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Production of Dormant Stages and Stress Resistance of Polar Cyanobacteria

Ph.D. Thesis

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ANNOTATION

Cyanobacteria are amongst the most abundant photosynthetic organisms in both the High Arctic and Antarctica, where they have colonized a wide range of terrestrial and aquatic habitats. Despite their success in these extreme environments, the mechanisms of their tolerance to multiple environmental stresses have been only partially understood. This thesis describes the survival strategies of *Phormidium* and *Microcoleus* species, which form extensive macroscopic mats and films in terrestrial habitats of the High Arctic, with the main focus on dormancy and tolerance to freezing and desiccation. The study shows that the species are highly tolerant to natural rates of freezing and desiccation in spite of the fact that they do not form any morphologically specialized or metabolically inert dormant cells under the studied conditions.

Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracovala samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

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Daria Tashyreva

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List of papers and author's contribution

The thesis is based on the following papers (listed chronologically):

I **Tashyreva, D.** and Elster, J. (2012) Production of dormant stages and stress resistance of polar cyanobacteria. In, Hanslmeier, A., Kempe, S., and Seckbach, J. (eds), *Life on Earth and other Planetary Bodies*, Cellular Origin, Life in Extreme Habitats and Astrobiology. pp. 367–386 (Book Chapter).

Daria Tashyreva was fully responsible for writing and revising the manuscript

II **Tashyreva, D.**, Elster, J., and Billi, D. (2013) A novel staining protocol for multiparameter assessment of cell heterogeneity in *Phormidium* populations (cyanobacteria) employing fluorescent dyes. *PLoS One* **8**: e55283 (IF on acceptation = 4.09).

Daria Tashyreva conceived, designed and performed the experiments, analyzed the data, and wrote and revised the manuscript.

III **Tashyreva, D.** and Elster, J. (2015) Effect of nitrogen starvation on desiccation tolerance of Arctic *Microcoleus* strains (cyanobacteria). *Front. Microbiol.* **6**: 1–11 (IF on acceptation = 4.0).

Daria Tashyreva conceived, designed and performed the experiments, analyzed the data, and wrote and revised the manuscript.

IV **Tashyreva, D.** and Elster, J. (submitted manuscript) Annual cycles of two cyanobacterial mat populations in hydro-terrestrial habitats of the High Arctic.

Daria Tashyreva participated in data collection in the field and designing the experiments, performed the laboratory experiments, analyzed the data, and wrote and revised the manuscript.

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1. INTRODUCTION

Cyanobacteria are very abundant and remarkably successful in both the High Arctic and Antarctica. They colonized various terrestrial and freshwater aquatic habitats in the Polar Regions, including lakes and streams, barren rocks and soils, ice-shelves and glaciers (Vincent, 2000). In these stressful environments, cyanobacteria frequently are the dominant autotrophic organisms with high productivity (Tang et al., 1997; Vincent, 2000). Some of the cyanobacterial taxa live in the most hostile places on Earth, such as the McMurdo Dry Valleys in Antarctica, where the conditions are considered to resemble closely those on Mars (Marchant and Head, 2007; Billi, 2008). The cyanobacterial dominance in the High Arctic and Antarctic is provided by their ability to grow at temperatures that are well below their optima, low grazing pressure and, more importantly, by their tolerance of a number of abiotic environmental stresses (Tang et al., 1997; Vincent, 2000).

1.1. Stress conditions in the Polar Regions

In general, the High Arctic and Antarctica are characterized by a short vegetative season, which is defined by the presence of water in a liquid state, low summer air temperatures, intense continuous light and high level of UV-radiation during the summer months, and absence of any light as well as constantly subzero temperatures during winter. However, polar habitats are highly variable, with seasonal and diurnal fluctuations of environmental conditions such as temperature, light intensity, and availability of water and mineral nutrients (Vincent and Howard-Williams, 1986; Vincent, 2000; Castenholz and Garcia-Pichel, 2002; Elster, 2002; Quesada and Vincent, 2012; Tashyreva and Elster, 2012). Various habitats provide a different degree of stress or protection to the organisms inhabiting them. Hence, it is reasonable to classify the polar environments with respect to the survival strategies of organisms.

The amount of water and its availability during the year defines the stability of a particular habitat, and affects the survival strategies of organisms. According to these, polar habitats can be conventionally grouped into three categories: aquatic (unfrozen water is present throughout the whole year), hydro-terrestrial (liquid water is normally available during the summer but is frozen in winter), and terrestrial (water availability is temporary and even changes throughout diurnal oscillations). The category of hydro-terrestrial environments was introduced as an intermediate between stable aquatic habitats and highly variable terrestrial habitats (Elster, 2002). The presence of liquid water during the whole year in stable aquatic environments (e.g. deep lakes) enables organisms to avoid both freezing and desiccation (Fritsen and Priscu, 1998; McKnight et al., 2000) as well as the seasonal fluctuation of salinity due to freezing and drying (Vincent and Quesada, 2012). Furthermore, due to its high heat capacity, water plays a role in maintaining a constant temperature regime (Tashyreva and Elster, 2012). The relatively favorable conditions in certain polar lakes allowed benthic communities of cyanobacteria to develop perhaps the thickest microbial

biofilms known to occur in natural environments: the biomass accumulation can reach a thickness of more than 90 centimeters (Vincent, 2000). In contrast to the lacustrine environments, organisms in terrestrial habitats (e.g. barren soils and rock surfaces) have to withstand freezing in winter as well as periodic or extended desiccation during summer (Davey, 1991).

1.1.1. Hydro-terrestrial habitats

I focused on shallow meltwater pools, streams and seepages, which are very common terrestrial or hydro-terrestrial habitats (depending on the amount of water) in the Polar Regions. These are relatively unstable environments, in which water is available during the spring thaw and in early summer, but these often dry out towards the end of vegetative season, and are completely frozen during winter (Vincent and Quesada, 2012). These habitats are also subject to rapid diurnal temperature fluctuations, drying-rewetting and repeated freeze-thaw cycles (Davey, 1989; Elster, 2002; see also Paper #4). Because the amount of water is relatively little, the habitats may be characterized by a very unstable thermal regime: within a short time period, the temperature may rise or fall by 15°C (e.g. rise from 5 to 20°C) depending on air temperature, and the intensity and angle of incident sunlight. Because most of these habitats are not shadowed, the organisms there are exposed to continuous sunlight in the summer months, which may exceed 2000 μmol m^{-2} s^{-2} on sunny days (Láska et al., 2012), and elevated levels of UV radiation (Vincent, 2000), but they are completely devoid of any light during the polar winter. Hydro-terrestrial habitats can span a broad range of nutrient conditions: while some are largely nutrient-deficient (e.g. glacial streams), the others are very rich in nutrients, e.g. pools associated with bird rookeries (Vincent, 2000).

1.1.2. Definition of stress and stress factors

Stress can be defined as a condition which has a significant negative effect on growth, metabolism and reproduction of living organisms, or as a treatment, which causes cell injury (Wesche et al., 2009). What exactly should be considered as a stress is rather a philosophical issue, and there is no common opinion in the literature on that question. Living organisms, especially bacterial cells, have very diverse requirements for their growth, and the optimal conditions differ greatly among microorganisms. The preferences for temperature range from around 0°C to 120°C, the optimal pH values fall in the range from 0.5 to >11, the preferred salinities are from essentially distilled water to saturated salt solutions, and the same is true for pressure, redox potential, chemical composition of environment and so forth (Rothschild and Mancinelli, 2001). For instance, the conditions which are considered to be optimal for a human being are stressful or even lethal for anaerobic thermophilic bacteria. Thus, strong deviations from optimal growth conditions may cause sublethal or lethal stress. Nevertheless, there are certain conditions that are considered to be harmful to any organism, some of which are commonly occurring in polar hydro-terrestrial environments. Desiccation, freezing and UV-radiation are potentially injurious or

even lethal for every kind of cell, and cannot be advantageous for the growth and physiology of any living organism (Rothschild and Mancinelli, 2001). Additional stress for cyanobacteria arises from high rates of photosynthetically active radiation (PAR) despite the fact that light is an essential factor for photosynthesis (Schwarz and Grossman, 1998).

Despite the low temperatures, only a few truly psychrophilic species of cyanobacteria have been found in Arctic and Antarctic ecosystems, specifically in Antarctic ponds with a constant temperature regime (Nadeau and Castenholz, 2000). Most of the polar cyanobacteria are psychrotrophic, i.e. they are tolerant to cold but have temperature optima >15°C (Tang et al., 1997; Waterbury, 2006). For polar cyanobacteria, cold conditions are rather suboptimal than stressful, because cells maintain their biological activities but do not achieve as high growth and photosynthetic rates as at warmer temperatures (Tang et al., 1997). The adaptations, which enable mesophilic cyanobacteria to function at suboptimal low temperatures, have been exhaustively reviewed (Nishida and Murata, 1996; Chattopadhyay, 2006; Morgan-Kiss et al., 2006; Margesin et al., 2007 and others), and will not be introduced here.

1.1.3. Cell dehydration

Liquid water is essential to the structure and function of living cells. Water plays a primary role in maintaining the structure and native conformation of lipid membranes, proteins and nucleic acids. It is not surprising that the removal of water from cells can severely damage, or even result in cell death. Water can be removed from cells through different ways: by the action of osmotic solutions, extracellular freezing or the direct effect of dry air (Potts, 1994). Dehydration of cells by air drying, i.e. the equilibration of an organism to the relative humidity of the surrounding atmosphere, is referred to as desiccation (Holzinger and Karsten, 2013).

1.1.3.1. Injuries by complete desiccation

The water deficit due to air drying is far greater than can arise if a cell is frozen or exposed to osmotic solutions (Potts, 1994). In the most extreme cases of desiccation, the water content in cells may decrease to residual or even undetectable amounts. The most detrimental injuries to cells occur when the water content falls below 0.1 g per 1 g of dry biomass. This important threshold corresponds to the minimum amount of water needed to form at least a monolayer around cell proteins and membranes (Potts, 1994; Alpert, 2006). This structurally bound water is essential for maintaining the arrangement of membrane lipid molecules in bilayers as well as for separating membranes from each other. Water is fundamental in protein folding, because of its role in defining hydrophobic attractions, thereby being responsible for the stability and three-dimensional structure of proteins. Finally, water is needed for maintaining the helical structure of double-stranded DNA and its native conformation (Mazur, 2004).

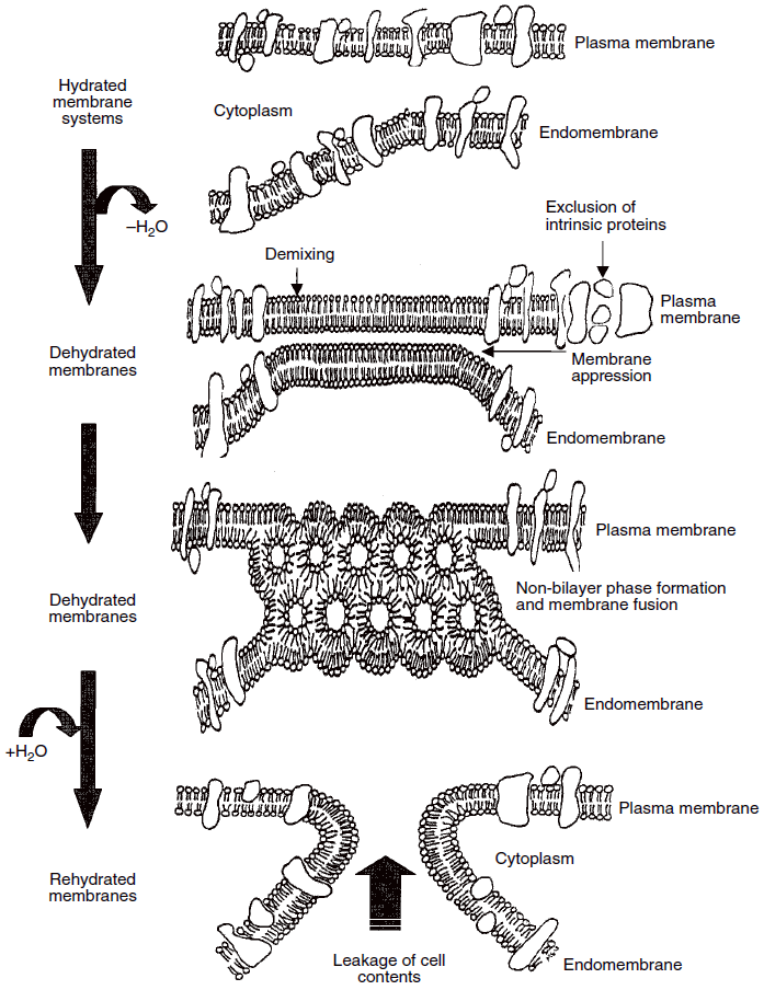

Fig. 1. Schematic drawing showing the effect of complete desiccation on cellular membranes. Removal of membrane-bound water leads to the formation of non-bilayer lipid structures (e.g. vesicles) and fusion between different membrane systems (Walters et al., 2002).

Upon removal of this water, cells of most organisms sustain lethal damage due to irreversible changes in the native structure of dehydrated nucleic acids and proteins as well as chemical modifications of proteins, sugars, and nucleic acids (Potts, 1994). Specifically, dried cell membranes are no longer separate from each other, which causes them to fuse, e.g. the plasma membrane fuses with photosynthetic membranes, and hence, the cell lipid membranes become largely disturbed upon rehydration (Fig.1; Walters et al., 2002). In addition, a fraction of lipids within a membrane may undergo phase transitions from the liquid crystalline state to the gel state, which would result in the separation of 'liquid' and 'solid 'membrane components when

water becomes available or in transient membrane leakage (Crowe and Crowe, 1992; Potts, 1999; Crowe et al., 2002). Damages to DNA molecules occur through alkylation or oxidation, crosslinking with sugars as well as base removal such as depurination (Billi and Potts, 2002; Shirkey et al., 2003).

1.1.3.2. Dehydration and incomplete desiccation

Incomplete desiccation of cells at higher RH values or dehydration due to freezing, i.e. when nucleic acids and proteins are fully hydrated, is also quite stressful. Dehydration-induced damage occurs mostly to nucleic acids and proteins. In large part, the damage reflects the accumulation of mutations and damages to biomolecules during the time when there is no cell growth (during desiccation). It is unlikely that repair mechanisms operate in dry cells, and this damage manifests upon rehydration (Potts, 1994). Damages to DNA and proteins as well as peroxidation of membrane lipids are mediated through reactive oxygen species (ROS), such as singlet oxygen, superoxide (O^{2-}) and hydroxyl (HO^{-}) radicals as well as hydrogen peroxide (Potts, 1994; García, 2011). ROS are normally produced in aerobic metabolism, but occur in much higher quantities under UV and intensive visible light (Shirkey et al., 2003). The toxic action of ROS cannot be eliminated in dry cells due to the absence of enzymatic activity. The fusion of membrane bilayers and the aggregation and precipitation of proteins may occur even as a result of incomplete desiccation and freezing (Strauss and Hauser, 1986; Crowe et al., 1990; Walters et al., 2002).

Finally, as the water level decreases, cells become exposed to increasing concentrations of solutes. It is believed that the action of hyperosmotic solutions causes injuries to cell membranes due to their extensive shrinkage during dehydration and abrupt expansion upon rehydration. Also, high concentrations of solutes are toxic for cells. The direct interaction of solutes with the membrane would cause the dissociation of proteins resulting from the suppression of intramembrane ionic interactions, alterations in intramembrane hydrophobic interactions, or charge neutralization (Steponkus, 1984). In cyanobacteria, exposure to high NaCl concentrations (e.g. 0.5 M) resulted in irreversible loss of the activities of photosystems I and II, and the Na^{+}/H^{+} antiport system. However, the effect was reversible when the cells were dehydrated without externally added NaCl (Allakhverdiev et al., 2001).

1.1.4. Injuries due to freezing

One of the truly remarkable properties of water is that it is able to remain liquid at temperatures which are well below 0°C. The 0°C temperature is actually not the freezing point of water, but is the melting point of ice. In fact, although it is not commonly known, pure water has a very low probability of freezing at temperatures higher than -38…-40°C (Wisniewski et al., 2002). Freezing at warmer temperatures has to be initiated by certain substances, which serve as templates for the forming of ice crystals. Such substances are termed ice-nucleators and include specific bacteria, various biological molecules and structures, and organic and inorganic debris (Pearce,

2001; Lundheim, 2002). In nature, water can supercool to low subzero temperatures inside organisms, which lack intrinsic ice-nucleators and, at the same time, are well insulated from the external environment, such as trees and some invertebrates (Pearce, 2001; Holmstrup et al., 2002). Hence, such organisms are able to avoid any ice formation inside their body tissues down to a certain subzero temperature. This strategy, however, has a disadvantage, because, when the temperature finally drops below the ice-nucleation point, ice is formed intracellularly (Fig. 2), which is believed to have a lethal effect on nearly all known types of cells (Mazur, 1984).

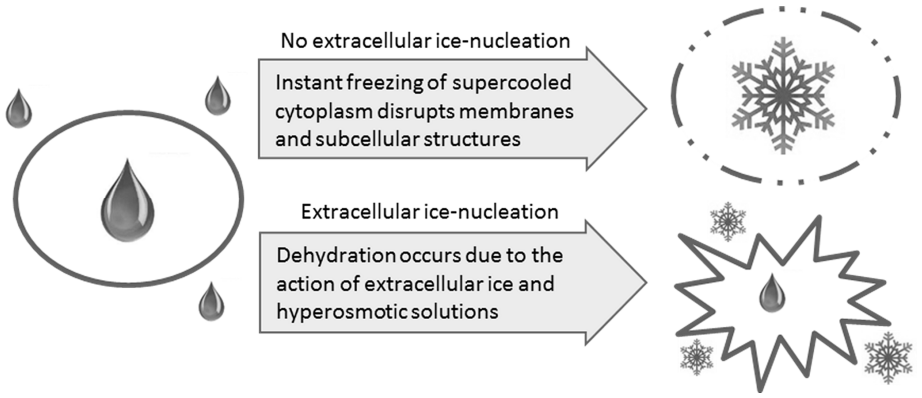

Fig. 2. Schematic drawing of physical events during freezing in the presence or absence of extracellular ice-nucleators.

Cyanobacterial cells in polar hydro-terrestrial habitats are very unlikely to supercool. That is because water in their natural habitats normally contains bacteria as well as various organic and inorganic particles that serve as very effective ice-nucleators (Lundheim, 2002; Franks, 2003; Christner et al., 2008). Therefore, freezing takes place at higher subzero temperatures (i.e. close to 0°C) in water that surrounds cyanobacterial communities. When the water freezes at temperatures around 0°C, the main stresses associated with freezing are cell dehydration and mechanical disruption of cell structures by ice crystals (Mazur, 1984; Thomashow, 1998; Tashyreva and Elster, 2012). Dehydration occurs when freezing is initiated in the extracellular medium (by various ice-nucleators). As water freezes, the osmotic pressure in unfrozen water fractures around ice crystals increases due to a concentration of solutes. Because intact plasma membranes are permeable to water and impermeable to extracellular ice, water outflows from cells along an osmotic gradient and freezes in the extracellular medium (Steponkus, 1984). Alternatively, in dilute water environments, dehydration is driven by the difference of vapor pressures between the extracellular ice and the supercooled water inside the cell, both being at the same temperature (Wharton et al., 2003). Dehydrated cells contain a residual amount intracellular water (10% or higher of its initial content, or at least 0.25 g g^{-1} dry mass), which does not freeze even at extremely low subzero temperatures (Mazur, 1984; Crowe et al., 1990). Therefore, freeze-induced dehydration results in severe though

incomplete water loss (discussed earlier) but, at the same time, prevents lethal intracellular freezing (Wharton et al., 2003).

In addition to the shrinking of cells due to the outflow of intracellular water along the osmotic gradient, the frozen cells are exposed to the mechanical pressure of ice. Cell plasma membranes are damaged since they are rigid at subzero temperatures, and therefore become increasingly sensitive to any deformation by expanding extracellular ice and the growth of sharp ice crystals (Mazur, 1984).

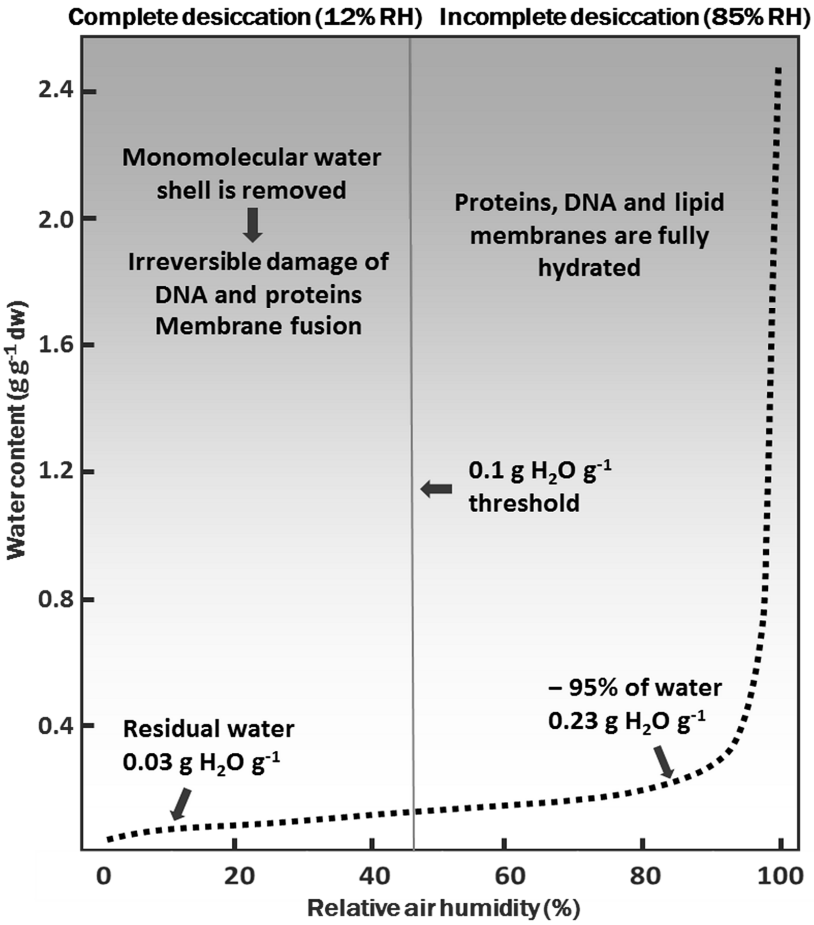

Fig. 3. The curve schematically shows the relation of water content in cells/ biomass to RH values (modified from Sun, 2002).

1.1.5. Dehydration under natural conditions

The conditions which lead to complete desiccation and severe dehydration in fact readily occur in natural terrestrial habitats (Billi and Potts, 2002; Belnap, 2003; Rajeev et al., 2013). Water loss from plant and bacterial cells is represented by a biphasic curve: during the first phase, the water loss follows an exponential function, while in the second phase water content decreases only a little, because the biomass is

closer to achieving equilibrium with the air (Sun, 2002). When cells are directly exposed to air, a substantial amount of water is lost already at very high air humidity values (Fig. 3). Assuming that the water content in cyanobacterial cells is ca. 80% (Potts, 1994), which gives 4 g H_2O g^{-1} dry mass (dm), drying at 85% RH results in a loss of as much as 95% of initial water content (to only 0.23 g H_2O g^{-1} dm). Natural freezing regimes cause the same rate of cell dehydration (to 0.25 g H_2O g^{-1} dm) as drying at 85% RH (Crowe et al., 1990). The action of air below 40 to 50% RH at 20°C is sufficient to cause the complete desiccation, i.e. drying to water content below 0.1 g H_2O g^{-1} dm (Potts, 1994; Sun, 2002; Alpert, 2005). However, the exact humidity values and amount of available water in natural microniches as well as in the immediate vicinity of communities and cells are not known (Potts, 1994; Sun, 2002; Belnap, 2003; Rajeev et al., 2013).

