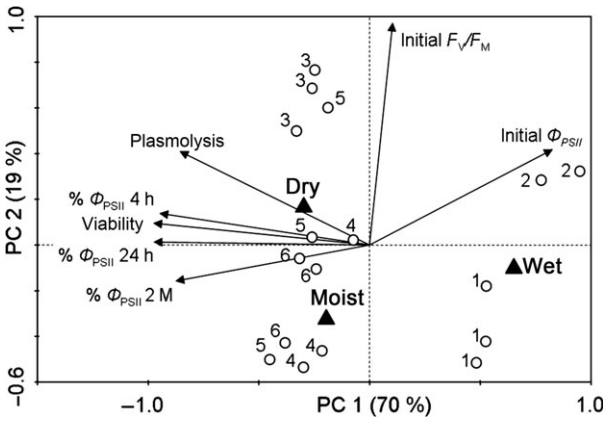

Fig. 6. PCA ordination plot of samples from populations 1–6 stressed by the incubation in 2 M sorbitol solution based on various measured parameters. PC1 and PC2, principal component axes with percentage of explained variability; viability, relative viability after the incubation in 2 M sorbitol compared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

to our results. However, this value is still much more negative than that in related freshwater algae (e.g. *Micrasterias* clearly plasmolyzed in 339 mM sorbitol; Affenzeller *et al.*, 2009). Notably, 2 M sorbitol represented a rather strong osmotic stress and led to high mortality and lack of the recovery of PSII quantum yield (Fig. 5, Table 2).

The dry-collected samples differed markedly from the wet ones in their reaction to osmotic stress (Fig. 6). In general, they were much more resistant to osmotic stress. For comparison, a true aeroterrestrial alga *Klebsormidium crenulatum* plasmolyzed at 800 mM sorbitol (–2.09 MPa; Kaplan *et al.*, 2012). *Klebsormidium nitens* plasmolyzed in 600 mM sorbitol (–1.67 MPa), but it showed an ability to acclimate to the actual osmotic conditions because after 24-h cultivation, the cells were no longer plasmolyzed even in 800 mM sorbitol. *Klebsormidium nitens* also fully recovered oxygen evolution after 3 h of recovery from a 2 M sorbitol treatment (Kaplan *et al.*, 2012). Thus, the field-desiccated (naturally hardened) *Zygnema* cells can be compared with the true aeroterrestrial algae on the basis of cellular osmotic potential and their ability to recover fluorescence parameters.

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

Sources of variation among samples

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

Another possible explanation for population differences might be a hidden genetic diversity. The genus *Zygnema* comprises more than 130 morphologically defined species (Kadlubowska, 1984), but the first molecular analyses showed that traditional systematics of the genus do not correspond with molecular phylogeny (Stancheva *et al.*, 2012). We hypothesize that the difference in physiological performance between populations 1 and 2 in the natural state (Fig. 3) might reflect species-specific characteristics. This is supported by morphology, because population 2 differed from the others, with wider filaments and different chloroplast morphologies (Fig. 2). Moreover, two phylogenetically distant genotypes were revealed within the same seepage pools in Svalbard recently (Pichrtová *et al.*, 2013). Namely, the published *Zygnema* G (Pichrtová *et al.*, 2013) was isolated from the site 1 in this study and *Zygnema* B from the site 2. However, this assignment cannot be proven as no molecular methods were applied in the present study. Nevertheless, our results show that the performance of the samples under osmotic stress conditions clearly depends on their natural hydration status *in situ*, regardless of possible genotype-related variability.

Role of pre-akinetes in *Zygnema* survival

Genkel and Pronina (1979) studied *Z. stellinum* in Belarus and concluded that resting cells – identical to the pre-akinetes we observed – play a role in survival during short periods of unfavorable conditions, whereas production of much more specialized cells, such as zygospores and par-

thenospores, is essential for winter survival. However, as such specialized cells have never been observed in the Arctic or Antarctic *Zygnema* (Hawes, 1990; Kim *et al.*, 2008, 2011; Holzinger *et al.*, 2009), we expect that pre-akinetes (McLean & Pessoney, 1971) play a key role for winter survival. This hypothesis is also supported by our observation: Frozen biomass collected in April 2012 consisted of viable pre-akinetes (M. Pichrtová & J. Elster, unpublished data).

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

Synthesis and accumulation of organic osmolytes (notably sugars and sugar alcohols) is one of the main mechanisms involved in the osmotic acclimation of algae (Bisson & Kirst, 1995), although sugar alcohols are missing in streptophyte algae such as *Klebsormidium* (Karsten & Rindi, 2010). These compounds osmotically equilibrate the cells with their medium (Karsten *et al.*, 2007; Oren, 2007) and as compatible solutes stabilize proteins and membranes by substituting water. They are involved not only in desiccation tolerance, but also in cold acclimation and subsequent freezing resistance (Nagao *et al.*, 2008). The only analysis of osmolytes in *Zygnema* revealed sucrose to be the dominant one – it represented 95% of extractable sugars in Antarctic samples (Hawes, 1990). Nevertheless, the author noted that the total content of soluble sugars in field samples was too low to decrease considerably the freezing point of the cells. To our knowledge, production of organic osmolytes has never been studied in *Zygnematophyceae* in detail, and the biochemical nature of cellular protection mechanisms in *Zygnema* remains unresolved.

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

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

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