1.1.6. Damages by photosynthetically active and UV radiation

DNA and proteins are primary targets of UV-B irradiation, because they absorb UV-B. DNA lesions caused by UV-B irradiation include single-stranded and double-stranded breaks, and DNA to protein cross-links (Ehling-Schulz and Scherer, 1999). Several proteins may be photooxidized by UV-B radiation since tryptophan, tyrosine, phenylalanine and histidine absorb radiation in the 290–315 nm range (Castenholz and Garcia-Pichel, 2002). Chlorophyll and phycobilins are photodamaged directly by UV-A and intensive photosynthetically active radiation (PAR) as these pigments absorb light both in the UV and visible parts of the light spectrum (Küpper et al., 2009). Although many biomolecules do not directly absorb UV-A and PAR radiation, damages occur through interaction with reactive oxygen species, which are produced in intermediate reactions under UV-A radiation and intensive PAR (Ehling-Schulz and Scherer, 1999; Castenholz and Garcia-Pichel, 2002). ROS cause injuries through cross-linking reducing sugars with amino acids, lipid peroxidation and create damage within nucleic acids, photosystem II proteins, ribulose-1,5-bisphosphate carboxylase, nitrogenase, and phycobiliproteins (Ehling-Schulz and Scherer, 1999; Potts, 1999). In addition to the ROS effect, damage to photosystem II is induced by excessive PAR and leads to inactivation of electron transport and subsequent oxidative damage of the reaction centers (Aro et al., 1993). Exposure to PAR and UV-radiation is particularly harmful when cells are inactive, e.g. frozen or desiccated, because ROS cannot be eliminated by enzymes, and accumulation of damages to DNA due to the cessation of any reparative activity.

1.2. Adaptations at macro- to microscales

Survival strategies in stressful environments involve avoidance of stress if possible, protection from damage if stress cannot be avoided, and reparation in case damages nevertheless occur. These strategies are realized through a complex set of mechanisms, which includes behavioral, physiological, structural and biochemical adaptations. Here, I will discuss the most common bacterial adaptions to

abovementioned stresses which occur in polar hydro-terrestrial environments with the main focus on cyanobacteria.

1.2.1. Avoidance and minimizing the extent of stress

As was discussed earlier, cyanobacteria in terrestrial and hydro-terrestrial environments cannot completely avoid desiccation and intensive solar radiation, yet they developed some strategies which allow them to minimize the action of these stress factors. In nature, cyanobacteria produce macroscopic colonies (e.g. *Nostoc*) or thick multilayered mats and films (many oscillatorians), in which cells and filaments are embedded in copious amounts of extracellular polysaccharides, or EPS (Vincent, 2000). Such community structure decreases the surface-to-volume ratio, which slows down water evaporation, and at the same time protects the cells beneath the surface of the colonies or mats against UV radiation and excessive PAR (Potts, 1994). The hydrophilic polysaccharides have an important ability of absorbing air moisture as well as retaining a water layer around the cells, which slows water loss from the cytoplasm (Knowles and Castenholz, 2008). For example, cells of the cyanobacterium *Nostoc commune* secrete significant amounts of polysaccharides, which comprise more than 60% of the dry weight of a colony (Hill et al., 1997). It was also hypothesized that EPS sheaths may prevent direct contact between the cell surface and ice crystals (Vincent, 1988). Indeed, EPS-amended cell suspensions of cyanobacteria better tolerate freezing, but the mechanisms of protection are not clear (Tamaru et al., 2005; Knowles and Castenholz, 2008).

Stress due to desiccation, UV radiation and PAR can be avoided through vertical migration of filaments within mats or beneath the soil surface. This behavior is typical for oscilllatorian cyanobacteria, because they are capable of a gliding movement. *Oscillatoria* and *Microcoleus* species demonstrate desiccation avoidance behavior by retreating to the subsurface under water limitation and active migration to the soil crust surface when water becomes available, as well as mobility within cyanobacterial mats (Pringault and Garcia-Pichel, 2004; Castenholz and Garcia-Pichel, 2012; Rajeev et al., 2013). Vertical migration of filaments to a water-saturated substrate might be a good solution to avoid dehydration during dry episodes in summer.

At the cellular level, osmotic adjustment is the means for avoiding or slowing down water loss. This happens during the onset of desiccation when water stress is mild, i.e. liquid water is not available and the relative air humidity is slightly less than 100%, but the mechanism is ineffective at lower humidity values (Hershkovitz et al., 1991; Walters et al., 2002). Osmotic adjustments prevent water loss and cell contraction by lessening the water potential difference between cells and the environment and by increasing the amount of dry matter in cells (Walters et al., 2002). In response to both mild desiccation and osmotic stress, terrestrial freshwater cyanobacteria accumulate non-reducing disaccharides such as sucrose and trehalose (Hershkovitz et al., 1991).

The alteration of cell wall thickness and their chemical composition is another way by which cells may prevent or at least slow down water loss. Most probably, such

a structural adaptation is typical for dormant cells, because the thickening of cell envelopes reduces exchanges with the environment (Caiola et al., 1996). For instance, the thick multilayered coating of bacterial endospores prevents their cores from dehydration, but the permeability of the cell envelopes is extremely reduced (Setlow, 2006). Among cyanobacteria, multilayered envelopes are a feature of akinetes, heterocysts, and certain species of coccoid cyanobacteria under stress conditions (Billi and Caiola, 1996; Kaplan-Levy et al., 2010).

1.2.2. Protection from damages

1.2.2.1. Solar radiation and oxidative stress

Protection against UVR and PAR is realized through both screening the radiation and eliminating ROS, which are produced under sunlight. Cyanobacteria exposed to UVR synthesize a number of pigments, which filter out or dissipate as heat both UV-A, UV-B and visible light. A combination of scytonemin, which is deposited in EPS sheaths, and intracellular mycosporine-like amino acid derivatives, creates an effective shield against UV radiation in the 250 to 400 nm range (Ehling-Schulz and Scherer, 1999; Castenholz and Garcia-Pichel, 2002, 2012; Fleming and Castenholz, 2007). Carotenoid absorption maxima are mainly in the visible spectral range (i.e. >400 nm), thus they mostly protect cyanobacteria from high doses of PAR and partly long-wave UV light. In cyanobacteria, carotenoids occur in the outer cellular membrane as well as in the thylakoids, which is defined by their functions in cells. Carotenoids bound to the outer membrane partly filter out the incident light by absorbing it followed by dissipation as heat. Specific thylakoid-bound carotenoids (e.g. myxoxanthophyll, echinenone, zeaxanthin) are effective through the thermal dissipation of the excitation energy of chlorophyll and scavenging of singlet oxygen produced by chlorophyll molecules (Castenholz and Garcia-Pichel, 2002, 2012).

Enzyme complexes, such as superoxide dismutase, peroxidase and catalase, degrade ROS molecules when cells are metabolically active as well as during desiccation provided that there is sufficient water available for enzymatic activity (Chen et al., 2012). In addition, tocopherols, ascorbates, and glutathione are used by cyanobacterial cells as non-enzymatic peroxide detoxifying compounds (Castenholz and Garcia-Pichel, 2002). Since chlorophyll and phycobilins act as photosensitizers (i.e. they produce ROS), cyanobacterial cells under intensive light may reduce their content by regulating pigment synthesis downward as well as their controlled degradation (Schwarz and Grossman, 1998; Rajeev et al., 2013). In the dry state, cyanobacteria tend to preserve chlorophyll; however the cells are able to deactivate the PS II reaction center and to cease energy transfer from phycobilisomes to PS II and down-regulate respiration, nitrogen metabolism and protein synthesis on the onset of desiccation (Potts, 1994; Katoh et al., 2004; Lüttge, 2011). Because biomolecules in dry and frozen cells become increasingly sensitive to damages, protection against solar radiation and detoxifying ROS are essential adaptations for surviving desiccation and dehydration as well.

1.2.2.2. Freezing and incomplete desiccation

Cyanobacteria in polar hydro-terrestrial environments cannot avoid the winter freeze, because the communities become frozen not only by low air temperatures but also by the permafrost from underneath. Adaptations to freezing and incomplete desiccation are discussed here together, because they both cause substantial cell dehydration but do not remove the hydration shell around biomolecules (Potts, 1994; Leslie et al., 1995).

Cyanobacterial cells acclimated to these stresses commonly accumulate compatible solutes, which are highly soluble organic molecules that in high amounts demonstrate compatibility with cellular metabolism (Klähn and Hagemann, 2011). Terrestrial cyanobacteria synthesize sucrose and trehalose as primary compatible solutes, but may also produce minor compounds such as glucosylglycerate or proline as secondary or tertiary solutes (Reed et al., 1984; Klähn and Hagemann, 2011). It turns out that sucrose and trehalose have multiple functions in cyanobacterial stress tolerance. In addition to preventing water loss via osmotic adjustment and the substitution of water molecules in completely desiccated cells, these sugars are also known to stabilize membranes and biomolecules in dehydrated cells (Steponkus, 1984; Strauss and Hauser, 1986; Hoekstra et al., 2001). It was hypothesized that compatible solutes are preferentially excluded from the surface of proteins, thus keeping the proteins preferentially hydrated. Hence, sufficient hydration prevents proteins from denaturation as well as from aggregation and/or cross-linking with other biomolecules (Hoekstra et al., 2001; Crowe et al., 2004). Sucrose and trehalose are also known for their ability to prevent fusion of membrane bilayers, which may occur even when the hydration shell around membranes has not been removed (Strauss and Hauser, 1986; Crowe et al., 1990; Walters et al., 2002). Glycerol and polyalcohols, which are involved in the freezing tolerance of many organisms (Tanghe et al., 2003), have not been reported as naturally produced compatible solutes in cyanobacteria.

Manipulating the lipid composition of cell membranes, such as increasing phospholipid:sterol ratios and the amount of unsaturated fatty acids, is a well-known adaptation of organisms to cold (Nishida and Murata, 1996; Margesin et al., 2007). Besides enabling normal cell functioning at low temperatures, these changes facilitate careful folding of the plasmalemmas when cells shrink due to dehydration, and provide greater elasticity to the expanding membranes during rehydration (Steponkus, 1984; Walters et al., 2002; Tanghe et al., 2003). Desaturation of membrane lipids increases membrane fluidity and prevents phase transition of the membrane lipids, which may reduce the risk of plasma membranes being damaged by the mechanical pressure of expanding ice (Mazur, 1984; Crowe et al., 2002).

A wide range of terrestrial and freshwater photosynthetic organisms from Antarctica, possibly including cyanobacteria, produce macromolecular ice-active substances, which are partly or entirely composed of proteins. These molecules incorporate themselves in growing ice and affect both the morphology and size of ice crystals (Raymond and Fritsen, 2004), thus possibly preventing the rupture of plasma

membranes and surface structures. Other ice-active compounds, which are common for cold-adapted organisms (Fuller, 2004), such as fish antifreeze proteins (depress freezing point and block ice-nucleation) or bacterial ice-nucleating proteins (initiate freezing at high subzero temperatures) have not yet been reported for cyanobacteria (e.g. Kieft, 1988).

1.2.2.3. Complete desiccation

Certain chemical substances are capable of replacing water in anhydrobiotic (i.e. completely desiccated) organisms. These substances bind to biomolecules at the positions where water molecules are normally present in hydrated cells, thus preserving the native structure of proteins, lipid membranes and nucleic acids as well as preventing their chemical cross-linking (Crowe and Hoekstra, 1992; Potts, 1994). Non-reducing sugars such as sucrose and trehalose are well documented water-replacing substances for many anhydrobiotic organisms, including cyanobacteria. It is commonly thought that accumulation of water-replacement molecules in response to desiccation is the key adaptation, which enables cells to survive complete desiccation (Potts, 1994; Leslie et al., 1995; Crowe et al., 2002). These sugars are also known to prevent cross-linking between reducing sugars and amino groups of proteins (Chen et al., 2003). Besides preserving the structure of membranes and preventing them from fusing, trehalose has an important function of maintaining membrane lipids in the liquid crystalline state. Without trehalose, phospholipids undergo transition to the gel state during desiccation, which leads to the temporal loss of membrane functions upon rehydration and, hence, leakage of cell content (Crowe and Hoekstra, 1992).

In addition to the sugars, species of *Nostoc* may employ extracellular polysaccharides with the same function for preserving cell surface structures (Hill et al., 1997; Tamaru et al., 2005). EPS improve desiccation and salt tolerance by retaining and absorbing moisture, eliminating ROS, and absorbing cations as well as by providing a matrix for the extracellular accumulation of trehalose (Lan et al., 2010).

1.2.3. Reparation and de novo synthesis

An essential part of the repair process from damages by ROS is rapid de novo synthesis of photosystem II proteins, which enables normal cell functioning of cyanobacteria under continuous intensive sunlight (Singh et al., 2010; Lüttge, 2011). Some species of cyanobacteria apparently do not synthesize MAAs or scytonemin; therefore they have to utilize only metabolic reparative mechanisms for UVR damage (Castenholz and Garcia-Pichel, 2002). Cyanobacteria employ a number of mechanisms for repairing DNA including photoreactivation, recombination and excision repair as well as de novo synthesis of PS II proteins (Sinha and Häder, 2002). Since the genomes of cells progressively accumulate damages during long-term desiccation, the harm may be reduced by increasing the number of chromosome copies present per cell (Potts, 1999). Vegetative cells of some cyanobacteria are naturally polyploid (Singh et al., 2010), e.g. *Anabaena* cells contain, on average, eight

genome copies per cell. However, the chromosomal content of akinetes increases up to 450 copies, with an average value of 119 genome copies per akinete (Sukenik et al., 2012). Histone-like proteins play important roles in maintaining nucleoid organization and regulate DNA repair following UV irradiation (Potts, 1999). While certain species of cyanobacteria (e.g. *Nostoc* and *Chroococcidiopsis*) show remarkable stability of their genomes during long-term desiccation (Shirkey et al., 2003; Billi, 2008), other species (e.g. *Anabaena*) are capable of repairing massive DNA damages within a short time following rehydration (Singh et al., 2013).

1.3. Dormancy

In order to survive severe growth-limiting stress, bacteria may enter an inactive non-replicative state, which is termed dormancy. I accept the broad definition given by Kaprelyants et al. (1993), which states that dormancy is "a reversible state of low metabolic activity, in which cells can persist for extended periods without growth and division". This definition implies that two principally different kinds of dormancy exist: (i) endogenous dormancy, which is a result of active and voluntary shutdown of metabolic activity, and (ii) exogenous dormancy, which is externally forced and directly maintained by environmental conditions (Rebecchi et al., 2007).

Exogenous dormancy occurs when the conditions cannot support even the minimal rate of metabolism. Exogenous dormancy is often maintained when not enough water is available for cells, namely when cells are frozen, desiccated or exposed to strong osmotic solutions. This kind of dormancy is initiated directly by these stress factors and maintained by them, and is immediately discontinued when favorable conditions return. Yet, the cells still must possess or acquire adaptations, which enable them to survive the exogenous stress (Sudo and Dworkin, 1973; Rebecchi et al., 2007). These adaptations were described in section 1.2.2 as "protection from damage".

Endogenous dormancy is a predictive mechanism, which is triggered by environmental signals, but is not directly induced by them (Rebecchi et al., 2007). Such a dormancy program is initiated well before conditions become inappropriate for growth. The transition of vegetative cells from an active into a dormant state is itself an active metabolic process (Kaprelyants et al., 1993). Traditionally, dormancy has mainly been connected with morphologically specialized cells such as endospores, myxospores, akinetes and cysts. These types of dormant cells are clearly morphologically distinct from their respective vegetative forms, which led to extensive investigation of their functions.

1.3.1. Overview of bacterial dormant cells

This chapter will cover the properties of akinetes, which are the only commonly accepted type of dormant cells in cyanobacteria, as well as endospores and cysts of eubacteria. Endospores are produced by a limited number of Gram-positive eubacteria belonging to the *Bacillus* and *Clostridium* genera though many of the species were recently reassigned to new genera (Logan and Vos, 2015). Cysts are formed by

several non-related groups of eubacteria, e.g. purple photosynthetic bacteria (Berleman and Bauer, 2004), methane-utilizing (Whittenbury et al., 1970) and non-symbiotic diazotrophic bacteria (Sudo and Dworkin, 1973). Akinetes are produced by cyanobacteria, namely by members of the orders Nostocales and Stigonematales (Kaplan-Levy et al., 2010). Although endospores, akinetes and cysts are quite different in properties and the way they are formed, they do share some common features. These include arrested growth and division, absent or much reduced cell metabolism, severe modifications of cell morphology and ultrastructure, and increased tolerance to environmental stress as compared to their vegetative forms.

1.3.1.1. Endospores of Gram-positive bacteria

Endospores are the most resilient type of bacterial structures being extremely resistant to a variety of harsh conditions (Setlow, 1995). Bacterial endospores exhibit phenomenal resistance to heat being able to tolerate temperatures up to 150°C (Mury and Popham, 2014), and survive treatment with a variety of chemicals including acids, bases, oxidizing agents and organic solvents (Setlow, 2006). *Bacillus* spores can withstand exposure to ionizing and UV radiation for more than 500 days, extended desiccation and multiple cycles of freeze-drying (Nicholson et al., 2000). The spores are normally able to survive a level of stress which would be manifold or several orders of magnitude stronger than what their vegetative forms could tolerate, e.g. spores are up to 50-fold more resistant to UV-C radiation than growing cells, and tolerate at least 40°C higher temperatures (Setlow, 2006). Spores are also extremely resistant to freezing, desiccation, freeze-drying as well as to multiple cycles of freezing-thawing and drying-rehydration (Nicholson et al., 2000).

The most remarkable adaptation of spores is their thick multilayered envelopes, which consist of the exosporium, coats, outer membrane, cortex, germ cell wall and inner membrane (Setlow, 2006). These layers are intended to protect the core (i.e. spore's protoplast) from a variety of environmental insults. The envelopes provide a mechanical barrier against lytic enzymes, oxidizing agents, acids and other toxic chemicals as well as protection against predation. The cores contain much less water than vegetative cells (e.g. 27% vs. 80%), but the biomolecules are still fully hydrated, and thick envelopes block any further evaporation of water (Setlow, 2006). High mineralization of spore envelopes with calcium and manganese is responsible for the resistance of spores against heat (Mury and Popham, 2014). Pigments located in the spore's outer layers protect UV-sensitive core molecules from damages (Setlow, 2006). A group of small acid-soluble spore proteins bind to the core DNA and dramatically change its structure and properties making the DNA molecules resistant to heat and UV radiation. Dipicolinic acid, which is present in cores in high amounts, immobilizes proteins by reducing their molecular motion, and thus protects them from denaturation by heat (Slieman and Nicholson, 2001; Setlow, 2006). In addition, spores bear powerful reparative machinery, which eliminates damages to DNA upon spore germination (Nicholson et al., 2000). To summarize, endospores are completely metabolically inert structures (Setlow, 1995), which are isolated from external stress

with their multilayered envelopes, and protected from the inside by small molecules, which stabilize core DNA and proteins.

It is not known which stress factors or suboptimal conditions are responsible for inducing sporulation in natural habitats (Nicholson et al., 2000). However, under laboratory conditions, spore formation has been clearly identified as a strategy by which bacteria escape from nutrient starvation (Setlow, 2006). Spore development comes at a cost: bacteria have to invest resources for forming the resting structures and time for transitioning into and out of the dormant state (Lennon and Jones, 2011). Therefore, sporulation is usually the terminal phase of population development when no more nutrients can be extracted from the environment. Since lack of nutrients is a common condition in natural habitats, bacteria increase the efficiency of nutrient transport when the resources are limited before undergoing sporulation (Ferenci, 2001; Smith and Brun, 2005; López and Kolter, 2010). In addition to improved nutrient scavenging, cultures of spore-forming bacteria release cannibalistic toxins that kill susceptible subpopulations, thus increasing nutrient abundance for the other subpopulations (López and Kolter, 2010).

1.3.1.2. Cysts of eubacteria

The function of encystment is long-term persistence under dry conditions as well as avoidance of nutrient starvation (Sadoff, 2001). Cysts are usually produced in old cultures (triggers are not clear) or in nutrient-limited media with added butyrate (Whittenbury et al., 1970; Sadoff, 2001; Berleman and Bauer, 2004). The common feature for cysts formed by different taxonomic groups of bacteria is their resistance to desiccation, under which the cysts retain viability for weeks up to several years. However, cysts are neither as heat resistant nor as radiation-resistant as endospores. They have a slightly elevated resistance to UV-radiation, and survive temperatures which are only several degrees higher than what vegetative cells can withstand (Whittenbury et al., 1970; Sudo and Dworkin, 1973; Berleman and Bauer, 2004). Cysts display no endogenous respiration; however, unlike endospores they start to respire immediately when nutrient substrate is added (Sudo and Dworkin, 1973). Not much is known about the mechanisms of cyst desiccation tolerance: the multilayered mineral-lipid envelopes (Sadoff, 2001) possibly protect them from water loss, but the exact mechanisms are not known.

1.3.1.3. Cyanobacterial akinetes

Akinetes have an overwintering and long-term resting function, which allows cyanobacteria to persist under rather suboptimal than stressful conditions, such as low temperature, lack of light and a decrease in nutrient supply (Livingstone and Jaworski, 1980; Kaplan-Levy et al., 2010). The ability of akinetes to survive cold and darkness is generally far greater than that of vegetative cells (Adams and Duggan, 1999). Akinetes may also be resistant to desiccation and freezing, but, unlike endospores, they are very sensitive to elevated temperatures (with one exception), UV-radiation and chemical treatment (Sutherland et al., 1979, 1985; Hori et al., 2003; Olsson-

Francis et al., 2009), or they may not be more freezing and desiccation tolerant than their vegetative forms (Adams and Duggan, 1999). It seems that akinetes cannot tolerate complete desiccation, at least when it is produced by freeze-drying under a vacuum (Olsson-Francis et al., 2009). The thickened cell walls and multilayered envelopes are likely to act as a barrier that prevents water loss from protoplasts as well as to protect the cells from mechanical damage by extracellular ice. The reduced water content (Potts, 1994) and increased amount of sugars (Kaplan-Levy et al., 2010) in akinetes may prevent intracellular freezing. However, there is no experimental proof to support these assumptions, and hardly any attempts have been made to study the mechanisms of their tolerance to freezing and desiccation.

Akinetes represent a "seed bank" that provides inoculum for summer seasons when conditions are optimal for growth (Kaplan-Levy et al., 2010). Unlike endospores and cysts, akinetes are not completely dormant as they maintain detectable rates of metabolic activity. For instance, mature akinetes are capable of incorporating radioactive sulfur, fixing CO_2 at 10% of the rate observed during exponential growth, and consume and evolve oxygen at 7% of vegetative cells, but they maintain only residual photosynthetic activity (Sudo and Dworkin, 1973; Thiel and Wolk, 1983; Sutherland et al., 1985; Sukenik et al., 2007). Akinetes are much bigger than their respective vegetative cells, deposit large amounts of storage polymers, contain multiple copies of the genome and accumulate numerous ribosomes (Sukenik et al., 2012). These properties of akinetes allow for immediate resumption of metabolic activity and protein synthesis, which ensures quick repopulation of niches thus giving akinete-producing species an ecological advantage (Kaplan-Levy et al., 2010). It seems that conditions which trigger the differentiation of akinetes, reflect their main ecological function, i.e. overwintering. Decreasing temperature or its strong diurnal fluctuations as well as reduced light availability have been reported to be the major triggers for akinete formation in many species (Moore et al., 2005; Kaplan-Levy et al., 2010; Mehnert et al., 2014). The role of nutrients in controlling akinete differentiation is controversial: while some species of cyanobacteria respond to a lack of nutrients by producing akinetes, others never form akinetes under nutrient-limiting conditions (reviewed in Kaplan-Levy et al., 2010).

1.3.2. Endogenous dormancy in non-spore-forming bacteria

Only a limited number of species are able to produce specialized dormant cells, yet non-spore-forming species successfully co-exist with those that produce spores (cysts, akinetes, etc.). Modern microbiology accepts that a vast majority of bacterial species are capable of entering a dormant state (Kaprelyants and Kell, 1993; Nyström, 2004; Sachidanandham and Yew-Hoong Gin, 2009; Navarro Llorens et al., 2010; Lennon and Jones, 2011; Rittershaus et al., 2013). Such dormant cells have markedly reduced metabolic activity, but these are rather morphologically similar to their vegetative forms. Detection of such dormant cells became possible with the development of single-cell analysis based on fluorescence staining, flow cytometry, cell sorting and microsensor techniques (Brehm-Stecher and Johnson, 2004; Nyström,

2004; Lidstrom and Konopka, 2010; Müller and Nebe-von-Caron, 2010; Carlquist et al., 2012). The properties of dormant cells of non-sporulating bacteria have been vastly investigated over the past 30 years, and seem to be even better understood than the properties of endospores, cysts and akinetes.

The conditions which commonly induce this kind of dormancy are starvation as well as a complex of factors occurring in stationary phase cultures, which include critically high concentrations of cell metabolites, nutrient starvation and critical cell density in populations (Ishihama, 1999; Lazazzera, 2000; Nyström, 2004; Lennon and Jones, 2011). By reducing the rate of metabolic activity and by arresting growth and division, cells avoid energy exhaustion that leads to cell death. Dormancy could be viewed as the final and most extreme stage of the starvation response when improved nutrient uptake and cannibalism cannot provide enough energy to support minimum cell requirements (del Giorgio and Gasol, 2008; Navarro Llorens et al., 2010). Unlike endospores, which are believed to be completely metabolically inert, such dormant cells have to preserve very low metabolic activity for cell maintenance and survival. These include regulation of osmotic pressure and pH, maintenance of an energized state of the plasma membranes, turnover of macromolecules, and repairing damages to DNA molecules (Lennon and Jones, 2011; Gefen et al., 2014). Therefore, the cells need to accumulate endogenous reserves before turning dormant, which are usually deposited in the form of intracellular inclusions of biopolymers (Rittershaus et al., 2013). Alternatively, energy can be gained by digesting some of the cell constituents (Nyström, 2004).

The dormant cells of non-spore-forming species are not morphologically specialized, yet they may differ to some extent from their vegetative forms: the cells often reduce their size and may become spherical (Nyström, 2004; del Giorgio and Gasol, 2008). However, these changes in size and shape are a result of starvation rather than an adaptation to it: reduction of cell size occurs due to continuous self-digestion of cell constituents, and the spherical shape is the result of several cell divisions without an increase in cell volume (Kolter et al., 1993; Nyström, 2004). Although dormant cells do not include obvious morphological differentiation, they have profoundly altered the physical and biochemical properties. These include manipulations of the structure and composition of cell envelopes, changes in cytoplasm properties, modification of macromolecules, synthesis of specific proteins, changes in the structure of nucleic acids and selective activation or inhibition of certain processes (Siegele and Kolter, 1992; Kaprelyants and Kell, 1993; Givskov et al., 1994; Nyström, 2004; del Giorgio and Gasol, 2008; Rittershaus et al., 2013, etc.).

A hallmark of dormant (stationary-phase, starved) cells is alteration of their envelopes, which reflect the need for protection and insulation from stressful environments. Similarly to spores, dormant cells of non-sporulating species increase the thickness of peptidoglycan (Fig. 4) and reduce cross-linking between the peptidoglycan layers (Mukamolova et al., 1995; Makinoshima et al., 2003; Suzina et al., 2004; Rittershaus et al., 2013). The cells produce hydrophobic surface molecules

that favor adhesion and aggregation, which are known for improving the survival rate (Kolter et al., 1993; Monier and Lindow, 2003). The cells usually increase the rigidity of their plasma membranes by converting fatty acids to their cyclopropane derivatives and increasing the degree of lipid saturation. These changes seem to stabilize the membranes against turnover and degradation, e.g. lipid peroxidation by ROS (Cronan, 1968; Mukamolova et al., 1995; Härtig et al., 2005; Muñoz-Rojas et al., 2006). A high content of cyclopropane fatty acids was shown to be the key factor which enables gram-negative bacteria to survive freeze-drying (Muñoz-Rojas et al., 2006; Li et al., 2009). It is commonly known that microorganisms are more resistant to freezing, freeze-drying and desiccation when the cultures are in their stationary phase of growth, i.e. when membrane fluidity is decreased (Siegele and Kolter, 1992; Morgan et al., 2006; García, 2011). These findings are somewhat in contradiction with the adaptations described above, where increased membrane fluidity was necessary for surviving freezing and desiccation (Steponkus, 1984; Walters et al., 2002; Tanghe et al., 2003). In addition, such cells are more resistant to antibiotics and heat treatments (Anuchin et al., 2009).

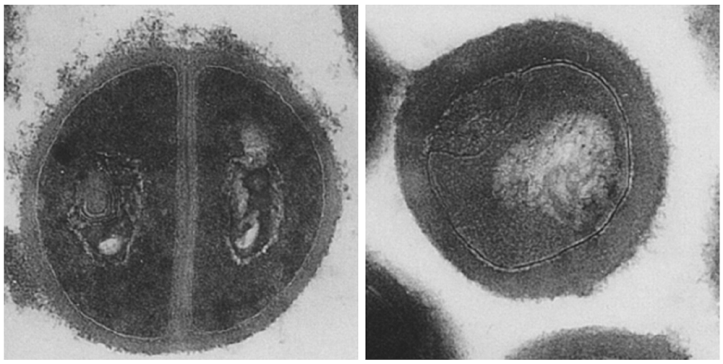

Fig. 4. Transmission electron micrographs of thin sections of *Micrococcus luteus* cells, a gram-positive non-spore-forming heterotrophic bacterium: cells in the exponential phase of growth (left) and after 4-month nutrient starvation (right); note the increased peptidoglycan thickness in the starved cell (Mukamolova et al., 1995).

Onset of the stationary phase triggers the expression of over a hundred proteins, which include molecular chaperones, enzymes and regulatory molecules as well as the modification of existing molecules (Tani et al., 2002; Navarro Llorens et al., 2010). These include enzymes, which are involved in self-digestion of proteins and lipids, elimination of ROS and synthesis of small molecules such as compatible solutes (Kolter et al., 1993). For instance, starved/stationary-phase cells accumulate trehalose, which is involved in heat and desiccation tolerance (Hengge-Aronis et al., 1991; Potts, 1994). Various chaperone and reparative proteins greatly contribute to the stability of RNA, DNA and other biomolecules, which seem to be one of the key adaptations against multiple stresses (Booth, 1998; Hengge-Aronis, 2002; Nyström, 2004).

Subsequently, the cells dramatically reduce the levels of rRNA and tRNA and block translation by modifying the structure of ribosomes. The ribosomes form dimers, which are a way to preserve the ribosome structure during long-term translational inactivity (Navarro Llorens et al., 2010). At the final stage of development, dormant cells are characterized by a markedly changed morphology and properties of their nucleoids, which are highly ordered and compact structures composed of DNA molecules and specific proteins bound to them (Frenkiel-Krispin et al., 2004). The DNA in such compact nucleoids forms a biocrystal, which is resistant to damages by UV and gamma radiation, iron and copper toxicity, thermal stress, and acid and base shock (Nair and Finkel, 2004). At this stage, nucleoids have a toroid or spherical shape, and carry no or very little transcription activity (Siegele and Kolter, 1992; Kolter et al., 1993; Robinow and Kellenberger, 1994; Frenkiel-Krispin et al., 2004). Recent research discovered that cytoplasm in dormant bacterial cells exists in a glassy state, which permits only the movement of small molecules, but restricts the motion of larger molecules, bodies and aggregates. Such cytoplasm properties preserve the subcellular architecture of dormant cells while allowing the diffusion of small proteins and signaling molecules (Parry et al., 2014).

1.3.3. Endogenous dormancy in non-akinete-forming cyanobacteria

Among non-spore-forming bacteria, dormancy has been widely explored for species associated with human microflora as well as pathogenic species, e.g. *Escherichia coli*, *Pseudomonas aeruginosa*, *Vibrio cholerae* and *Salmonella* species (Lennon and Jones, 2011). Similar studies on endogenous dormancy in cyanobacteria are rare, and these mostly explain the starvation-induced response as an acclimation rather than dormancy (Hardie et al., 1983; Schwarz and Grossman, 1998; Bhaya et al., 2000; Kumar Saha et al., 2003; Klotz et al., 2015). Most of the research conducted in this area described the mechanisms of avoiding oxidative stress, which occurs under intense light and starvation conditions. Cyanobacteria, as organisms with an oxygenic type of photosynthesis, are subject to stronger oxidative stress than other aerobic microorganisms. When anabolism is repressed under nutrient limitation, additional stress occurs due to the functioning of the photosynthetic apparatus. Cyanobacteria cope with oxidative stress by modifying the photosynthetic apparatus, down-regulation of photosynthesis and production of ROS-detoxifying enzymes (Schwarz and Grossman, 1998; Kumar Saha et al., 2003). Starvation and excessive light induce controlled degradation of phycobilisomes, which constitute the major light-harvesting antennae in cyanobacteria, as well as chlorophyll a, the pigment in the reaction centers and core antenna of photosystems I and II. Bleaching due to pigment decomposition seems to be a common response to nitrogen, sulfur, iron, phosphorus and inorganic carbon starvation, which has been described for all studied cyanobacterial species. In addition to reducing light harvesting, starved cells decompose phycobilisomes to gain energy and macronutrients. Similarly to the other eubacteria, cyanobacteria significantly improve the efficiency of nutrient uptake, release extracellular enzymes and digest intracellular reserves before slowing down

their metabolic activity (Hardie et al., 1983; Fresnedo and Serra, 1992; Billi and Caiola, 1996; Görl et al., 1998; Schwarz and Grossman, 1998; Bhaya et al., 2000; Schwarz and Forchhammer, 2005).

These changes are accompanied by an almost complete degradation of the thylakoid membranes and in some cases concurrent accumulation of glycogen granules and/or poly-β-hydroxybutyrate (PHB) inclusions (Wanner et al., 1986; Fresnedo et al., 1991; Billi and Caiola, 1996; Kumar Saha et al., 2003; Schwarz and Forchhammer, 2005). Starved and stationary-phase cells of unicellular cyanobacteria (e.g. *Synechocystis*) synthesize DNA-binding proteins similar to those of *E. coli*, which are essential for protecting bacterial DNA under stress. They maintain a slow turnover of proteins involved in photosynthesis and redox homeostasis, but do not synthesize proteins participating in the translational machinery (Schwarz and Forchhammer, 2005). *Synechocystis* cells in stationary-phase cultures synthesize the Chl-binding protein involved in the dissipation of light energy thus protecting PSII against photooxidative stress (Foster et al., 2007). Starved *Synechocystis* cultures expressed genes involved in chaperone function and oxidative stress protection, and various genes encoding proteins with unknown functions (Zhang et al., 2008).

Long-term nitrogen-starved cells of *Synechococcus* and *Chroococcidiopsis* can be considered as dormant: they suppress oxygen evolution and anabolic processes to undetectable levels, preserve only residual rates of photosynthesis and significantly reduce oxygen consumption. At the same time, the cells maintain their membrane potential and retain the capacity to regenerate when placed in nutrient-rich media (Billi and Caiola, 1996; Görl et al., 1998; Sauer et al., 2001).

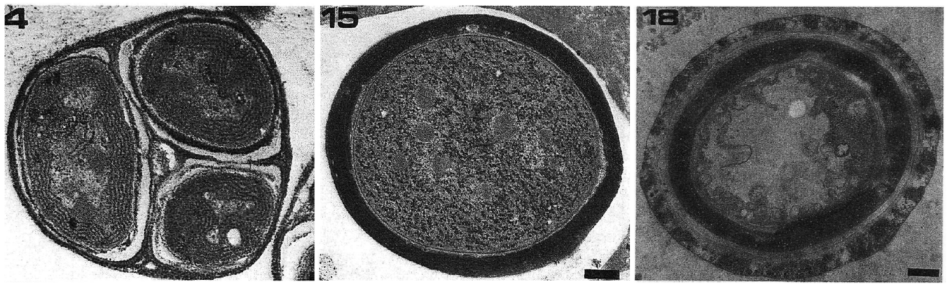

Fig. 5. Transmission electron micrographs of *Chroococcidiopsis* sp. cells in exponential (left) and late stationary (middle) phases of growth, both cultured in complete medium; cell stored in nitrogen-free medium for 4 months (right). Figures are taken from the paper by Billi and Grilli Caiola, 1996.

However, very little is known about the stress resistance of starved/stationary-phase cyanobacterial cells nor is there much information regarding their morphology, ultrastructure and the biochemical properties responsible for stress resistance. Aged and nitrogen-starved cultures of *Chroococcidiopsis* formed mostly single cells instead of multicellular aggregates. Such cells were metabolically inactive and surrounded by

thick multilayered envelopes, formed of sporopollenin-like compounds, acid- and beta-linked polysaccharides, lipids and proteins (Fig. 5; Billi and Caiola, 1996; Caiola et al., 1996). Similar observations have not yet been reported for other cyanobacterial species.

1.4. Cross talk between endogenous dormancy and stress tolerance

The action of a stress factor may trigger (i) a specific response, which is directed to cope specifically with this particular stress or (ii) a general stress response, which improves resistance to multiple environmental insults. While the specific stress response tends to eliminate a stress agent and/or to mediate repair of cellular damage that occurred due to its action, the general stress response makes cells broadly stress resistant in such a way that damage is prevented rather than needing to be repaired (Hengge-Aronis, 2002).

Examples of specific stress responses may include osmotic adjustment via intracellular accumulation of compatible solutes, synthesis of UVR-screening pigments and ROS-scavenging enzymes, desaturation of membrane phospholipids in response to decreasing temperature, etc. When cellular damages occurring due to different stresses are similar, acquisition of tolerance to one of the stresses may, to some extent, improve tolerance to the others. As discussed above, desiccation (i.e. air drying), freezing and osmotic stress result in considerable loss of cellular water. Hence, when cells are pre-adapted to osmotic stress, they may better survive freezing and desiccation (Tanghe et al., 2003; Lan et al., 2010). However, protection from each of these stresses may require different chemical substances as well their amount and spatial distribution. For instance, freeze tolerance is provided by a number of diverse chemical substances (e.g. sugars, alcohols, amino acids) that are completely ineffective against complete desiccation (Crowe et al., 1990). Although trehalose is involved both in osmotic and desiccation tolerance, dry cells also require its presence on the outer side of the plasma membrane and hence, must possess trehalose transporters to deliver it to the extracellular/periplasmic space (Crowe et al., 2004). In addition, the trehalose concentration required to stop membrane fusion under freezing and incomplete desiccation is too low to prevent leakage of cellular content upon rehydration after complete desiccation (Crowe et al., 2002). Finally, additional specific adaptations may be required to cope with each of these stresses (e.g. Tanghe et al., 2003).

In contrast to the specific response, a general stress response makes cells resistant to a broad range of stresses thus allowing for survival from stresses that have not been encountered yet (Hengge-Aronis, 2002). Most often, the general stress response is initiated in stationary-phase cultures, specifically by metabolic alteration and quorum sensing molecules in critically dense cultures (Lazazzera, 2000) or by lack of carbon, nitrogen, phosphorus or other nutrients (Navarro Llorens et al., 2010). Stationary-phase and starvation-mediated responses may be governed by a single (e.g. *E. coli*) or several sigma factors, subunits of RNA polymerase, which replace

vegetative sigma subunits under stress conditions (Hengge-Aronis, 2002; Navarro Llorens et al., 2010). These sigma factors profoundly alter the cell properties by expressing genes that are not expressed during growth phases: for example, the action of RpoS, the sigma factor of *E. coli*, triggers the expression of 10% of the genes when there is the transition to the stationary phase. The genes, which belong to the RpoS regulon, are responsible not only for cessation of growth and metabolism, but also for resistance to multiple stresses, such as heat shock, desiccation, oxidative and osmotic stress, resistance to UVR and acids, etc. Heat shock induces another type of general stress response, which is governed by an alternative sigma factor, but also renders cells resistant to multiple stresses (Hengge-Aronis, 2002; Navarro Llorens et al., 2010).

The adaptations provided by stationary-phase/starvation response (leading to dormancy) and by specific stress responses (i.e. acquiring specific stress tolerance) are not identical, although the resistance mechanisms may overlap. The role of specific and general responses are also different: while specific adaptations enable cell growth and metabolism under suboptimal conditions and/or ensure quick recovery from stress, dormancy represents a mechanism which tends to prevent any cellular damage by careful conserving the cellular components, isolating them from the environment and cutting off energy expenses (Hengge-Aronis, 2002; Nyström, 2004). Finally, the general stress response usually provides cells with stronger resistance than any specific stress response, i.e. cells pre-adapted to a particular stress through exposure to non-lethal levels are less resistant than dormant cells (Kolter et al., 1993). For example, a short starvation episode provided *E. coli* with stronger osmotic tolerance than treatment with non-lethal hyperosmotic solutions (Jenkins et al., 1990).

Examples of mechanisms which are common for specific and general responses include synthesis of trehalose (Hengge-Aronis et al., 1991), ROS-scavenging enzymes (McCann et al., 1991) and activation of membrane osmoporters (Pichereau et al., 2000). However, in other cases, the mechanisms may be different or even contrasting, e.g. increased membrane fluidity favors survival of freeze-thawing and desiccation in vegetative cells, while the opposite (i.e. rigid membrane) is involved in tolerance to these stress factors in dormant cells (see above). To summarize, a general stress response that leads to dormancy makes the cells resistant by folding and modifying the macromolecules as well as protecting them with chaperone proteins, while a specific stress response rather involves reparation, de novo synthesis and biochemical adjustment.

1.5. *Phormidium* and *Microcoleus*

1.5.1. Taxonomic position

The objects of the research introduced in this thesis included species of *Phormidium* and *Microcoleus*, the closely related genera of filamentous non-heterocystous cyanobacteria within the Oscillatoriales order. Both *Phormidium* and *Microcoleus* are polyphyletic genera according to molecular analyses (Strunecký et

al., 2013). The taxonomy of Oscillatoriales has been recently extensively revised, e.g. a large group of *Phormidium* species was included into *Microcoleus*, and new genera were established (Strunecký et al., 2013; Komárek et al., 2014). Thus, *Phormidium* species, which were the primary objects of this research, currently fall into both *Phormidium* and *Microcoleus*. Specifically, the strains *Phormidium* sp. 816 CCALA, 845 CCALA and 850 CCALA (Fig. 6; see Paper #3) have been identified as *Microcoleus* species using the polyphasic approach (Strunecký et al., 2013) while the field populations clearly belonged to Group VIII of *Phormidium* according to cytomorphological criteria (see Paper #4). Most of the published studies, which addressed the ecology, stress tolerance and survival strategies of these cyanobacteria, studied not yet sequenced strains, used the old nomenclature and/or identified the species only according to their morphological traits. Therefore, this review includes representatives of both genera and refers to the original names used in the publications.

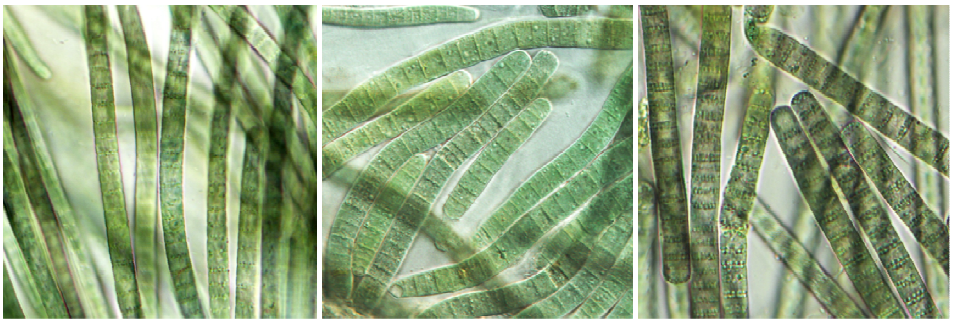

Fig. 6. *Microcoleus* sp. 816 CCALA, previously *Phormidium* cf. *autumnale*, isolated from a stream in the vicinity of a glacial moraine in Northern Sweden, Lapland, Abisko (left); *Microcoleus* sp. 845 CCALA, previously *Phormidium* sp., isolated from a stream with moss carpets on the Svalbard archipelago (middle); and *Microcoleus vaginatus* 858 CCALA, previously *Phormidium* sp., isolated from a small pool in a moraine on the Svalbard archipelago (right).

1.5.2. Distribution in polar habitats

Phormidium and *Microcoleus* species have colonized many types of habitats in both Polar Regions: they often dominate cryoconite communities, constitute a major component of periphyton in lakes and rivers, form thick benthic mats in ponds and lakes, and represent primary colonizers in fellfield soils, but are normally absent in snow fields and endolithic habitats (Vincent, 2000; Komárek et al., 2008). *Phormidium* and *Microcoleus* species form extensive macroscopic films and mats in seepages and shallow meltwater streams and ponds, the most common hydro-terrestrial habitats in the high Arctic and Antarctica (Vincent et al., 1993; Komárek et al., 2008). These are thick multilayered structures, often with vertical stratification, which are composed of filaments embedded in an EPS matrix and various mineral particles and organic debris. Although cyanobacteria are the most abundant organisms

in these communities and constitute the majority of their biomass, the mats also contain diatoms, green algae, microscopic fungi, rotifers, nematodes and a variety of heterotrophic bacteria (de los Rios et al., 2004; Stal, 2012). Being exposed to continuous sunlight, such communities are usually highly pigmented (Fig. 7) due to the presence of sun-screening carotenoids, xanthophylls and possibly scytonemin (Vincent et al., 1993; George et al., 2001).

Fig. 7. Dark *Microcoleus* mat in a shallow meltwater stream next to bird rookeries (left); orange-pigmented *Phormidium* mat in a shallow seepage (right).

1.5.3. Tolerance to PAR, UVR and oxidative stress

The degree of tolerance to UVR and PAR as well as the protective mechanisms may vary among oscillatorian species. These include avoidance of radiation exposure by self-shading, migration to the soil subsurface or within cyanobacterial mats (Castenholz et al., 1991; Quesada et al., 1999; Pringault and Garcia-Pichel, 2004; Quesada and Vincent, 2012), protection with ROS-quenching compounds (e.g. carotenoids) and ROS-detoxifying enzymes (Vincent et al., 1993; Quesada and Vincent, 1997; Roos and Vincent, 1998), efficient DNA-reparation mechanisms and de novo synthesis of PS II proteins (Lüttge, 2011). *Phormidium* and *Microcoleus* mats and crusts may produce UVB-screening mycosporine-like amino acids and rarely scytonemin. Alternatively, these species do not produce any pigments which absorb radiation in the UV-region (Quesada et al., 1999; George et al., 2001; Castenholz and Garcia-Pichel, 2002; Pattanaik et al., 2008; Lan et al., 2010; Bhandari and Sharma, 2011). In desert soil crusts, desiccated *Microcoleus vaginatus* preserves the integrity of the photosynthetic apparatus under extremely intense light, which enables activation of photosynthesis within minutes after rehydration (Harel et al., 2004). The character and extent of the damages to DNA, which occur due to UVR exposure, and the reparative mechanisms seem to be universal in all cyanobacteria probably including *Phormidium* and *Microcoleus* species (Castenholz and Garcia-Pichel, 2002).

1.5.4. Freezing and desiccation tolerance

Most of the adaptations to UVR and PAR intercept with the mechanisms of desiccation and freeze tolerance since the action of both factors results in oxidative stress and damages to nucleic acids and other macromolecules (e.g. Chen et al., 2012; Rajeev et al., 2013). The mat-like structure of communities can be considered as a self-protection strategy, which allows for the retention of water under low air humidity values (Potts, 1994). Terrestrial species of *Phormidium* and *Microcoleus* are surrounded with thick EPS sheaths, which improve desiccation and salt tolerance by absorbing and retaining moisture, absorbing cations and eliminating ROS (Vincent, 2000; Lan et al., 2010; Chen et al., 2012; Helm and Potts, 2012). In response to mild water stress, *Phormidium* laboratory cultures and *Microcoleus* desert soil crusts accumulate compatible solutes such as sucrose and trehalose (Hershkovitz et al., 1991; Chen et al., 2006). A recent study on the genome-wide response of *Microcoleus vaginatus* desert crusts reported that the onset of desiccation promoted the expression of multiple genes which are involved in the synthesis of trehalose and sucrose, chaperone proteins that reduce desiccation-induced aggregation of cell proteins, chaperone chlorophyll-binding proteins, ROS-scavenging catalases and ROS-quenching carotenoids, enzymes that reverse protein oxidation, and phycobilisome-degrading enzymes. Rehydration activated genes responsible for DNA repair, synthesis of PS II proteins, chlorophyll and allophycocyanin, uptake and assimilation of various nutrients and activation of respiration (Rajeev et al., 2013).

Although the ability of *Phormidium* and *Microcoleus* to survive freezing and desiccation has been well documented (Vincent and Howard-Williams, 1986; Davey, 1989, 1991; Hawes et al., 1992; Šabacká and Elster, 2006; Chen et al., 2012, etc.), the mechanisms and the rates of their tolerance remain poorly understood:

1. In part, this is caused by the fact that precise damages from incomplete desiccation and especially freezing-thawing remain unknown even for other micro- and macroorganisms though many hypotheses have been suggested. Hence, the precise molecular and genetic mechanisms underlying freeze and desiccation tolerance cannot be elucidated (Hoekstra et al., 2001; Tanghe et al., 2003; Harel et al., 2004; Muldrew et al., 2004).

2. Although the tolerance mechanisms of microorganisms, including cyanobacteria, are generally well studied, many of these have not yet been confirmed for *Phormidium* and *Microcoleus* species. For instance, it is not known whether desaturation of membrane lipids is involved in their freezing and desiccation tolerance, in which compatible solutes protect them against freezing, nor whether macromolecules (DNA, proteins) are preserved during the action of stresses or are repaired upon thawing and rehydration, etc. Terrestrial freshwater *Phormidium*-dominated mats from Antarctica produced ice-binding protein-containing substances, which affected the morphology and size of ice crystals. However, the exact function of these substances is not known nor which organisms produce them in the mixed mat communities (Raymond and Fritsen, 2004). The mechanisms of tolerance can greatly

differ among other taxonomic groups of cyanobacteria, e.g. *Nostoc* and *Chroococcidiopsis* species preserve genome integrity in a desiccated state (Shirkey et al., 2003; Billi, 2008), while *Anabaena* repairs the massive damages to the genome which accumulated during desiccation (Singh et al., 2013).

3. The exact functions of certain structural and chemical changes are not clear. As discussed above, the protective substances, which are common for many organisms, may have multiple functions in stressed cells. For instance, *Phormidium* and *Microcoleus* species synthesize sucrose and trehalose in response to water stress (Hershkovitz et al., 1991; Rajeev et al., 2013), but the exact role of these sugars have not been studied for this group of cyanobacteria. It has been suggested that sucrose acts as a compatible solute under hyperosmotic stress (Chen et al., 2003), and both sugars prevent cells from water loss under high RH, i.e. close to 100% (Hershkovitz et al., 1991). However, their role in depressing the freezing point, stabilizing membranes and macromolecules in frozen and partially desiccated cells as well their water-replacing function in completely dry cells have not been documented yet. As pointed out by Crowe et al. (1990), freezing and desiccation do not necessarily require the same compatible solutes for preserving the native structure of macromolecules. In addition, it is not known which concentration of these sugars in cyanobacterial cells and their ratio are required to provide each of the aforementioned functions. The same is true for the role of the EPS, which may protect cells in multiple ways (Lan et al., 2010). It has been hypothesized that EPS sheaths may protect cyanobacteria by preventing ice nucleation in the immediate vicinity of the cell surface (Vincent, 1988); however, no studies have tested this role in freeze tolerance.

4. It is not known whether the desiccation and freezing tolerances are species-specific and/or habitat-specific, or if all *Phormidium* and *Microcoleus* species share the same degree of stress tolerance regardless of the environments they inhabit. It seems that desiccation tolerance is a common feature of terrestrial and desert-dwelling isolates, yet no studies have investigated the tolerance of aquatic isolates. Also, there have been no comprehensive studies on tolerance mechanisms of a particular strain or natural community, i.e. it is not clear whether a studied strain/community possesses a full complex of adaptive mechanisms, or the sets of adaptations differ among strains/communities.

5. The degree of drying that cells are able to withstand has not been investigated yet either in terms of survival rate of cells or extent of water loss. Other genera occurring in hot and cold deserts, such as *Nostoc* and *Chroococcidiopsis*, are known to withstand complete desiccation, i.e. the conditions when the hydration shell of biomolecules is lost (Hawes et al., 1992; Caiola et al., 1996; Billi and Potts, 2002; Tamaru et al., 2005). Desert communities are subject to extreme water stress under natural conditions, which may potentially result in the nearly complete loss of all cellular water (Potts, 1994; Belnap, 2003; Rajeev et al., 2013). However, the water content in dry cells of *Phormidium* and *Microcoleus* species has not been measured in

either field or laboratory studies. Hence, it is not clear which rate of dehydration these species are able to tolerate, and whether they survive complete desiccation.

6. Furthermore, the conditions that lead to the development of desiccation (and freezing) tolerance are not clear. While *Nostoc* and *Chroococcidiopsis* possess constitutive desiccation tolerance (i.e. present under optimal conditions), the survival of many species of microorganisms is strongly dependent on environmental conditions and require pre-adaptation (Morgan et al., 2006). The study by Rajeev et al. (2013) gave an indication that the tolerance of *Microcoleus* could be induced by slow dehydration as natural desert crusts expressed multiple genes on the onset of desiccation.

1.5.5. Dormancy

Exogenous dormancy, defined as the absence of photosynthesis and respiration in a desiccated state, has been well documented in *Phormidium* and *Microcoleus* species (Brock, 1975; Harel et al., 2004; McKnight et al., 2007; Lan et al., 2010; Rajeev et al., 2013). In contrast, virtually nothing is known about endogenous dormancy in this group of cyanobacteria. Similarly to the studies on unicellular species, the changes which accompany nutrient starvation in *Phormidium* cultures are explained as acclimations to nutrient deficiency. These include increased efficiency of nutrient uptake followed by cessation of growth and degradation of phycobilisomes, small soluble proteins and thylakoid membranes (Fresnedo et al., 1991; Fresnedo and Serra, 1992). Available data suggest that, under prolonged darkness, at least some *Phormidium* species do not form any dormant cells, either morphologically specialized or metabolically inert. Instead, their cells rely on a balanced self-digestion of cell constituents in the case of complete darkness (Montechiaro and Giordano, 2006; Montechiaro et al., 2006) or maintain constant photosynthesis provided that at least a little light (<1 μmol m^{-2} s^{-2}) is available, e.g. in ice-covered lakes during polar winters (McKnight et al., 2000; Henshaw and Laybourn-Parry, 2002; McMinn and Martin, 2013).

2. AIMS AND TASKS

The main aims of the research were to:

(i) characterize the strategies which enable survival of *Phormidium* and *Microcoleus* species in terrestrial habitats of the High Arctic;

(ii) discover whether populations exist in a dormant state under natural conditions;

(iii) identify whether the species produce any specialized resistant or dormant cells under natural conditions, and describe their morphology, ultrastructure, metabolic status and stress resistance;

(iv) describe the conditions which enhance stress tolerance and/or lead to production of dormant cells.

3. SUMMARY

As discussed above, dormancy in non-akinete-producing cyanobacteria has received very little attention, with there being no information on dormancy among *Phormidium* and *Microcoleus* species. As no morphologically specialized cells have ever been encountered, I assumed that any presumptive dormant cells might not be visually different from their respective vegetative cells. Hence, the criteria for identifying the dormant cells had to be defined. I accepted that dormant cells of cyanobacteria would generally possess the properties similar to those in non-sporulating heterotrophic eubacteria: reduced or absent metabolic activity displayed as a low rate/absence of respiration and decreased ATP content; arrested growth and ability to multiply; restricted transcription and translation expressed as condensed nucleoid and decreased RNA content; and normally enhanced resistance to environmental insults. The visible properties might optionally include changes in ultrastructure (e.g. thickness of cell envelopes, degradation of subcellular structures), deposition of storage polymers and changes in biochemistry (e.g. accumulation of cryo- and osmoprotective substances). These and other issues were discussed in **Paper I**, which summarized the stress factors acting in various polar habitats, the adaptations and survival strategies of polar cyanobacteria and the types of dormancy among different groups of eubacteria. In this Introduction, the review has been significantly broadened and complemented with new data from recently published research.

Since dormant and/or stress resistant cells were not expected to have a distinct morphology, I needed a method which would allow for the detection of dormant (according to the aforementioned criteria) and resistant cells, i.e. those which survive stress treatment. Moreover, I needed an approach for evaluating the population stress response by using the cell survival rate as well as their state after stress treatments. The traditional plating method, which is widely used for unicellular bacteria, could not be used to assess the number of viable cells in cultures of filamentous cyanobacteria. All available studies evaluated the stress response of *Phormidium* and *Microcoleus* by 'bulk' measurements of respiration and photosynthesis in natural crusts and films or in laboratory cultures. This approach provided only an average on the state of physiologically heterogeneous populations without considering the properties of single cells. As widely discussed in the literature, bacterial populations display high heterogeneity in their morphology, viability and physiological activity, especially under stressful conditions; similar behavior is also typical for filamentous cyanobacteria (Fig. 8; unpublished results). For evaluating cell viability, injuries and metabolic activity, I employed multicolor staining with a combination of fluorescent dyes, which allowed for simultaneous estimates of cellular respiration activity (CTC), membrane and nucleoid integrity (SYTOX Green and DAPI), and detection of photosynthetic pigments fluorescence along with morphological observations. According to the staining results, five types of cells could be distinguished: (i) active

and intact, or live; (ii) injured but active; (iii) metabolically inactive but intact, or dormant; (iv) inactive and injured, or dead; (v) non-nucleoid-containing dead cells. The method has been introduced in **Paper II**.

Although each of these dyes has been used routinely for research on heterotrophic bacteria, only DAPI had been systematically used for cyanobacteria. A few studies employed SYTOX Green for unicellular species while CTC staining has not previously been used in cyanobacterial research. Also, the combination of these three fluorescent dyes had never been used simultaneously on any species of microorganisms. The paper describes the staining procedures (conditions, staining time and concentrations), spectral properties of the dyes and photosynthetic pigments, interaction between the dyes, proper handling of cyanobacterial samples and interpretation of the staining results. The method enables an accurate assessment of dead and live cells in both natural samples and laboratory cultures, and detects cell injuries associated with plasma membranes. Of the aforementioned criteria for dormant cells, the method indicates cell viability, respiration rate and the shape of nucleoids. The method was subsequently used in both laboratory experiments and field observations.

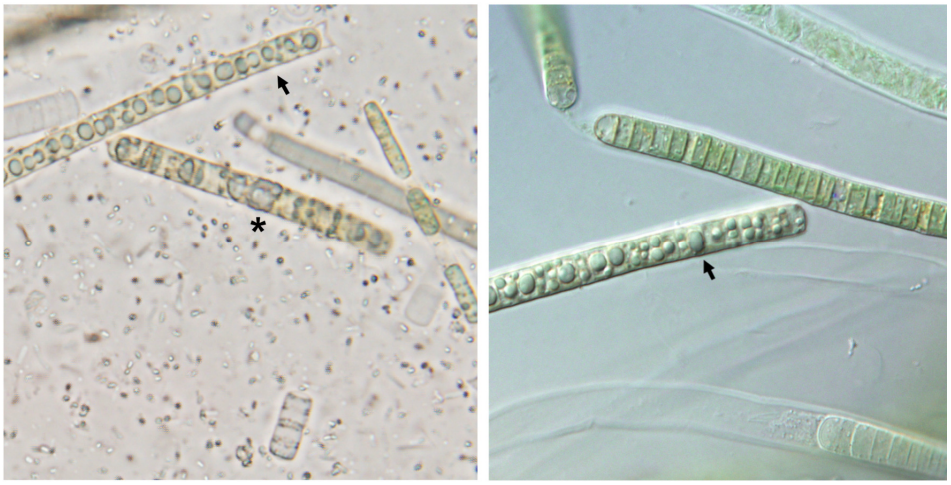

Fig. 8. Batch culture of *Microcoleus* sp. grown for 16 months (left) and 7 months (right) in full BG-11 medium; the cells have different size, shape, pigmentation and intracellular inclusions – presumably cyanophycin (*asterisk*) and liquid oil-like inclusions (*arrows*).

Freezing, desiccation and intensive UVR and PAR were considered as the main stress factors acting in terrestrial habitats of the High Arctic. In **Paper III**, I studied the desiccation tolerance of three *Microcoleus* strains, isolated from shallow seepages and streams in Svalbard and Abisko, under controlled laboratory conditions. As mentioned earlier, no study had investigated the degree of water loss which *Microcoleus* species are able to tolerate, the proportion of cells that survive

desiccation treatments, injuries that cells sustain during desiccation and rehydration, and the environmental factors which induce/improve desiccation tolerance. The strains were grown as thin biofilms in liquid medium to provide consistent conditions for growth and even water loss during subsequent desiccation in contrast to previous studies that desiccated thick pieces of material.

The first aim was to test whether the strains had the ability to tolerate complete (at 12% RH) or/and incomplete desiccation (at 85% RH). The second aim was to determine whether their desiccation tolerance was a constitutive or inducible trait: the former implies that the cells tolerate desiccation at any stage of growth and under any conditions, while the latter requires pre-acclimation under particular conditions. Since freezing and incomplete desiccation are similar vectors in terms of dehydration rate, acclimation to cold was selected as a possible inducer of a specific stress response, while nitrogen starvation was employed to invoke the general stress response. Finally, injuries, metabolic activity and state of nucleoids prior to desiccation and after rehydration were monitored with the fluorescence staining method.

Laboratory experiments showed that, despite expectations, the species were neither able to tolerate complete desiccation nor possessed constitutive desiccation tolerance since no cells in the optimally grown cultures survived the desiccation treatments. Acclimation to cold (the specific response) improved tolerance to incomplete desiccation very poorly while nitrogen starvation (the general stress response) strongly increased the survival rate of cells, up to 65%. The cells in nitrogen-starved cultures were not dormant as they maintained respiration and had unfolded nucleoids; the cells surviving incomplete desiccation resumed respiration immediately upon rehydration and carried no injuries associated with plasma membrane integrity. The cells did not alter their size and shape in response to cold temperature, nitrogen starvation and subsequent desiccation-rehydration. Moreover, the cells which survived the desiccation treatments were not different from non-decayed dead cells. Degradation of photosynthetic pigments, changes in the structure of sheaths and accumulation of small intracellular inclusions were the only morphological traits that differed among the treatments.

Paper IV describes a set of observations over the full annual cycle of two *Phormidium* populations in the High Arctic (Central Spitsbergen, Svalbard Archipelago). The main objectives of this study were to investigate the survival strategies of natural communities in hydro-terrestrial habitats and to find whether the populations produced any specialized dormant or stress-resistant cells under natural conditions. The study described the viability, morphology and ultrastructure of cells and filaments over the full annual cycle including the growth season (i.e. water is available) and winter, and monitored environmental factors (temperature, irradiance, water conductivity, macronutrient content).

Terrestrial *Phormidium* populations did not develop endogenous dormancy under the natural conditions of the High Arctic. Based on the results of the respiration assay, nucleoid morphology and evidence of cell fission it is apparent that the cells

remained active whenever liquid water was available. In contrast to polar aquatic and non-polar terrestrial habitats, these populations cannot avoid being frozen, hence they experience periods of exogenous dormancy during winter. The overwintering cells were well adapted to freezing stress as at least 85% of the cells survived winter; they possessed intact membranes and resumed respiration within minutes after thawing. The cells did not have altered morphology when observed any time during the year; any changes in ultrastructure included only degradation of thylakoids due to either excess or the absence of light. Although the thickness and structure of the cell walls did not change, the trichomes were surrounded with EPS sheaths, whose thickness increased remarkably towards the end of the vegetative season. Thus, protection from freezing stress was likely provided by thick sheaths and presumably by biochemical adjustments. Decreases in temperature and nitrogen/phosphorus deficiency did not seem to play any role in acquiring freezing tolerance, because these factors remained relatively constant throughout the vegetative season. More probably, the production of sheaths and possibly biochemical adjustments were triggered with growing salinity as water conductivity had increased by several times over the course of summer or, alternatively by lack of other nutrients than those we measured. Finally, the populations disseminate under more favorable conditions during spring melt by fragmenting their trichomes into hormogonia and releasing them from their sheaths into the environment.

4. CONCLUSIONS

Dormancy

Phormidium/*Microcoleus* natural communities exist in a state of exogenous dormancy when water is not available in the liquid state, specifically during winter freezing and seasonal drying. In the frozen state, the cells, by definition, cannot maintain metabolic activity since 0.25 g g^{-1} dm of non-freezable water (Crowe et al., 1990) is too low to sustain any measurable enzymatic activity (Sun, 2002). Accordingly, the cells become metabolically inactive when desiccated to a similar (e.g. 0.23 g g^{-1} dm) or lower water content. However, neither the whole communities nor individual cells entered a state of endogenous dormancy under any studied laboratory or natural conditions. Due to the short summers in the High Arctic and frequent desiccation episodes in terrestrial habitats, such a strategy seems to be advantageous for slow-growing cyanobacteria, because the transition to dormancy and exit from it requires time and resources. In contrast, exogenous dormancy is discontinued immediately after thawing and rehydration thus expanding the periods for growth. Similarly to heterotrophic bacteria, the endogenous dormancy in cyanobacteria is rather expected to develop as a response to severe long-term nutrient limitation or in old batch cultures. The fact that nitrogen-depleted cells in the laboratory experiments did not show signs of dormancy can be explained by the insufficient duration of the treatments: cyanobacterial cells are very rich in nitrogen, which is stored in the form of phycobilisomes and cyanophycin deposits, and hence, complete exhaustion of their intracellular resources happens over longer periods of nitrogen deprivation. For instance, many cells in *Microcoleus* cultures stored in nitrogen-free media for 10 months as well as the cells in old batch cultures showed spherical nucleoids and markedly decreased respiration (Fig. 9; unpublished results).

Conditions which improve stress resistance

Nevertheless, nitrogen depletion triggered a general stress response that provided the laboratory cultures with strong tolerance to incomplete desiccation. Since the optimally grown cultures failed to survive the drying treatments, we may assume that *Microcoleus* species do not possess constitutive desiccation tolerance. Accordingly, they might not possess constitutive freezing tolerance, because the natural freezing regimes cause the same rate of cell dehydration as the incomplete desiccation regime employed in the laboratory experiments (Crowe et al., 1990). Instead, *Microcoleus*/*Phormidium species* require certain suboptimal conditions for acquiring these tolerances; while low temperature and light conditions are not the cause, increasing salinity is likely to trigger desiccation and freezing tolerance in nutrient-sufficient environments. Alternatively, desiccation and freezing tolerances may be induced by a lack of micronutrients (not yet tested).

Adaptations to stress

Survival under freezing and desiccation conditions was not attributed either to any morphologically/ultrastructurally specialized cells or to a small number of non-

specialized resistant cells. Instead, a high percentage of cells in natural populations and laboratory cultures survived freezing and desiccation. In both the laboratory and field experiments, no cells were observed with a morphology different from the vegetative cells except for changes in pigments and thylakoid membranes as well as accumulation of storage polymers. The cells neither increased the thickness of their cell walls nor produced any additional cell envelopes for surviving winter stresses. However, the EPS sheaths seemed to be an important factor for surviving both winter stress and seasonal drying since (i) their thickness increased remarkably towards the end of the vegetative seasons and was correlated with water availability in the habitats, and (ii) in frozen samples, the proportion of dead cells within sheathless trichomes was much higher than in ensheathed ones. Presumably, certain biochemical adjustments, which are yet to be investigated, provide resistance to freezing and desiccation in *Phormidium*/*Microcoleus* species.

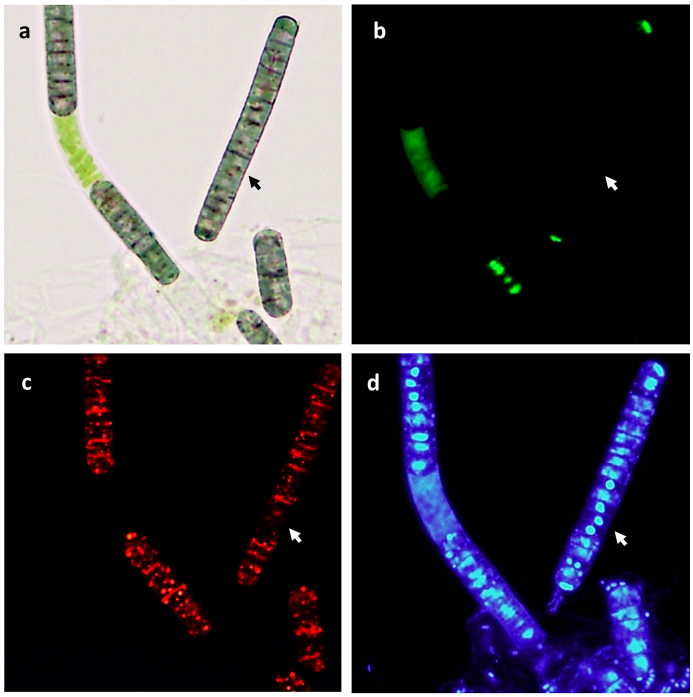

Fig.9. Batch culture of *Microcoleus* grown for 10 months in full BG-11 medium (a), stained with SYTOX Green (b), CTC (c), and DAPI (d). Presumable dormant cells (arrows) have intact membranes (SYTOX Green-negative), decreased respiration (CTC-negative), and spherical nucleoids (DAPI-stained).

Survival strategy

The slow growth rates and high stress resistance pre-determine the perennial character of natural *Phormidium*/*Microcoleus* populations, which means that populations are not established with a few "seeds" every spring but most of the cells

remain viable over the winter. During the spring and early summer, the populations are not under freezing and desiccation stress. In this period of relatively favorable conditions, the populations disseminate by producing hormogonia, releasing them from sheaths into the environment. This is followed by synthesis of new EPS sheaths and the assembly of filaments into mat-like structures. These adaptations, together with the presumed active migration of trichomes into more hydrated conditions, slow down water loss thereby extending the periods for growth and preventing complete desiccation, a condition which *Phormidium* and *Microcoleus* species are not able to survive.

Future prospects

As discussed above, the injuries due to freezing, desiccation, osmotic stress and solar radiation in cyanobacterial cells are only partially understood, especially in *Phormidium* and *Microcoleus* species. Tolerance mechanisms have been investigated mostly in *Nostoc* and *Chroococcidiopis*, while those in other cyanobacterial taxa remain largely unknown. The mechanisms and rate of stress tolerance have to be compared among different species of *Phormidium* and *Microcoleus*, their isolates from various types of habitats and climatic zones as well as with other cyanobacterial species. Since the tolerance mechanisms have been studied for different isolates and natural communities of *Phormidium* and *Microcoleus*, it is important to describe a full set of adaptions which provide stress resistance to a particular species or population. Furthermore, environmental factors other than those described here are yet to be tested for their role in inducing freezing and desiccation tolerance.

Although endogenous dormancy was not encountered under the studied conditions, it is very likely that *Phormidium* and *Microcoleus* species enter a state of endogenous dormancy in response to long-term nutrient starvation as is the case with the majority of studied bacteria. Hence, it is important to study the properties of such dormant cells as well as their resistance to various kinds of environmental stresses.

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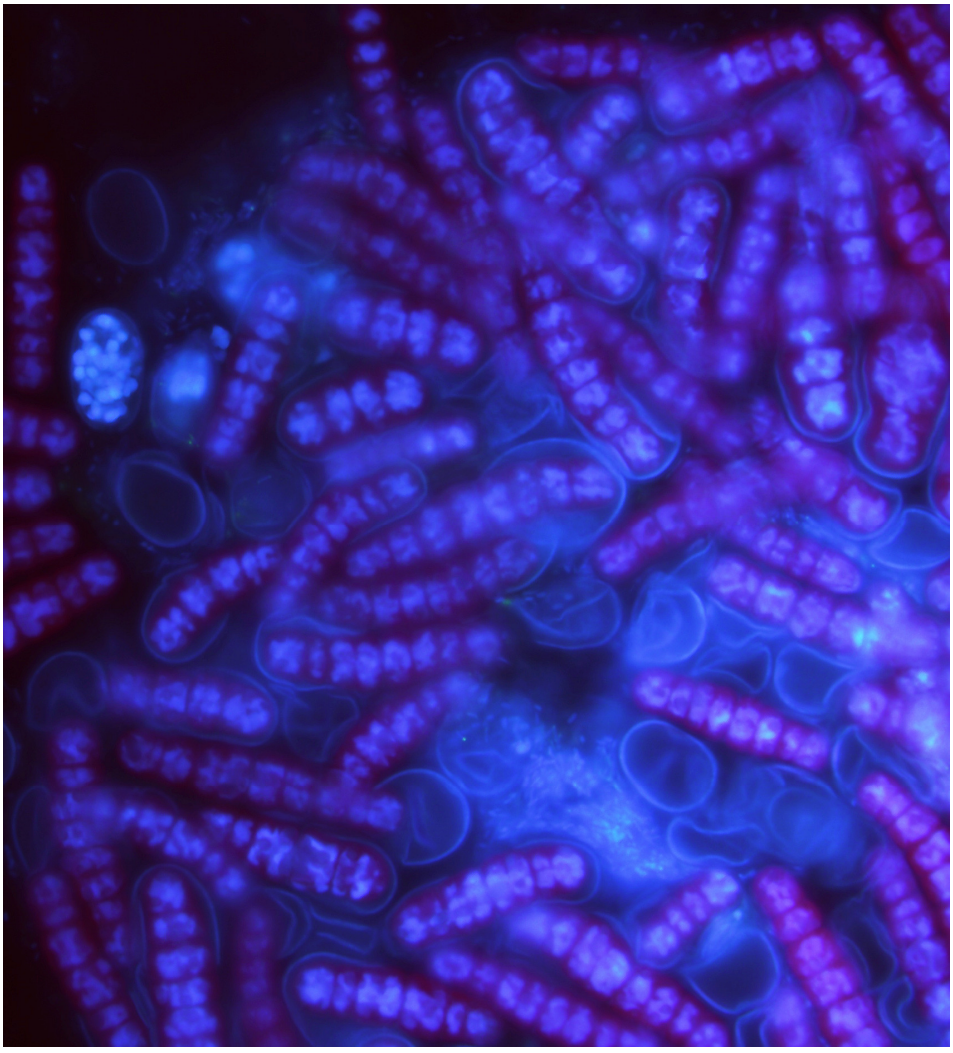
6. ORIGINAL PAPERS



PRODUCTION OF DORMANT STAGES AND STRESS RESISTANCE OF POLAR CYANOBACTERIA

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ABSTRACT

Cyanobacteria represent the major component of the autotrophic community in many different types of habitats in both the Arctic and Antarctic. Their dominance is attributed mainly because of their high tolerance to the extreme polar environments. Low temperatures and desiccation are the main forms of physical environmental stressors. During freezing-melting and desiccation periods, the cells are exposed to radical dehydration effects which can be quite damaging. Polar cyanobacteria have evolved a diverse range of protective strategies in order to avoid, or tolerate, the various stresses. The most widespread adaptation to environmental stress is dormancy. Dormancy can be subdivided into diapause and quiescence. Diapause (the cyanobacterial akinete) is endogenously controlled: it is connected to external stressors but is not directly induced by them. Akinetes are more resistant to various insults and commonly considered as overwintering stages. However, the majority of cyanobacteria in the Polar Regions survive winters without the production of akinetes. This suggests that other alternative mechanisms contribute to survival during stressful conditions. Quiescence (the decrease of metabolic activity under exogenous control) is the transformation into a resistant state, with hardly visible morphological differentiation of the cell. It has been suggested that starvation and entrance into the stationary phase can induce changes in the ultrastructure (*e.g.*, thickening of cell walls) and biochemistry (*e.g.*, sucrose and trehalose accumulation, changes in composition of fatty acids, secretion of extracellular polysaccharides) of the stressed cells. We accept that the stationary-phase and/or starvation-induced cells can represent alternative dormant stages of polar cyanobacteria which do not produce akinetes. This overview summarizes the present knowledge about production of dormant stages and stress resistance of polar cyanobacteria. It is clear that we still have paucity of information on this topic and that further research is necessary.

Key words: cyanobacteria, polar environment, dormancy, stress resistance, desiccation tolerance/injuries, freezing tolerance/injuries, avoidance strategies, akinete, quiescence, diapause, sucrose, trehalose, extracellular polysaccharides, ice-activesubstances.

A NOVEL STAINING PROTOCOL FOR MULTIPARAMETER ASSESSMENT OF CELL HETEROGENEITY IN *PHORMIDIUM* POPULATIONS (CYANOBACTERIA) EMPLOYING FLUORESCENT DYES

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ABSTRACT

Bacterial populations display high heterogeneity in viability and physiological activity at the single-cell level, especially under stressful conditions. We demonstrate a novel staining protocol for multiparameter assessment of individual cells in physiologically heterogeneous populations of cyanobacteria. The protocol employs fluorescent probes, i.e., redox dye 5-cyano-2,3-ditolyl tetrazolium chloride, 'dead cell' nucleic acid stain SYTOX Green, and DNA-specific fluorochrome 4′,6-diamidino-2-phenylindole, combined with microscopy image analysis. Our method allows simultaneous estimates of cellular respiration activity, membrane and nucleoid integrity, and allows the detection of photosynthetic pigments fluorescence along with morphological observations. The staining protocol has been adjusted for, both, laboratory and natural populations of the genus *Phormidium* (Oscillatoriales), and tested on 4 field-collected samples and 12 laboratory strains of cyanobacteria. Based on the mentioned cellular functions we suggest classification of cells in cyanobacterial populations into four categories: (i) active and intact; (ii) injured but active; (iii) metabolically inactive but intact; (iv) inactive and injured, or dead.

Key words: cell staining, fluorescence, DAPI staining, fluorescent dyes, pigments,signal filtering, fluorescence microscopy, cell membranes.

INTRODUCTION

Bacterial populations, including pure cultures in laboratory studies, display high heterogeneity in morphological and physiological activity. It has been widely discussed that individual cells in microbial populations differ substantially in growth rate and in resistance to various stresses [1],[2], [3] that result in a significant cell-to-cell discrepancies in viability and physiological state, becoming more pronounced under stressful conditions. In natural microbial communities this variability is high due to the non-homogeneous physical character of natural environments, irregularity in nutrient distribution and competition between species [4], [5]. Population-based methods, such as respiration measured by the overall oxygen uptake or estimation of photosynthesis performance, provide averaged information on the population's physiological state without considering the properties of single cells, and may result in faulty interpretation of population development and its stress response. Therefore, a versatile approach that estimates multiple physiological parameters at the single-cell level is required for reliable information on the state of the cells in inhomogeneous populations.

The use of fluorochromes for physiological assessment of bacteria provides accurate information about the state of individual cells in populations [6], [7]. A number of fluorescence-based assays that reflect various physiological functions are available for detecting cell viability and activity, such as assessment of membrane integrity and potential, intracellular pH, respiration intensity, intracellular enzymatic activity, etc. [7], [8], [9]. In studies of physiological heterogeneity in populations of microorganisms the fluorochrome staining techniques are often based on detection of only one particular cell function, although multiparameter techniques for bacteria and yeasts have also been established [10], [11], [12], [13]. In cyanobacterial research similar studies, including those where the application of fluorescence dyes are used, are rare and mostly concern unicellular species [14], [15].

The cell is a complex system that responds to a fluctuating environment by modifying its structural organization and by changing its multiple physiological parameters. We consider that a living, healthy and active cyanobacterial cell is primarily characterized by plasma membrane and genome integrities, detectable metabolic activity, and significant content of pigments for effective photosynthetic performance. Under stressful conditions, and due to apoptosis, cells may sustain one or several kinds of damage to their subcellular structures, and changes in their physiological activities.

For the detection and estimation of metabolic activity an assay based on energy dependent processes is required. Respiration is closely bound to the cellular activity [16] and accurately reflects overall energy metabolism of cells. Therefore, detecting respiration is preferable to indirect techniques based on active transport of fluorochromes into the cells, fluorogenic assays for intracellular enzymatic activity, or analysis of photosynthetic performance. Such an estimate may be achieved by

employing tetrazolium salts that act as artificial electron acceptors in reaction with the respiratory chain, therefore directly competing with molecular oxygen, and this reaction detects metabolically active cells [17].

The loss of plasma membrane integrity provides a good estimate for bacterial cell viability as it plays a key role in the operation of the whole cell. The maintenance of its integrity is one of the main features discriminating dead or severely injured cells from living cells. Fluorescence assays intended for estimating membrane integrity are based on the passive exclusion of particular dyes (e.g. propidium iodide, SYTOX Green) by cells with structurally integral membranes.

The presence of genetic material is another inherent prerequisite of viability. In cyanobacterial cell, DNA is organized as a compact structure (nucleoid), which is usually located at the center of the cell. The absence of nucleoid or its visibly severe degradation is an obvious feature of non-viable cells. The image of nucleoid morphology may indicate the level of their metabolic activities, since in metabolically inactive cells the irregularly shaped nucleoid may acquire nearly spherical shape due to inhibition of protein synthesis [18]. In addition, the degradation of photosynthetic pigments, either phycobiliproteins or chlorophyll, may also serve as a marker for viability and activity of cyanobacterial cells since it occurs as a response to various stress conditions, and is associated with cell death [15], [19]. In our study we used a cell-permeable dsDNA-specific stain 4′,6-diamidino-2-phenylindole (DAPI) to confirm the presence of nucleoids, and to reveal their shape and localization in SYTOX Green-negative cells.

We present here a novel staining protocol for a multiparameter cell assessment of physiologically heterogeneous populations of cyanobacteria employing three fluorescent dyes combined with microscopy image analysis. Our method allows simultaneous estimation of cellular respiration activity, membrane and genome integrities, and detection of photosynthetic pigment content along with morphology observation. The staining protocol has been adjusted for filamentous species of the genus *Phormidium* (Oscillatoriales). To our knowledge the aforementioned fluorescent probes (and their combinations) have not been previously employed in estimating viability and metabolic activity of filamentous cyanobacteria. Therefore, we tested the efficacy of each fluorescent probe and the specific staining procedure (i.e., incubation time and staining concentration). Simultaneous application of several fluorescent markers required a careful selection of fluorochromes with particular spectral properties, a detailed investigation of their interaction, and their interference with broad-spectrum autofluorescence of the cells (pigments and surface structures). Also, we describe proper handling of the samples and their staining and de-staining procedures due to certain characteristics of the *Phormidium* species, i.e., specific cell-to-cell connections, production of sheaths, and tight structure of natural communities.

MATERIALS AND METHODS

Cyanobacterial material and culture conditions

All laboratory experiments (i.e., concentrations, incubation time, interaction of dyes) were conducted with the *Phormidium autumnale* CCALA 845 strain isolated in 2008 by Snokhousova et Elster from a sample collected in a stream with moss carpets in the Svalbard archipelago (77°00′ 15°20′E), and currently maintained in the Culture Collection of Autotrophic Organisms (CCALA), Institute of Botany, Academy of Sciences of the Czech Republic. Maintaining cultures in liquid or agar BG-11 medium at a continuous photon flux density of 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (white light) at +18°C were optimal conditions for the given strain (unpublished data). The incubation time in order to reach the phase of active growth ranged from 15 to 25 days. Because the culture tends to grow in aggregates, and due to the filamentous nature of this strain, estimation of the logarithmical growth phase using optical density of the culture or dry weight increment was not possible. Therefore, the decision regarding the growth phase of the cultures was made on the basis of microscopy observations of the cell morphology (intensive blue-green color, well-pronounced thylakoids, uniform morphology of cells, absence of visible inclusions, absence of necridic cells, and evidence of cell fission). The cultures used for the experiments were at their active growth phase in order to prevent errors in cell counts since "old" cultures are physiologically heterogeneous.

Several strains of cyanobacteria in different stages of growth were also tested with the staining protocol: namely, *Phormidium* species isolated from temperate, tropical and polar environments (CCALA 771, 816, 845, 849, 850, 861, 881), *Calothrix sp.* CCALA 034, *Merismopedia glauca* CCALA 099, *Chroococcidiopsis cubana* CCALA 041, *Oscillatoria limosa* CCALA 134, and *Aphanothece clathrata* CCALA 013.

Besides the examination of laboratory cultures, samples of two natural *Phormidium*-dominated crusts were collected from streaming and stagnant shallow Arctic waters in the Svalbard archipelago in June and September of 2011 and, immediately after collection, used for the fluorescence assays. Both crusts were dominated by *Phormidium autumnale* species identified on the basis of 16S rRNA sequence [20]. We also examined 2 natural samples (mixed epilithic community) collected in the Lužnice River, the Czech Republic in the middle of September 2012. Cyanobacteria in these samples, according to their morphological criteria, were identified as *Phormidium* and *Lyngbya*.

No specific permits were required for the field studies (both in the Czech Republic and in Svalbard). The Svalbard Act of 15 June 2001 No.79 Relating to the Protection of the Environment in Svalbard, states: "The collection of fungi and seaweed for private use is permitted. The collection of flora for research or teaching purposes is permitted where this does not make significant inroads into the local

populations of the flora involved." We confirm that the field studies did not involve endangered or protected species.

Sample preparation

The laboratory cultures were prepared as follows: cyanobacterial biomass was removed from the growth medium, washed 3 times by shaking and careful draining of liquid with a micropipette, and then resuspended in 300 μL of sterile fresh BG-11 medium in Eppendorf tubes. During the staining procedure the samples were incubated at room temperature in the dark.

The samples of natural crusts were prepared by cutting them into pieces of approximately 1 cm^2, then thoroughly washed of sand, and transferred to microplates filled with water collected at the sampling sites. The plates were wrapped in aluminum foil, and incubated during the staining procedure at *in situ* outdoor temperature (+2 to +6°C). The soft biomass from the river samples was resuspended in filtered river water, and incubated at +15°C.

Properties of fluorescent dyes

Membrane state. SYTOX Green stain provides several advantages over other dyes used for the same purpose due to its spectral properties and relative non-toxicity to living cells. SYTOX Green is a high affinity nucleic acid stain that does not penetrate living cells, yet it passively diffuses into cells with compromised membranes where it preferentially binds to DNA resulting in >500-fold enhancement in fluorescence emission [21]. The nucleoids of the cells with compromised membranes fluoresce in green color due to the dye uptake. Its bright fluorescence in the green spectral region (absorption and emission maxima at 502 and 523 nm, respectively) allows its observation simultaneously with orange-red fluorescence of CTC (emission maximum at 630 nm), and red autofluorescence of photosynthetic pigments.

Respiration. For single-cell evaluation the cell-permeable 5-Cyano-2,3-Ditolyl Tetrazolium Chloride (CTC) and 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-tetrazolium chloride (INT) redox dyes are the most convenient. These dyes are reduced from soluble colorless form into their corresponding colored (INT) or fluorescent (CTC) insoluble formazans that accumulate intracellularly. The formazan crystals are viewed as intracellular opaque dark-red deposits under transmitted illumination, or as red fluorescent spots (excitation and emission maxima at 488 and 630 nm) when using epifluorescence microscopy. INT was tested as a cheap non-fluorescent analogue of CTC dye; its use may also reduce the cost for an additional microscopy filter cube.

Nucleic acids. The DAPI stain was selected for labeling nucleoids due to its brightness and stability; in addition, the probe (Ex/Em maxima at 358/461) is easy to combine with other probes with higher excitation wavelengths. If present, the nucleoids are stained with DAPI in blue (or white-blue) in both living and dead

cells [7]. The diffused character of DAPI staining in the nucleoid area, or a whole-cell DAPI signal gives evidence of an extensive damage to genetic material [15].

The properties of the employed fluorescent dyes are summarized in Table 1. The redox dye INT was purchased from Sigma-Aldrich Co. (USA); the redox dye CTC, DNA-binding fluorochrome DAPI, and cell-impermeant nucleic acid dye SYTOX Green were obtained from Life Technologies Corporation (USA). Stock solutions of fluorochromes were prepared and stored according to instructions suggested by the manufacturers.

Tab. 1 Properties of fluorescent dyes

Fluorochrome	Cell function	Reaction	Detection*
DAPI	Presence/shape of nucleoids	Stains dsDNA in both living and dead cells	Blue-white (blue) fluorescence of nucleoids
SYTOX Green	Membrane integrity	Stains cells with damaged membranes	Green fluorescence of nucleoids
CTC	Respiratory rate	Accumulation of formazan crystals in active cells	Red-orange fluorescence of CTC-formazan
INT	Respiratory rate	Accumulation of formazan crystals in active cells	Purple deposits under transmitted light

* The colors of the dyes' fluorescence were given by the filter sets that were employed in microscopy observations.

Investigation of the optimal staining procedure of laboratory samples

SYTOX Green. To study the minimal SYTOX Green staining concentration, inactivated controls were prepared by immersing the biomass in hot water (10 min at +90°C). They were also prepared by treating the biomass for 60 minutes with 30% (v/v) ethanol water solutions prior to the staining procedure. The samples were then washed free of fixative solution, resuspended in BG-11 medium, and examined within several hours after the fixation procedure. Cell suspensions were stained at final concentrations of 0.05, 0.1, 0.2, 0.3, 0.5, 0.7, 1, 2, and 5 μM of SYTOX Green for 30 min protected from light. The lowest dye concentration that all cells exhibited bright and even fluorescence of their nucleoids was considered as a minimal effective staining concentration.

The maximum staining concentration was optimized by staining non-fixed samples with SYTOX Green solution concentrations of 0.1, 0.2, 0.3, 0.5, 0.7, 1, 1.5, 2, 3, 4, 5, and 10 μM for 30 min in the dark. For each dye concentration, the number of SYTOX Green positive cells and total cell counts were enumerated. At a particular concentration, the SYTOX-positive cell counts were significantly higher compared

with the preceding lower concentration of the dye. This lower concentration was considered as maximum.

To find the optimal SYTOX Green incubation times, the samples were stained with a dye concentration (1 μM) in the range between the minimum and maximum staining concentrations. Aliquots of stained suspensions were sampled for investigation after 15, 30, 60, 90, 120, and 150 min of incubation. The minimal staining time of 15 min was set according to the manufacturer's recommendation; the maximum staining time was estimated using a similar technique as for the maximum staining concentration.

CTC and INT. When investigating the effect of incubation time on dye reduction we used concentrations of 5 mM CTC and 0.01% w/v INT that was previously employed for direct enumeration of respiring bacteria in environmental and laboratory samples [15], [17]. Subsamples of cell suspensions were collected for microscopy examination after 10 min of staining, and then, in 30 min intervals, during the 2 hour incubation. Cyanobacterial biomass was then fixed with 3% (v/v) formaldehyde water solution and used for slide preparation. The rate of CTC and INT reduction was judged from the quantity and size of red formazan spots in the light field; for CTC the dye red-orange fluorescence in the dark field was also observed. Inactive control was prepared by treating samples with 3% (v/v) formaldehyde water solution for 60 minutes; partially inactivated samples were obtained with subletal formaldehyde concentration of 0.1% (v/v). Additionally, cultures were continuously incubated for 24 hours with CTC or INT dyes to investigate their damaging effect on cells, as previously observed in our preliminary experiments.

The effect of CTC concentrations on dye reduction was conducted with cyanobacterial biomass stained for 30 min in the dark with concentrations of 0.01, 0.05, 0.1, 0.5, 1, 2.5, 5, 7.5, 10 and 20 mM. After incubation, the samples were treated with 3% (v/v) formaldehyde water solution to stop CTC reduction. The quantity, size and pattern of deposition of red formazan spots were observed as well as formazan fluorescence.

DAPI. Five μg/mL DAPI concentration, as suggested by the manufacturer, was used in all the experiments. The samples were stained for 15 to 30 min and then rinsed 3 times with BG-11 medium.

Assessing CTC, DAPI and SYTOX Green interaction, and sequence of staining

Final concentrations of 5 mM CTC, 5 μg/mL DAPI and 1 μM SYTOX Green were used for all the treatments. The effect of SYTOX Green staining on CTC reduction was studied as follows: laboratory samples were dyed with SYTOX Green, after 30 minutes they were washed 3 times, and soaked in fresh BG-11 medium for 15 min to remove excess dye adsorbed on the surfaces of cell walls and sheaths. The biomass was then transferred for staining into a CTC solution for 30 minutes, rinsed in BG-11 medium, and used for microscopy observations. The percentage of respiring cells after SYTOX Green treatment was calculated and the CTC fluorescence pattern

was observed; the results of CTC-SYTOX co-staining were compared with the results from samples stained with CTC only. Similarly, to assess the effect of CTC on SYTOX Green counts, 3 sets of samples were prepared: (i) SYTOX Green was used as a single dye; (ii) samples were stained with CTC prior to SYTOX Green; and (iii) SYTOX Green-dyed samples were post stained with CTC. The number of DAPI-positive cells after CTC-SYTOX Green treatment was compared with the cell counts when DAPI alone was used.

Protocol for CTC, SYTOX Green and DAPI co-staining

Both laboratory and natural samples were stained with SYTOX Green dye in final concentration of 1 μM (10 μM for natural samples) and incubated for 30 min (60 min for natural samples) protected from light. The biomass was then washed 3 to 5 times with sterile BG-11 medium (laboratory samples) or ambient water from the sampling sites (natural samples), and soaked for 15 to 30 min to remove excess dye as necessary. In order to estimate respiratory activity, the samples were post-stained with either INT at final concentrations of 0.01% and 0.03% (w/v), or with 5 mM CTC for 30 min in the dark. After removal of the redox dye solutions, samples were counterstained with DAPI for 15 min (30 min for natural samples) at a final concentration of 5 μg/mL. The samples were then washed thoroughly of the DAPI solution and immersed in fresh media. The same protocol was used for all of the studied laboratory cultures and field samples.

Microscopy examination and filter spectral properties

To analyze the samples stained with fluorochromes a small aliquot of cyanobacterial suspension was trapped between a glass slide and a 24×24 mm square cover slip; the edges were sealed with wax or nail polish to prevent water evaporation. An Olympus BX53 microscope equipped with a 100 W ultrahigh-pressure mercury arc lamp (Olympus) was used with ×20 and ×40 objectives. The optical system for fluorescence observations included 5 UIS2 fluorescence mirror units (Olympus, Japan): U-FUN filter cube with a 360–370 nm band pass excitation filter and 420 nm long pass cutoff filter for DAPI fluorescence observation; U-FBWA cube with 460–495 nm excitation and 510–550 nm emission filters for SYTOX Green fluorescence, and U-FRFP cube (535–555 nm excitation, 570–625 nm emission ranges) for observing CTC-formazan (CTF) fluorescence. In addition, 3 optional mirror units (excitation filter/emission filter/dichromatic mirror) were made for CTF (425–445 nm/570–625 nm/455 nm), DAPI (360–370 nm/460–510 nm/420 nm), and phycobiliproteins (565–585 nm/600IF/595 nm) fluorescence analyses. The additional mirror units were needed due to the presence of photosynthetic pigments that have broad excitation and emission spectra, mainly in red region. These filters were used in order to avoid overlap between dyes fluorescence (CTC-formazan and DAPI) and pigments autofluorescence. The light absorption and fluorescence emission curves of SYTOX Green and DAPI dyes were redrawn with the permission from www.lifetechnologies.com web source. CTC-formazan graph was taken from the

www.olympusmicro.com web source. The formation of INT formazan was observed as optically dense purplish deposits visible with transmitted bright field microscopy.

Digital image analysis and cell counting

Series of several dark-field images were acquired to record the fluorescence of each of the signals required; and a bright-field image was taken for the total cell counts and detection of INT deposits. The images were captured with an Olympus DP72 microscope digital camera (Japan) at 1024×1360-pixel resolution at a constant shutter, offset and ISO parameters for each filter set. These settings were adjusted to obtain images with the same fluorescence intensity as were seen in microscope eyepieces; a Differential Interference Contrast filter set was used for the bright field image for the enhanced view of the cell septa.

All experiments were run in triplicate on each of the 3 batch cultures grown under the same conditions, i.e., each of the cultures was sampled 3 times during the active growth phase in 2 to 4-day intervals. In experiments investigating concentrations and incubation time, the entire biomass used for staining was observed except the case of SYTOX Green incubation time and SYTOX Green maximum concentration. In two latter and all other experiments, at least 800 randomly chosen cells in 15 to 20 fields of view were counted for each replication.

Image processing was carried out in Adobe Photoshop CS4 program as follows: both bright field image and the corresponding fluorescent image of a microscope view were divided into 20 large squares by applying a grid; 3 to 5 squares were selected for each image, and within these all the cells were counted. Cell counting was performed semi-automatically by using mouse-click counter software (Desktop Counters 2.0, Freelabs); each mouse click marked a counted cell with a dot.

Statistical Analysis

All statistical analyses of the data were performed in Statistica v.10.0 software (StatSoft, USA). Most of the data did not pass a normality and equal variance test due to uneven distribution of stained cells between microscope fields of view and unequal number of observations per group, which is due to the filamentous nature of the studied strain. Therefore, the Kruskal-Wallis 1-way analysis on ranks at the significance level of 5% was applied to compare the mean of the percentage of stained cells between several groups.

RESULTS AND DISCUSSION

Application of fluorescent dyes

SYTOX Green dye; the effect of staining time and concentration.

The minimal effective staining concentration of SYTOX Green dye for laboratory samples of *Phormidium autumnale* was between 0.2 and 0.3 μM in 3 replicates. At this dye concentration all nucleoids in alcohol and heat-pretreated cells

exhibited bright and even fluorescence in the green spectral region, which was distinctly visible with a 510–550 optical cutoff filter set (Fig. 1a). Further staining with higher concentrations of the dye (up to 5 μM) did not increase fluorescence intensity. This treatment, as long as it passes through the membranes of the dead cells, confirmed the applicability of SYTOX Green dye to *Phormidium* cultures, since it is also the inactive control.

The mean percentage of SYTOX Green-positive cells in exponentially growing cultures totaled from 0.73% to 1.42%, and was not significantly different at each of the dye concentrations between 0.3 and 3 μM ($p>0.05$). However, in all treatments, pair wise comparisons revealed a significant rise in percentage of stained cells at concentrations of 4 μM or higher in contrast to each of the preceding concentrations ($p<0.05$). Thus, the optimal staining concentration for SYTOX Green dye for the 30-minute incubation period for laboratory samples grown under optimal conditions is in the range of 0.3 to 3 μM.

One μM of SYTOX Green dye concentration was selected to approximate the minimal and maximum incubation time required for reliable discrimination between cells with intact vs. compromised membranes. A 15-minute incubation time ensured complete penetration of the dye into the cells with permeabilized membranes. There were no differences ($p>0.05$) in proportion of SYTOX Green-positive cells between subsamples stained for 15, 30 and 60 minutes; the mean percentage of stained cells increased significantly ($p<0.05$) in the period between 60 and 90 min. Repeated experiments provided identical results.

Excessive dye concentrations and prolonged incubation periods resulted in heterogeneous staining of cell populations in addition to the increase in proportion of stained cells (Fig. 1b). In these experiments we show that the majority of cells exhibited dim fluorescence of their nucleoids starting at the 3 μM concentration of the dye (30 min incubation) and after 60 min of incubation at 1 μM concentration. At higher concentrations and longer staining periods, the fluorescence increased intensity and varied considerably from cell to cell, which made it difficult to discriminate between stained and non-stained cells. Eventually, at concentration higher than 10 μM the nucleoids of all living cells were brightly stained with SYTOX Green dye.

For the samples of natural population of *Phormidium autumnale*, a SYTOX Green concentration of at least 6 μM was required to label all nucleoids of formaldehyde-pretreated cells; that is 20 times higher than the laboratory samples. At this dye concentration the incubation time sufficient for the penetration of SYTOX Green stain into dead cells exceeded 60 minutes (Fig. 1r). Apparently, this discrepancy may be explained by the tight structure of natural crusts and markedly increased thickness of sheaths enveloping the filaments, which retard the diffusion of the dye into the cells as well as the low (+4°C) ambient temperature. Therefore, we expect that the optimal concentrations of SYTOX Green dye and incubation time may vary significantly from sample to sample depending on the interspecific differences in permeability, state of the cultures, and on conditions the cultures were grown and incubated during the staining procedure. In general, the total labeling of cells in living

cultures or inhomogeneous patterns of their staining apparently indicate either excessive dye concentration or inappropriately long incubation time; and accordingly, insufficient dye concentrations or incubation time results in incomplete labeling of cells in permeabilized control samples.

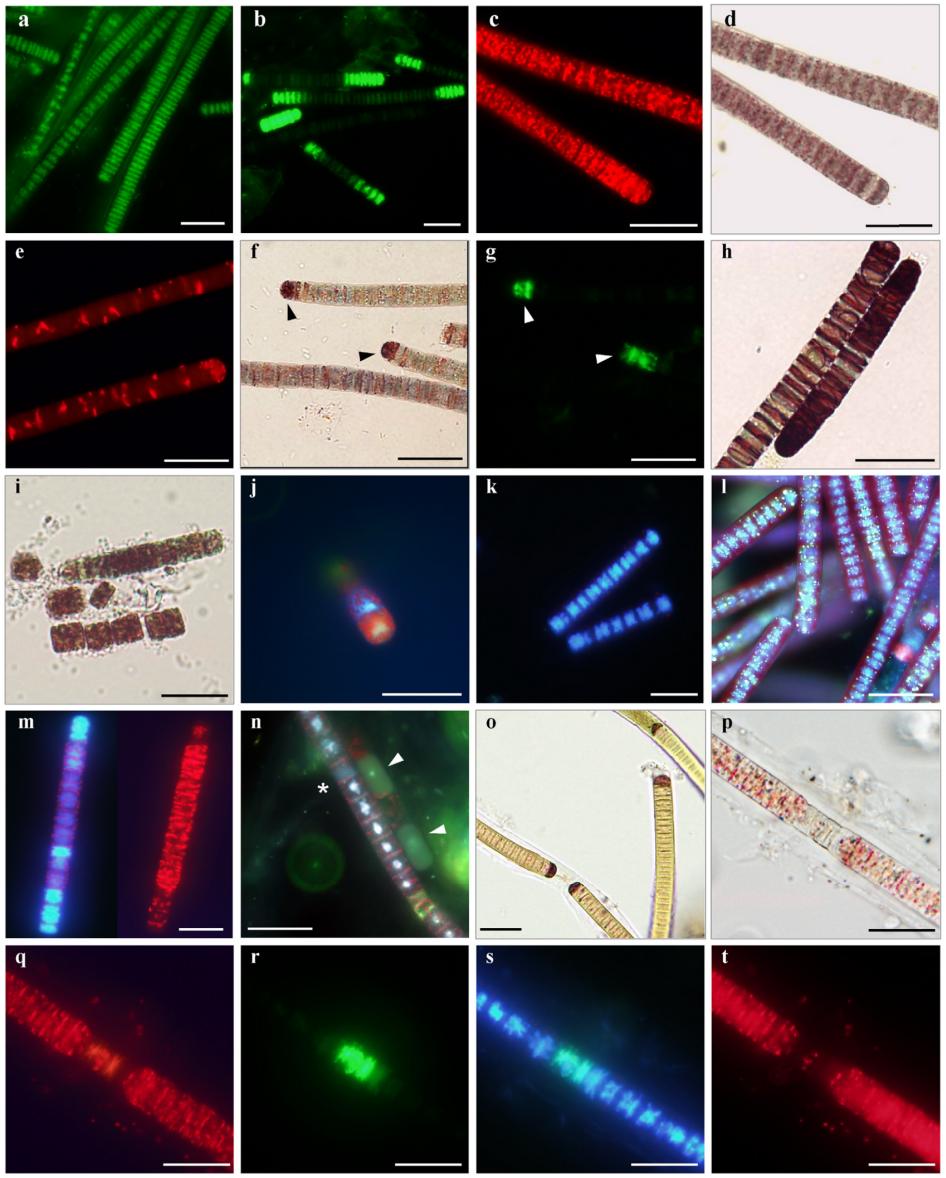

Fig. 1. *In situ* detection of cellular functions in cyanobacterium *Phormidium autumnale*. a–l. Cultures of *Phormidium autumnale* 845 CCALA grown on BG-11 medium. **a.** SYTOX Green staining of formaldehyde-pretreated filaments; **b.** variable staining of live samples after 30 min incubation with 5 μM SYTOX Green; **c–**

d.fluorescence and bright field image of CTC-stained filaments in active phase of growth;**e.** partially de-activated cells stained with CTC; **f–g.** INT-treated samples post-stained with SYTOX Green, cells that accumulated INT-formazan (**f**, *arrowheads*) were also SYTOX-positive (**g**, *arrowheads*); **h.** INT-stained filaments in logarithmic phase of growth; **i.** disintegration of filaments after 24-h incubation with CTC; **j.** pattern of CTC-formazan deposition; **k.** DAPI-stained nucleoids in living filaments; **l.** yellow-green metachromatic inclusions in DAPI-stained filaments viewed with a long pass emission filter; **m.** reduced fluorescence intensity of DAPI-stained nucleoids in cells accumulated CTC-formazan (natural samples); **n.** cells from old laboratory cultures simultaneously stained with DAPI, CTC and SYTOX Green under UV-illumination, extensively damaged cells lack SYTOX Green and DAPI staining of nucleoids (*arrowheads*) or have whole-cell DAPI signal (*asterisk*); **o–t.** Field-collected samples of mats in July and September; **o.**accumulation of INT-formazan by the terminal cells of filaments; **p.** a filament with thick sheath stained simultaneously with CTC (**q**), SYTOX Green (**r**), and DAPI (**s**), and showing pigment autofluorescence (**t**). Scale bars are 20 μm.

Application of CTC and INT dyes.

Incubation for 30 min with 0.01, 0.05 and 0.1 mM CTC concentrations were insufficient to invoke the accumulation of any visible formazan deposits, and they were not visible with incubation time that extended for up to 120 min. Among the concentrations analyzed, 0.5 mM of CTC was the lowest concentration that formazan deposits were produced. These deposits were notably smaller than at higher CTC concentrations (starting from 1 mM) that resulted in low fluorescence intensity. The differences in quantity, shape and size of the formazan deposits, as well as the pattern of their deposition, were hardly discernible between 1 and 20 mM.

After 10-min incubation of living laboratory samples with 5 mM concentration of CTC dye, about 99% of the cells in all replicates contained deposits of CTC-formazan visible both by epifluorescence microscopy (Fig. 1c) and under transmitted light (Fig. 1d). Further 2-hour incubation did not lead to any significant increase in the proportion of CTF-containing cells ($p>0.05$). There was no visible difference between observations in quantity, shape and location of formazan inclusions within the cells for any of the incubation times. However, at incubation periods of 30 min or longer, the CTC-formazan deposits appeared larger, and exhibited brighter fluorescence compared with the deposits produced with 10-min incubation. The same staining protocol was sufficient for penetrating CTC into cells from natural samples even though the filaments were enveloped by thick dense sheaths (Fig. 1p, 1q).

The 1-hour treatment of laboratory *Phormidium* suspensions with 3% (v/v) formaldehyde prior to CTC staining led to a complete suppression of formazan accumulation; this provided a convenient negative control for the absence of any abiotic CTC reduction with the components of the medium used. A short-time pretreatment of samples with subletal formaldehyde concentration (0.1% v/v) resulted

in a decrease in the proportion of formazan-containing cells. More important, the CTC-formazan deposits within the cells that retained respiratory activity were markedly different in their numbers, size and shape than from those in non-inactivated cells. Cells in formaldehyde-pretreated samples contained notably fewer sites of CTC reduction per cell, and typically formed larger and more elongated formazan crystals (Fig. 1e) in contrast to the small round formazan spots deposited uniformly throughout the active cells (Fig. 1c). However, the intensity of CTC-formazan fluorescence was not affected in metabolically less active cells. We observed a similar pattern of CTC-formazan deposition within the cells in the old *Phormidium* batch culture (cultivated for 3 to 6 months) and in certain proportion of filaments from field-collected samples collected at the end of the vegetative season on Svalbard (data not shown).

The incubation of the samples with INT revealed that after 10 min only terminal cells had incorporated INT-formazan crystals, whereas other cells within the filaments of neither laboratory nor field-collected samples contain significant formazan deposits (Fig. 1f, 1o). Further 2-hour incubation of laboratory samples resulted in progressive accumulation of INT-formazan from the polar ends across the whole filament. In contrast, subsequent incubation of natural population of *Phormidium* (collected at the end of the vegetative season) for the same amount of time did not lead to any further formazan accumulation, even when the INT concentration was increased to 0.03% (w/v).

Apparently, permeability of the studied cells to INT differs substantially from permeability to CTC. Since the production of formazan crystals is irreversible, the amount of formazan deposited within the cells represents the cumulative respiratory history of those cells [22]. Since the uneven penetration of INT into cells did not reveal differences in their actual respiration rate, we have not observed any visible difference in the amount of formazan accumulated in respiring cells after prolonged incubation of laboratory cultures (>120 min). Evidently, the CTC technique has several other advantages over INT staining when investigating filamentous cyanobacteria. CTC, along with confocal microscopy, may be readily employed in studies of structure and distribution of physiological activity within cyanobacterial communities due to its ability to fluoresce upon reduction. In addition, the production of dense opaque INT-formazan deposits (Fig. 1h) occludes any observations on morphology, intracellular structure, and pigment autofluorescence of cyanobacterial cells as compared with poorly colored and less opaque CTC-formazan (Fig. 1d, 1p).

Toxicity of tetrazolium salts.

In our studies we discovered that both INT and CTC are toxic to filamentous cyanobacteria, observed from the massive lysis of previously living cells that accumulated visible amounts of formazan during incubation. After an 8-hour continuous incubation with INT and a 24-hour incubation with CTC, the filaments in laboratory cultures disaggregated into single cells (Fig. 1i), and the density of the

cultures had dramatically decreased. Similar lysis of cultures occurred, with a delay (after 48 h), when, after a short-time incubation (<60 min), the biomass was washed out of the dye solutions and transferred into a fresh medium. Apparently, the rate of cell lysis positively correlated to the amount of formazan deposited within the cells, and was also connected to a difference in properties of CTC and INT. We also observed that both CTC and INT formazans had well-marked peripheral pattern of deposition (Fig. 1j) within the cells of the filamentous as well as in unicellular cyanobacteria (data not shown).

Tetrazolium salts were frequently reported to be toxic to mixed groundwater and seawater bacterial communities as well as to pure laboratory cultures [17], [23], [24]; however, the mechanism of their toxicity was not explained in these works. As shown for groundwater bacteria [24], the toxicity of CTC and INT dyes was restricted only to those isolates that took up and reduced the dyes into their respective formazans.

As discussed before, the reduction sites of tetrazolium dyes in the electron transport chain of bacteria are strongly species-specific; they are defined by the ability of a particular dye to enter the cells and on the experimental conditions. In studies of *Lactococcus lactis* the electron transport chain components involved in tetrazolium violet reduction included NADH dehydrogenases and menaquinones; in both cases the accumulation of formazan occurred entirely in the cell membranes [25]. The sites of CTC and INT reduction in the aerobic respiratory chain of *E. coli* K-12 were associated with membrane-bound succinate and NADH dehydrogenases. INT was additionally reduced at ubiquinone and possibly cytochromes $b_{555,\ 556}$ [26]. The CTC dye was also reduced by the components of plasma membrane ETC rather than by intracellular reductases, and the deposition of the CTC-formazan inflicted damage on plasma membranes [27]. Therefore, it is likely that the toxicity of insoluble tetrazolium formazans may be attributed to a mechanical disruption of the cell membrane produced by the formazan crystals being deposited in close proximity to or within (INT) the inner membrane (CTC and INT). The intramembrane formation of INT-formazan may explain more rapid and severe damage, which INT-formazan created in the studied cells in comparison to CTC-formazan. The fact that formazan-accumulating cells sustain similar damage upon centrifugation (e.g. 10 min at 5000 rpm) may also support the hypothesis of cell injury induced by mechanical disruption of membranes by formazan crystals. However, this does not rule out other mechanisms of spreading their toxicity since water-soluble formazan of XTT terazolium dye (2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carbox-anilide inner salt) was also toxic to bacterial isolates upon its reduction [24].

SYTOX Green interaction with CTC, INT and DAPI dyes.

Since we discovered that deposition of INT and CTC formazans possibly inflicted damage to plasma membranes of studied cyanobacteria, a careful investigation of their effect on staining with membrane-impermeant SYTOX Green dye was required. As we expected, in all replications, the number of SYTOX Green-

positive cells in laboratory samples pre-stained with CTC for 30 min was significantly higher than in samples where SYTOX Green was used as a single dye ($p<0.05$). The proportion of cells labeled with SYTOX Green was 17.5% to 19.3% in CTC-stained samples as compared to 2.18% to 3.24% in non-stained samples. Furthermore, the uneven pattern of SYTOX Green staining within CTC-treated cells was similar to that of cells incubated with excessive concentrations of SYTOX Green dye (Fig. 1b). The samples stained with INT displayed high variability in the SYTOX Green counts. However, most of the cells that incorporated INT-formazan were also distinctly fluorescent with SYTOX Green indicating that an accumulation of INT-formazan did have an effect on the integrity of plasma membranes (Fig. 1f, 1g). For the above mentioned reasons INT dye was not used in any further examination.

In order to employ CTC and SYTOX Green dyes simultaneously, we attempted to stain samples with SYTOX Green, prior to the incubation with CTC, using the same concentrations and staining procedure. The SYTOX Green counts in CTC-treated samples did not differ significantly from the reference samples stained with SYTOX Green only ($p>0.05$), provided the biomass was thoroughly washed free of the dye solution before CTC staining. Soaking the biomass in fresh medium for 15 to 30 minutes was sufficient to remove residuals of surface-bound SYTOX Green dye. Natural samples required longer time evidently due to the increased density and thickness of their sheaths. Following this procedure some fluorescence of nucleoids was still detectable; however, it was many times less intense when compared to that of non-living control samples. Insufficient washing procedures resulted in populations that were inconsistently stained with SYTOX Green upon further accumulation of CTC-formazan.

There was no effect of SYTOX Green treatment on the ability of studied cells to reduce CTC, i.e., the quantity of formazan crystals and their shape and size were similar to those in the control samples. Despite slightly lower intensity of formazan fluorescence, and the deposits appearing less opaque under transmitted illumination, the fluorescence was still clearly distinguishable from the characteristic formazan deposits produced by partially inactivated cells. In some of the other oscillatorian strains that we tested more CTC-formazan was deposited at the cross walls after SYTOX Green treatment, yet in some strains deposition of CTC-formazan and intensity of its fluorescence were not affected (data not shown).

Five µg/mL concentration of DAPI applied for 15 to 30 min was sufficient to label cell nucleic acids in both laboratory and natural cell populations. Therefore, other concentrations and incubation times were not studied. At 5 µg/mL concentration all observed cells exhibited bright DAPI fluorescence of their nucleoids (Fig. 1k) except for few, apparently, damaged cells. Preceding incubation of samples with SYTOX Green, CTC, INT, or combinations thereof had no effect on DAPI counts, nor did this influence the character of the DAPI staining; yet in some cases we observed interference of the DAPI signal with CTC-fluorescence (see below).

Protocol for co-staining of laboratory and natural samples.

Phormidium species isolated from temperate, tropical and polar environments (CCALA 771, 816, 845, 849, 850, 861, 881), also *Calothrix sp.* CCALA 034, *Merismopedia glauca* CCALA 099, *Chroococcidiopsis cubana* CCALA 041, *Oscillatoria limosa* CCALA 134, *Aphanothece clathrata* CCALA 013, at different stages of growth were successfully stained using the protocol developed for the laboratory samples. The efficiency of the staining protocol was evaluated according to the following criteria: (i) all cells in inactivated controls were brightly stained with SYTOX Green and lacked CTC-formazan deposits; (ii) all cells were stained with DAPI except the apparently damaged cells; (iii) SYTOX-positive cells in living samples were, in general, clearly distinguishable from SYTOX-negative cells; (iv) CTF-containing cells were not selectively stained with SYTOX Green, or the number of SYTOX-positive cells did not increase after staining with CTC; (v) filaments (Oscillatoriales) were not disaggregated into single cells or short fragments after staining procedure; (vi) SYTOX Green treatment prior to CTC did not suppress formazan reduction; (vii) most of the cells in actively growing controls were not stained with SYTOX Green and accumulated formazan deposits. Using this approach, the staining protocol established for actively growing laboratory cultures was also valid for the same cultures at later phases of growth (data not shown).

Although the developed protocols were suitable for all studied cultures, we expect that different organisms may require different staining protocols. If studied the culture/sample does not meet the aforementioned criteria, then staining concentrations, incubation time and/or different washing procedure should be tested. The reported optimal concentrations and incubation times may vary substantially depending on the nature of the samples [14], [15], [17], [21], [23], [24],[28], [29]. In initial experiments, it is worth to try several dye concentrations and incubation times to determine those that yield optimal staining. Following the above mentioned criteria, we performed optimization experiments for natural samples. The staining protocol was primarily developed for one of the field-collected Svalbard samples, and was suitable for all 4 studied natural populations of cyanobacteria.

Spectral interaction of fluorescent probes

The number of fluorescent dyes that may be simultaneously introduced into a specimen is generally limited by the relatively narrow spectrum of visible light. Therefore, a careful selection of fluorescent probes and microscopy filters is required so no overlap in their fluorescence signals (including pigment and cell surface autofluorescence) exists. This will provide reliable criteria for their discrimination if interference between signals cannot be avoided.

Autofluorescence of photosynthetic pigments.

Phycobiliproteins, as accessory light-harvesting pigments of cyanobacteria, have wide absorption and emission spectra that significantly restricts employing other

fluorochromes. The pigments typical for Oscillatoriales allophycocyanin (APC), C-phycocyanin (C-PC), and optionally C-phycoerythrin (C-PE), absorb light between 500 and 700 nm [30], [31], [32], [33]. The tight energetic coupling between phycobiliproteins of the phycobilisome [31], results in almost complete lack of fluorescence from C-PC and C-PE [31], [34], [35]. The emission of intact phycobilisomes arises mainly from far-red-emitting APC forms with a peak between 670 and 675 nm [34] (Fig. S1b) along with red fluorescence of chlorophyll *a,* mostly in the 675–695 nm range [32], [36], [37].

Light in the 565–585 nm range is sufficient to excite both C-PE and C-PC, and to some extent APC (Fig. S1a) and corresponds to the absorption peak of intact phycobilisomes from *Phormidium percisinum* grown under white light [38]. The composition of phycobiliproteins is dependent on the quality and the intensity of light as well as on nutrient conditions [33]; therefore, longer wavelength excitation may be useful for cultures containing predominantly APC pigments.

CTC fluorescence.

The selected 425–445 nm excitation range corresponds to the side absorption peak of CTC-formazan, and ensures its exclusive excitation since the absorption spectra of phycobiliproteins, SYTOX Green, and DAPI are not included in this region (Fig. S2a, S2b). The red fluorescence of chlorophyll *a* (excitation peak at 425 nm) was filtered out with a 625 nm cutoff filter. The fluorescence of formazan crystals was then readily observed against the low background noise given by the properties of the filter set used. The 530–550 nm excitation range was suitable for the observation of natural samples due to the lack of background noise and brighter fluorescence of CTC-formazan; but was not applicable to laboratory cultures apparently due to the difference in the composition of the phycobiliproteins.

SYTOX Green and DAPI fluorescence.

Green fluorescence from SYTOX Green-stained nucleoids was selectively observed with a 510–550 nm band pass barrier filter as the only signal available under given 460–495 nm excitation range (Fig. S2a).

Long-wave UV light (360–370 nm) required for the maximum excitation of dsDNA-bound DAPI (Fig. S2b) invoked concurrent excitation of CTC-formazan and SYTOX Green dye as well as photosynthetic pigments. A 420IF barrier filter was inappropriate for the observation of exponentially growing cells due to the shielding of the DAPI signal by intense pigment autofluorescence. However, it was useful for the simultaneous observation of CTC-formazan, DAPI, and SYTOX Green fluorescence in cells from old cultures (incubated for 3 to 6 months) with low photosynthetic pigment content (Fig. 1n). The intensity of DAPI fluorescence was low with a 420–460 nm barrier filter even though the emission maximum of DAPI (461 nm) extended into this range. The 460–510 nm barrier filter ensured bright fluorescence of DAPI-labeled nucleoids (Fig. 1k), and sufficiently eliminated signals of the other fluorochromes, including green-yellow fluorescence of DAPI-

accumulating metachromatic inclusions (Fig. 1l), apparently intracellular polyphosphates [7].

Overlaps in fluorescence signals.

The optimal combination of filter sets allowed for individual detection of fluorescence signals from all the fluorochromes employed. Nevertheless, in certain cases we observed an overlap in dye emission spectra, and their interference with autofluorescence of cell components. We detected autofluorescence of the entire filament surface with both 510–550 nm and 575–625 nm emission filters when samples were excited with blue or blue-violet light, yet the fluorescence signals from SYTOX Green-stained nucleoids and CTC-formazan crystals due to their higher intensity and their characteristic staining pattern contrasted vividly with the autofluorescence background. The fluorescence of SYTOX Green-labeled nucleoids of dead cells is visible with 425–445 nm/575–625 nm filter set, although the SYTOX Green dye absorbs light of this spectrum poorly (Fig. S2a). This overlap did not hinder the detection of the CTC-formazan signal because of the lack of respiratory activity in severely injured or dead cells. Although the data obtained from www.olympusmicro.com indicates that CTC-formazan is not excited by light longer than cca. 525 nm, it was reported that light up to 560 nm was optimal to excite CTC-formazan incorporated into bacterial cells [23], [24]. Thus we observed the red fluorescence of CTC-formazan with 565–585 nm/600IR nm filter set, provided the cells lacked photosynthetic pigments absorbing light in this range (Fig. 1t). In addition, the presence of CTC-formazan within the cells suppressed the DAPI fluorescence in some of the filaments in the natural samples. The fluorescence of such DAPI-labeled nucleoids was notably lower, yet still detectable (Fig. 1m). It seems that the structures within the cells stained with DAPI may come, at some point, into close spatial proximity of CTC-formazan deposits resulting in dye-to-dye interaction, which we observed in some of the natural samples. This interaction may be explained by the absorption of photons, emitted by DAPI, or by Forster energy transfer due to the overlap of the DAPI emission with the CTC-formazan absorption [29].

Sample handling during staining procedure and microscopy observation

The filamentous architecture of oscillatorian cyanobacteria and their tight cell-to-cell connections require certain precautions during the staining procedure and preparation of samples. We recommend avoiding extensive breakage of colonies into fine suspension, and long centrifugation at high speed since the cells usually sustain damage at the site of the filament disruption. We observed that after such treatment the polar cells of the filaments turn SYTOX Green-positive, and accumulate higher amounts of CTC-formazan, apparently due to injuries to their plasma membranes.

Dispersal of tight *Phormidium* colonies is required to permit the dyes a direct contact with the cells. Because the cells, which incorporate CTC-formazan, become increasingly sensitive to any mechanical manipulations, removal of staining solutions should be done through careful draining of the liquid with a micropipette rather than

centrifugation. If the staining solution cannot be separated in this manner it is possible to use gentle centrifugation (e.g. 1-2 min at 1000 rpm) or cell filtration.

The placement of an organism into an environment different in composition from the growth medium may result in an increase or a decrease in its respiratory activity. Therefore, as a solution for staining and washing we used complete or diluted BG-11 medium and incubated the samples under the same conditions as those used for cultivation. We also stress that many experiments require careful consideration of conditions for incubation in the context of aims and desirable results. This is especially true, for example, when the effect of osmotic, freezing, or nutrient stress is being studied. Once the staining procedure is completed, a different solution can be used for microscopy observation to eliminate background fluorescence. It is also worth noting that cyanobacterial cells, usually larger than other bacteria, may easily be destroyed during the preparation of microscopy slides, necessitating the spreading of the biomass onto the slide carefully, and sealing the edges of the cover slips to prevent an increase of tension between the glass surfaces as the water evaporates.

The damaging effect of excitation light on fluorochromes should also be considered in microscopy observations. To minimize the extent of pigment photobleaching and light-induced degradation of fluorescent probes, the excitation light of longer wavelengths is recommended to be applied first. The observation of phycobiliproteins, which absorbs light in the long-wave region, should be followed by SYTOX Green (blue excitation), CTC-formazan (blue-violet), and DAPI (UV) fluorescence observations. The bleaching effect of short-wave (UV) light through de-coloration of CTC-formazan may be advantageous in the case when the investigation of cell morphology and intracellular structure is required. Alternatively, an optical system based on light-emitting diodes (LED) may be employed for fluorescence microscopy observations in order to provide higher contrast and to reduce damages produced by mercury lamp irradiation, i.e., phototoxicity and heat effect on a specimen, and photobleaching of the fluorescent dyes. LED light sources with a flexible modular design (e.g., Colibri LED light source system from Carl Zeiss MicroImaging) allow rapid changes in the excitation light of the required wavelength, making this system suitable for multicolor fluorescence microscopy.

Interpretation of data and limitations of the method

As discussed, the cell physiology and activity do not appear as discrete variables – they are rather represented as a continuum of various physiological processes [5]. Apparently, there is a variety of states between obviously ‘dead’ and ‘living’ cells in microbial assemblages. Such cell states may display reduced overall metabolic activity or its particular processes, and the structural and functional integrity of their cellular compounds may be different. For this reason, the cells in the populations must be grouped into broad artificial categories depending on the operational definitions given for cell activity and viability. It should be mentioned that no single viability test provide reliable information of the cell state, and the classification of the cells into

particular groups is usually determined by the combination and sensitivity of selected assays.

Although the criteria for cell death and viability have been broadly debated [1], the absence of genetic material in the nucleoid area or its obvious disintegration provides a clear evidence of cell non-viability [39]. However, the degradation of DNA may occur with a delay after the cessation of physiological processes and the loss of membrane integrity, which prevents certification of viability of nucleoid-containing cells [39], [40].

Tab.2. Classification of cells according to the selected criteria

Physiological state of a cell	CTC reduction	SYTOX-positive	DAPI positive	Photosynt. pigments	Fig. 2, marked as
Active and intact (living healthy cell)	+	—	+	+	f
Injured but active (living injured or apoptic cell)	+	+	+	+ / —	g
Metabolically inactive but intact (presumably dormant cell)	—	—	+	± / —	h
Inactive and injured (nucleoid-containing dead cells)	—	+	+	± / —	i
Non-nucleoid-containing dead cells	—	—	—	—	j

The use of 'dead cell' stains (SYTOX Green) that reveal the structural disintegration of plasma membranes, is often considered useful for detecting non-viable cells, yet a positive reaction to such staining may indicate cell injury that can be subsequently followed by recovery or by death of the cell. In certain cases, algal communities do not show bimodal distribution of SYTOX Green-positive and SYTOX Green-negative cells. Instead, they show a more complex staining feature that is displayed as variability of fluorescence intensity from SYTOX Green-stained nucleoids [14]. Nevertheless, the SYTOX Green dye may be employed to discriminate between live, injured and dead cells by selecting different thresholds of intensity of the fluorescence signal. Respiration rate measurement, in general, may provide a good estimate for cell activity; it also indicates cell viability to some extent as respiration is attributed only to live cells. However, this assay should be interpreted in conjunction with other cellular functions since injured cells can still maintain significant rates of respiration.

The amount of formazan incorporated into cells does not necessarily correspond to the actual respiratory activity, e.g., intensive and rapid CTC reduction may reflect

the increased permeability of the membrane barrier of injured cells, unlike permeability of intact cells that display slower accumulation of CTC-formazan [23].

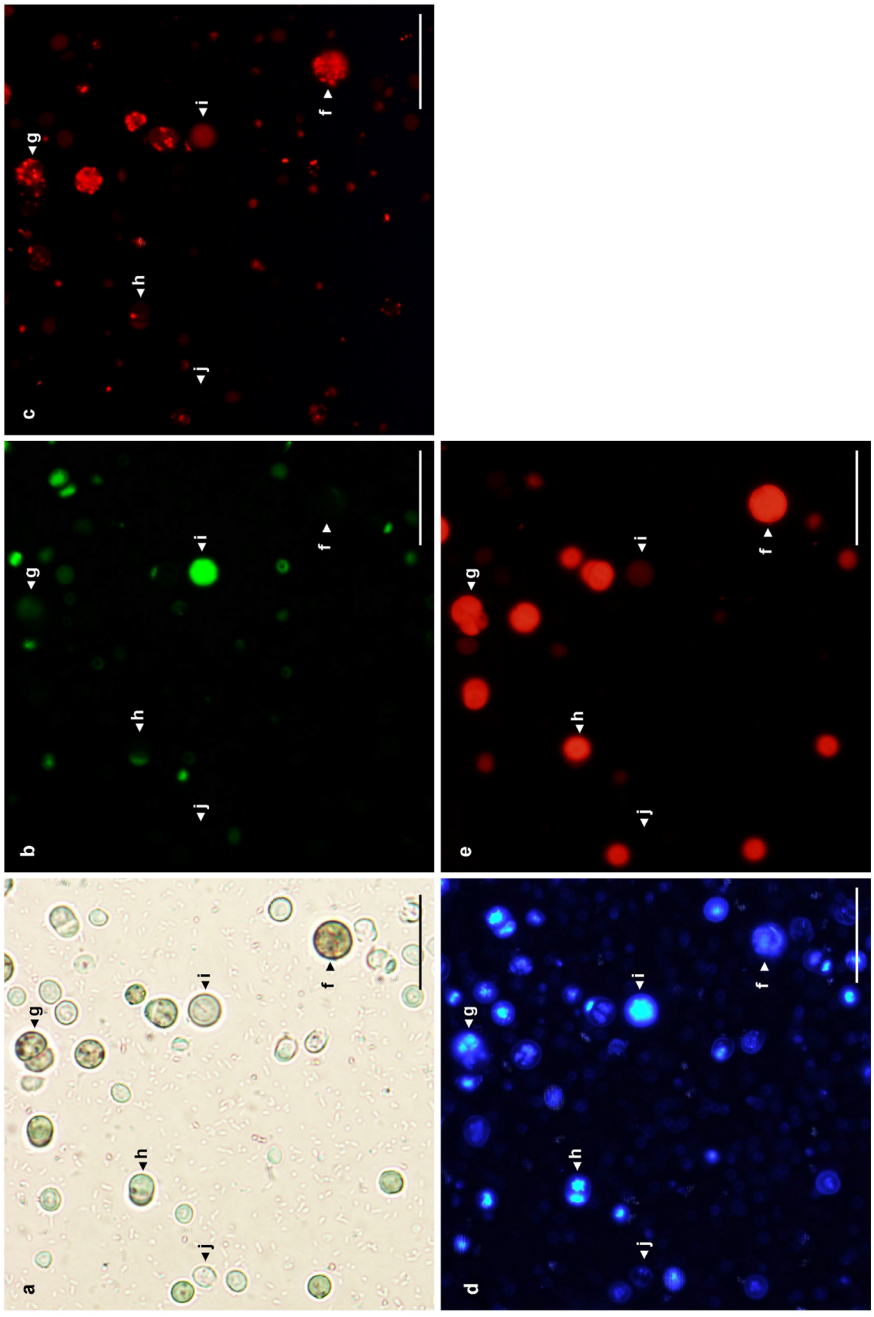

Fig. 2. Living 3-month-old *Chroococcidiopsis* 041 CCALA laboratory culture (**a**) simultaneously stained with SYTOX Green (**b**), CTC (**c**) and DAPI (**d**) dyes, and showing pigment autofluorescence (**e**).

The lack of phycobiliproteins or changes in their composition is influenced by environmental conditions, and may be attributed to cells in different physiological states. A living and active cell is expected to have significant amounts of phycobiliproteins. Indeed, the high content of accessory photosynthetic pigments may indicate an abundance of nitrogen sources in the environment that accumulate to form phycobiliprotein aggregates. Availability and composition of phycobiliproteins are also determined by the intensity of light or by its spectral properties. It was shown that non-viable cyanobacterial cells, after a long-term desiccation, undergo pigment bleaching [15]; their rapid decay upon cell death was also observed in marine phytoplankton [14]. However, phycobiliproteins can still be present in dead, injured, or metabolically inactive cells. In this case, their autofluorescence is detectable, but the output is possibly lower and of different wavelengths; e.g., stress and unfavorable conditions provoke the dissociation of phycobilisome subunits [34], [35]. This results in increased fluorescence emission from C-PE and C-PC, and a drop in chlorophyll autofluorescence. To summarize, the presence and intensity of pigment fluorescence may provide additional information regarding the state of the cells and their stress response, although it cannot serve, by itself, as a robust marker of activity level and viability of cyanobacterial cells. Accurate and detailed data regarding the physiology of the photosynthetic apparatus and its connection to the cell state may be obtained by employing chlorophyll fluorescence analysis [41], [42], which, should not be used in combination with fluorescent probes because they generally have negative effects on the cell physiology and may interfere with the chlorophyll fluorescence signal.

We propose that cells in cyanobacterial populations can be conventionally classified into several categories: (i) active and intact; (ii) injured but active; (iii) metabolically inactive but intact; (iv) inactive and injured, or dead. Through the use of a set of physiological markers employed by this method (respiratory rate, membrane integrity, the presence and integrity of a nucleoid, photosynthetic pigments), it is possible to distribute the cells among the above mentioned groups (Table 2, Fig. 2). Criteria for classifications of cells in heterogeneous bacterial populations have also been previously suggested in the papers by Caron and Badley, 1995 [43], Kell et al., 1998 [1] and Del Giorgio and Gasol, 2008 [5].

CONCLUSION

The method introduced is useful for studies of cyanobacterial population development and stress response. Our method allows rapid multiparameter assessment of the state of individual cells that may display substantial multimodal variability in both natural and laboratory populations. Although it provides a simplified view of physiological heterogeneity in populations of cyanobacteria (since it covers only limited spectrum of cell functions), our method allows a quick estimate on the condition of a large number of individual cells, and discriminate living, injured, dormant and dead cells according to the suggested criteria. Other than those described here, investigations of single-cell physiology include a number of advanced

techniques summarized in reviews by Lidstrom and Konopka, 2010 [3], and Müller and Caron, 2010 [7].

SUPPORTING INFORMATION

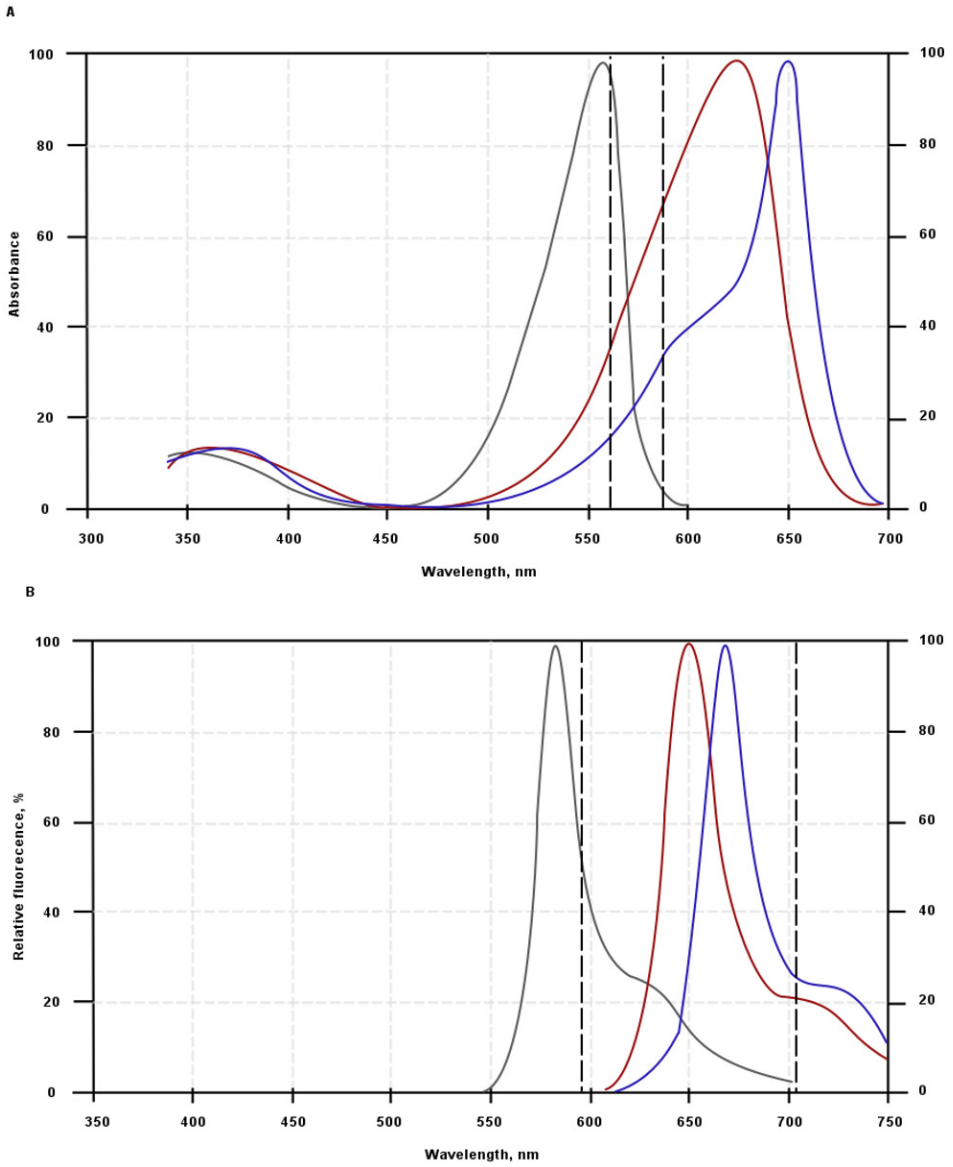

Fig. S1. Absorption (**a**) and emission (**b**) spectra of C-phycoerythrin (grey), C-phycocyanin (red), and allophycocyanin (blue). The excitation and emission ranges of microscopy filters are denoted as spaces between dotted lines. The graphs were modified from Mimuro et al. [32], Ying and Xie [44], and Teale and Dale [30].

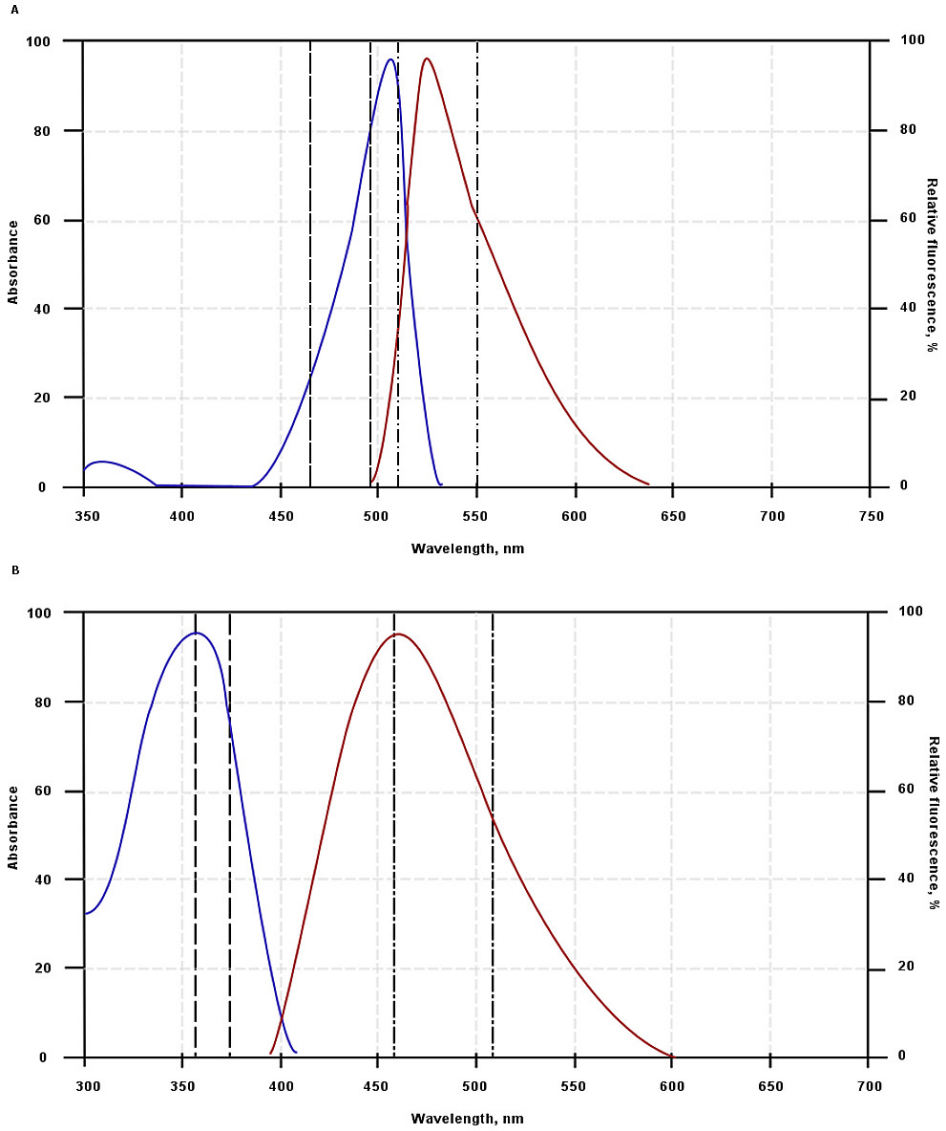

Fig. S2. Spectral profiles of the fluorescent probes used. The curves represent absorption and emission properties of SYTOX Green (**a**) and DAPI (**b**). The excitation (left chart) and emission (right chart) ranges of microscope filters are denoted as spaces between dotted lines. The light absorption and fluorescence emission curves of SYTOX Green and DAPI dyes were redrawn with the permission from www.lifetechnologies.com web source.

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This paper is in memoriam of Prof. Patrizia B. Albertano, the founder of the Centre of Advanced Microscopy and the leader of the Laboratory of Biology of Algae at the University of Rome 'Tor Vergata'. She died on March 14th, 2012.

AUTHOR CONTRIBUTIONS

General supervision of the research group: JE. Conceived and designed the experiments: DT. Performed the experiments: DT. Analyzed the data: DT DB. Contributed reagents/materials/analysis tools: DT JE DB. Wrote the paper: DT.

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EFFECT OF NITROGEN STARVATION ON DESICCATION TOLERANCE OF ARCTIC MICROCOLEUS STRAINS (CYANOBACTERIA)

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ABSTRACT

Although desiccation tolerance of *Microcoleus* species is a well-known phenomenon, there is very little information about their limits of desiccation tolerance in terms of cellular water content, the survival rate of their cells, and the environmental factors inducing their resistance to drying. We have discovered that three *Microcoleus* strains, isolated from terrestrial habitats of the High Arctic, survived extensive dehydration (to 0.23 g water g^{-1} dry mass), but did not tolerate complete desiccation (to 0.03 g water g^{-1} dry mass) regardless of pre-desiccation treatments. However, these treatments were critical for the survival of incomplete desiccation: cultures grown under optimal conditions failed to survive even incomplete desiccation; a low temperature enabled only 0–15% of cells to survive, while 39.8–65.9% of cells remained alive and intact after nitrogen starvation. Unlike *Nostoc*, which co-exists with *Microcoleus* in Arctic terrestrial habitats, *Microcoleus* strains are not truly anhydrobiotic and do not possess constitutive desiccation tolerance. Instead, it seems that the survival strategy of *Microcoleus* in periodically dry habitats involves avoidance of complete desiccation, but tolerance to milder desiccation stress, which is induced by suboptimal conditions (e.g., nitrogen starvation).

Key words: CTC dye; SYTOX Green; cyanobacteria; desiccation tolerance; viability; fluorescence staining; nitrogen starvation.

INTRODUCTION

Terrestrial cyanobacteria are often considered to be desiccation tolerant organisms. Some taxa of cyanobacteria have evolved a remarkable ability to resist desiccation stress (Caiola et al., 1996; Potts, 1999). This ability has allowed them to colonize the most hostile places on Earth.

Numerous studies have uncovered that cyanobacteria cope with desiccation stress through a complex of physiological, biochemical, structural, and morphological adaptations. Mechanisms contributing to this include: modifying the structure and composition of cell envelopes (Caiola et al., 1996), decreased respiration (Potts, 1994), down-regulation of photosynthesis (Harel et al., 2004), producing enzymes eliminating reactive oxygen species (Chen et al., 2012), accumulating sugars which stabilize the lipid membranes (Hershkovitz et al., 1991; Sakamoto et al., 2009; Klähn and Hagemann, 2011), secreting extracellular polysaccharides that serve as a physical barrier during desiccation that absorb and retain moisture (Hill et al., 1997; Tamaru and Takani, 2005), synthesizing UV-absorbing and sun-screening pigments (Roos and Vincent, 1998; Potts, 1999; Gao and Ye, 2007), and the presence of multiple copies of the genome together with an efficient DNA reparation system (Ehling-Schulz and Scherer, 1999; Potts, 1999).

The closely related genera *Phormidium* and *Microcoleus* (Oscillatoriales) are among the most frequently recorded cyanobacterial genera in hot and cold deserts worldwide (Vincent, 2000; Wynn-Williams, 2000), and have often been mentioned as desiccation tolerant organisms (Davey, 1989; Hershkovitz et al., 1991; Hawes et al., 1992; Harel et al., 2004; Šabacká and Elster, 2006; Chen et al., 2012; Olsson-Francis et al., 2013). The taxonomy of Oscillatoriales has been recently revised to include some *Phormidium* species within the *Microcoleus* genus (Strunecký et al., 2013). Therefore, in this manuscript we consider *Phormidium* and *Microcoleus* as synonyms, and refer to their original names used in publications.

Some studies have suggested that *Phormidium* might respond to drying differently than the extremely desiccation tolerant and well-studied *Nostoc* and *Chroococcidiopsis*. These latter two genera were found to withstand regular drying-rewetting cycles, tolerate rapid water loss to nearly zero water content (Caiola et al., 1996; Tamaru and Takani, 2005), preserve the structural integrity of their cell structures after many years of storage in a dry state (Potts, 1996; Billi, 2008), and resume respiration and photosynthesis within minutes after rewetting (Davey, 1989; Wynn-Williams, 2000). In contrast to *Nostoc* colonies, *Phormidium*-dominated mats from the Antarctic showed a very slow recovery from extreme desiccation; the population survived due to migration and the multiplication of a few surviving middle-layer trichomes (apparently partially hydrated), rather than recovering the bulk biomass (Hawes et al., 1992). Hot desert dwelling *Oscillatoria* and *Microcoleus* species also demonstrated desiccation avoidance behavior as they actively migrated to the soil crust surface when water became available and retreated to the subsurface under water limitation (Pringault and Garcia-Pichel, 2004; Rajeev et al., 2013).

The few mechanisms discovered of *Phormidium/Microcoleus* desiccation tolerance include accumulating trehalose (Hershkovitz et al., 1991; Chen et al., 2012), secreting exopolysaccharides (Chen et al., 2012), stabilizing the photosynthetic apparatus (Harel et al., 2004), and accumulating UV-protecting pigments (Quesada and Vincent, 1997). A recent study on the desert crust-forming cyanobacterium *Microcoleus vaginatus* reported the expression of genes involved in the oxidative and osmotic stress response, the desaturation of membrane lipids, and the production of EPS at the onset of desiccation. Rehydration activated the genes responsible for cell signaling and DNA repair followed by upregulation of anabolic pathways (Rajeev et al., 2013).

Taken together, it is likely that *Phormidium*/*Microcoleus* evolved a combined strategy for surviving dry periods including both avoidance and partial tolerance to desiccation, rather than the ability to tolerate complete desiccation. However, it is not known whether desiccation tolerance is their constitutive trait as in some groups of mosses (Oliver et al., 2005), or if it develops under particular conditions (e.g., suboptimal light and temperature, osmotic stress, or nutrient starvation), as in many species of yeasts and bacteria (Morgan et al., 2006). While some of the mechanisms have been described, there is very little information about their limits of desiccation tolerance in terms of water content in dry cells, the survival rate of cells, damage that cells sustain upon desiccation, and rehydration, and environmental factors inducing their resistance to drying.

In many previous studies that have addressed desiccation tolerance of *Phormidium*/*Microcoleus*, the conditions of desiccation, water content in dried material, and methods for quantification of viable cells were often not described. Most of the studies evaluated the survival and stress response of *Phormidium* and *Microcoleus* at the population level, e.g., 'bulk' measurement of respiration/ photosynthesis measured by oxygen evolution/uptake, recovery of photosynthesis, or growth tests (Davey, 1989; Hawes et al., 1992; Chen et al., 2003; Harel et al., 2004; Šabacká and Elster, 2006; Rajeev et al., 2013). For instance, such an approach often overlooks the number of cells that survive and their physiological state upon rehydration. A decrease in respiration or/and photosynthesis intensity upon rehydration, for example, may be attributed to a reduction of those functions in every cell, complete inactivation of a subpopulation while the others remain fully active, or to the differential loss of these in a few subpopulations. The importance of studying microbial populations at the single-cell level has often been stressed in recent years (Davey and Winson, 2003; del Giorgio and Gasol, 2008; Lidstrom and Konopka, 2010; Tashyreva et al., 2013).

The investigation of desiccation tolerance of filamentous cyanobacteria is generally complicated by the structure of the populations they form: cultures form tight colonies during standard cultivation in a liquid medium (e.g., in Erlenmeyer flasks). The conditions across such a colony can be markedly different in terms of light spectrum and intensity, nutrient availability, and concentration of cell

metabolites. In addition, cultivation on agar plates generates a water content gradient, under which the filaments on the top of a biofilm are directly exposed to air. This approach generates physiologically heterogeneous populations, and, in addition, cannot ensure uniform drying of such a colony/biofilm.

In order to resolve the above-mentioned methodological complications, we employed cultivating cyanobacteria in thin biofilms on glass slides immersed into dishes with a liquid medium. Such a cultivation method provides significantly more homogeneous conditions in comparison to traditional cultivation methods, making it possible to vary only one of the cultivation parameters by placing glass slides into different conditions with the other conditions remaining constant. Drying the thin biofilms helped to eliminate the development of desiccation tolerance directly induced by slow dehydration of a thick layer (Hershkovitz et al., 1991; Chen et al., 2012). Detecting desiccation survivors and investigating some of their cellular function was carried out by direct cell counts in combination with staining them with three fluorescent dyes to visualize the presence, location and shape of nucleoids, track membrane integrity, and detect respiration.

For our experiments, we selected three strains of *Microcoleus* inhabiting terrestrial habitats in the Arctic. The strains were isolated from ephemeral melt water streams and pools that often become completely dry and frozen in late summer.

In this study, we endeavored to determine: (1) whether desiccation tolerance is a constitutive property or if it is inducible by suboptimal conditions, i.e., low temperature and nitrogen depletion, (2) whether the strains are able to tolerate complete desiccation defined as water content below 0.1 g H_2O g^{-1} dry biomass (Alpert, 2005) and/or incomplete (85% RH) desiccation regimes, and (3) which proportion of cells survives desiccation and what their physiological state upon rehydration is.

MATERIALS AND METHODS

Cyanobacterial Strains

The experiments were conducted with strains *Microcoleus* sp. 816 CCALA (previously *Phormidium* cf. *autumnale*) isolated from a stream in the vicinity of a glacial moraine (Northern Sweden, Lapland, Abisko, 69°21′N 18°49′E); *Microcoleus vaginatus* 858 CCALA (previously *Phormidium* sp.) isolated from a small pool in a moraine (Svalbard archipelago, 77°00′N 15°20′E); and *Microcoleus* sp. 845 CCALA (previously *Phormidium* sp.) isolated from a stream with moss carpets (Svalbard archipelago, 77°00′N 15°20′E). All strains (isolated by Šnokhousová et Elster) are currently maintained in the Culture Collection of Autotrophic Organisms (CCALA), Institute of Botany, Academy of Sciences of the Czech Republic, with a taxonomical revision of these strains carried out by Strunecký et al. (2013). The strains were previously shown to be non-diazotrophic by the acetylene reduction method (unpublished data).

Cultivation

Cyanobacterial cultures were pre-cultivated for 15 days in Erlenmeyer flasks in liquid BG-11 medium (Rippka et al., 1979) at +20°C and a continuous photon flux density of 70 μmol m^{-2} s^{-1} (white light). The biomass was harvested and used as an inoculum for subsequent cultivation in biofilms.

A piece of the biomass was smeared over both sides of a glass microscope slide (76 mm × 26 mm); the filaments readily attached to the glass surface. Six glass slides were placed in an upright position in a rectangular glass dish (13 cm × 10 cm), and kept upright with a plastic holder. The dishes were filled with BG-11 medium so that it entirely covered the slides, and closed with a transparent lid, allowing gas exchange in a similar way as a Petri dish. The medium was continuously mixed with a magnetic stirrer at a low frequency (Topolino, IKA). The light source was located over the dishes, and light from the bottom was reflected with aluminum foil placed under the dishes. After 2 weeks of cultivation, half of the cultural medium was replaced with fresh BG-11 medium.

Previous studies have suggested that cyanobacteria are psychrotolerant but not psychrophilic (Tang et al., 1997). Although cyanobacteria in the Polar Regions are often subjected to high solar irradiances, it is known that low-light conditions are preferable for the growth of cyanobacteria (Sinetova et al., 2012; Jodłowska and Śliwińska, 2014). In our experiments (unpublished data), we found that two polar strains of Microcoleus yielded the highest biomass and chlorophyll a content at +20°C and 70 μmol m^{-2} s^{-1} in full BG-11 medium. Therefore, we consider these light and temperature conditions to be optimal.

After 18–30 days of cultivation, biofilm samples from each of the six slides were collected for microscopic examination in order to study their morphology and viability. The biomass was considered suitable for subsequent experiments if the cells were of intense blue–green color, with well-pronounced thylakoids, uniform in morphology, having evidence of cell fission, lacked any visible cell inclusions, and containing only a small number of dead or decaying cells. The homogeneity of these cultures, in terms of cell viability and respiration activity, was tested with multicolor fluorescence staining (see below).

Induction of Desiccation Resistance (Pre-desiccation Treatment)

Two of the glass slides from each of the dishes were transferred into a dish filled with nitrogen deficient BG-11_0 medium (standard BG-11 medium lacking $NaNO_3$), and incubated at +20°C and continuous light of 70 μmol m^{-2} s^{-1} for 2–3 weeks. Another two slides were kept in the original dish, which was placed at +4°C (70 μmol m^{-2} s^{-1} of light), and incubated for a week. The biomass from the remaining two slides had no pre-desiccation treatment and was directly used in desiccation experiments. Hereafter, these will be referred to as ‘control’ or ‘optimally grown’ biomass.

Desiccation and Rehydration

Desiccation of the samples was carried out in two regimes: complete drying over silica gel and incomplete drying at 85% RH at 20°C. For both tests, several patches of cyanobacterial biofilm (ca. 1 cm × 1 cm) sampled throughout both sides of the two slides were placed in a drop of culture medium inside three Petri dishes, and spread over the surface so that no folds were formed. Any excess liquid was removed with sterile filter papers. The temperature and humidity were measured with a digital thermo-hydrometer (KlimaGuard, TFA, Germany). The device was calibrated over P_2O_5 (0% RH) and saturated solutions of LiCl (11.3% RH) and KCl (85% RH).

Complete desiccation was achieved by placing dishes with biofilms in a stream of sterile air for 15 minutes and their subsequent storage in a closed chamber over silica gel for 20 days at low light (<10 μmol m^{-2} s^{-1}). The RH over the silica gel fluctuated from 10 to 13%. Partial drying was carried out by placing the dishes for 20 days at low light (<10 μmol m^{-2} s^{-1}) in a closed chamber over a saturated solution of KCl, which kept the relative air humidity at a constant 85%.

The films were rehydrated with a drop of sterile distilled water for 20 min while protected from light. The rehydrated biomass formed a thick suspension after being detached from the glass surface. Half of this suspension was then transferred into an Eppendorf tube with BG-11 medium for fluorescence staining, while the other half was used for a growth test.

Viability Tests

Cell viability and physiological activity were evaluated with fluorescence staining. SYTOX Green dye (Life Technologies, USA) was used to track damage to the plasma membrane, 5-cyano-2,3-ditolyl tetrazolium chloride, or CTC (Sigma-Aldrich Co., USA) was used to assess respiration activity, and 4′,6-diamidino-2-phenylindole, or DAPI (Life Technologies, USA) was employed to observe the presence, shape, and location of nucleoids. The samples were treated according to the staining protocol that we previously described (Tashyreva et al., 2013) with 1 μM SYTOX Green for 30 min, 4 mM CTC solution for 30 min, and 5 μg ml^{-1} DAPI for 15 min. All the samples were observed with standard light microscopy prior to staining with fluorescent dyes. According to the staining results, the cells were grouped into three categories: (i) live and intact: CTC and DAPI-positive, SYTOX Green-negative; (ii) injured: CTC, DAPI, and SYTOX Green-positive; (iii) dead: CTC-negative, SYTOX Green, and DAPI-positive, or all negative. The viability test was done prior to desiccation and 20 min after rehydration.

A growth test was carried out with samples that underwent desiccation in order to confirm the staining results. The non-stained half of the biomass suspension was spread onto a BG-11 agar surface, and cultivated for 3–5 weeks under the same conditions as in pre-cultivation. In order to track any hidden growth, the dishes were periodically observed under a microscope under transmitted light (magnification 200×). The results of the growth test were expressed as having a presence or absence of growth.

Fluorescence Microscopy

An aliquot of the stained sample was placed between a glass slide and a 24 mm × 24 mm cover slip; the edges were sealed with nail polish to prevent water evaporation. An Olympus BX53 microscope equipped with a 100 W ultrahigh-pressure mercury arc lamp (Olympus) was used with 400× magnification. The optical system for fluorescence observations included four UIS2 fluorescence mirror units (excitation filter/emission filter/dichromatic mirror): U-FBWA cube for SYTOX Green (460–495 nm/510–550 nm/505 nm), and combined mirror units for DAPI (360–370 nm/460–510 nm/420 nm), CTC-formazan (425–445 nm/570–625 nm/455 nm) and phycobiliprotein (565–585 nm/600IF/595 nm) fluorescence observation. A U-FUN filter cube (360–370 nm/420IF nm/410 nm) was additionally employed for observing the fluorescence of the DAPI-stained polyphosphate inclusions.

Cell Counts

The biomass for fluorescent staining prior to desiccation was randomly sampled from both sides of every two slides in each treatment. After the staining procedure, the biomass was used to prepare several microscopy slides, where 2–5 fields of view were photographed from each of them, resulting in a total of 15–20 fields of view observed per sample.

A series of several dark-field images were acquired to record the fluorescence of each of the signals required and a bright-field image was taken for total cell counts. The images were captured with an Olympus DP72 microscope digital camera (Japan). Each image within a series was divided into 12 squares by applying a grid in GIMP v.2.8 program, where all cells within 1–3 squares were counted and distributed among three groups according to the staining results. For each of the experiments, 1100–1400 cells were counted in total.

Statistical Analysis

The effects of the treatment, strain, sampling time (i.e., before and after desiccation), and their interactions on the proportion of dead cells were tested by repeated measures analysis of variance (ANOVA) using S-plus ver. 4.5 (Statistical Sciences, 1999). The repeated-measure factor (the qualitative independent variable) was the within-subjects factor, while the dependent quantitative variable on which each participant (one replicate of a strain) measured was the dependent variable (in proportion of dead cells). Tests of normality and equality of variances were performed and the data were found to be non-normal. Therefore, the dependent variable (proportion of dead cells) was arcsin transformed before using ANOVA.

Estimation of Water Content

Ten pieces of the biofilms of strains 816 and 845 CCALA were placed on thin squares of aluminum foil and desiccated in the same way as in the experiments. The

samples were weighted on analytical-grade scales before and after oven drying for 5 h at 102°C. The water content was expressed per unit of dry mass.

RESULTS

The general scheme of the experimental procedure is shown in Figure 1. All experiments were run in triplicate, i.e., pre-cultivation and cultivation in biofilms, subsequent pre-desiccation treatments, desiccation at both regimes, and viability tests were run three times separately for each of the strains.

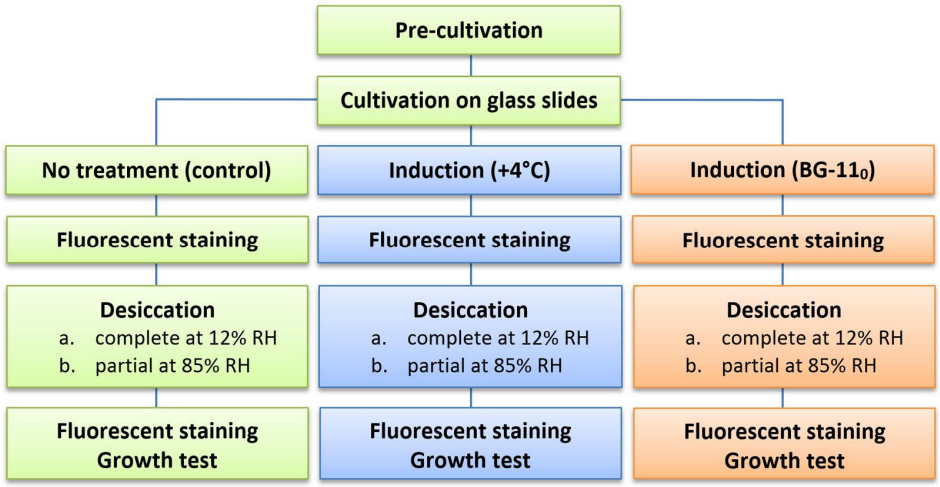

FIGURE 1. Flow diagram of the experimental procedure for each of the strains.

Morphology and Viability of Cells in Biofilms

Cyanobacterial cultures grown on glass slides formed thin biofilms, with loosely arranged filaments (Supplementary Figure S1). This cultivation regime provided consistent conditions for growth in terms of nutrient concentrations, light spectrum and intensity, and gas exchange.

Under optimal conditions, the cells were uniform in size and morphology, had an intense blue–green color and fluorescence of phycobiliproteins, well-pronounced thylakoids, lacked cell inclusions, and were arranged in long filaments (Figure 2A). The viability of these cells was confirmed by the lack of SYTOX Green staining (Figure 2B) and the accumulation of numerous small CTC-formazan deposits within each cell (Figure 2C). The cultures had a low percentage of dead (0.9–4.6%) and injured cells (0–2%) in different replicates/strains (Figure 3A). Most of the injured and dead cells occurred at the polar ends of filaments, possibly because of mechanical disruption of filaments during the staining procedure (Figure 2B).

All the cultures kept for a week at +4°C were well-pigmented, but variable to some extent in morphology and size. In most of the replicates, the cells had granulated cytoplasm apparently due to the accumulation of cyanophycin (irregularly shaped

granules), and often formed necridic cells, resulting in the splitting of trichomes into short fragments (Figure 2D). There were higher numbers (Figure 3A) of injured (0–4.2%) and dead cells (2.4–13% in different replicates/strains). The dead cells mostly occurred singly or in rows within filaments and were often represented by necridic cells (Figure 2E). Injured cells, both SYTOX and CTC-positive, were often adjacent to necridic cells (Figures 2E, F).

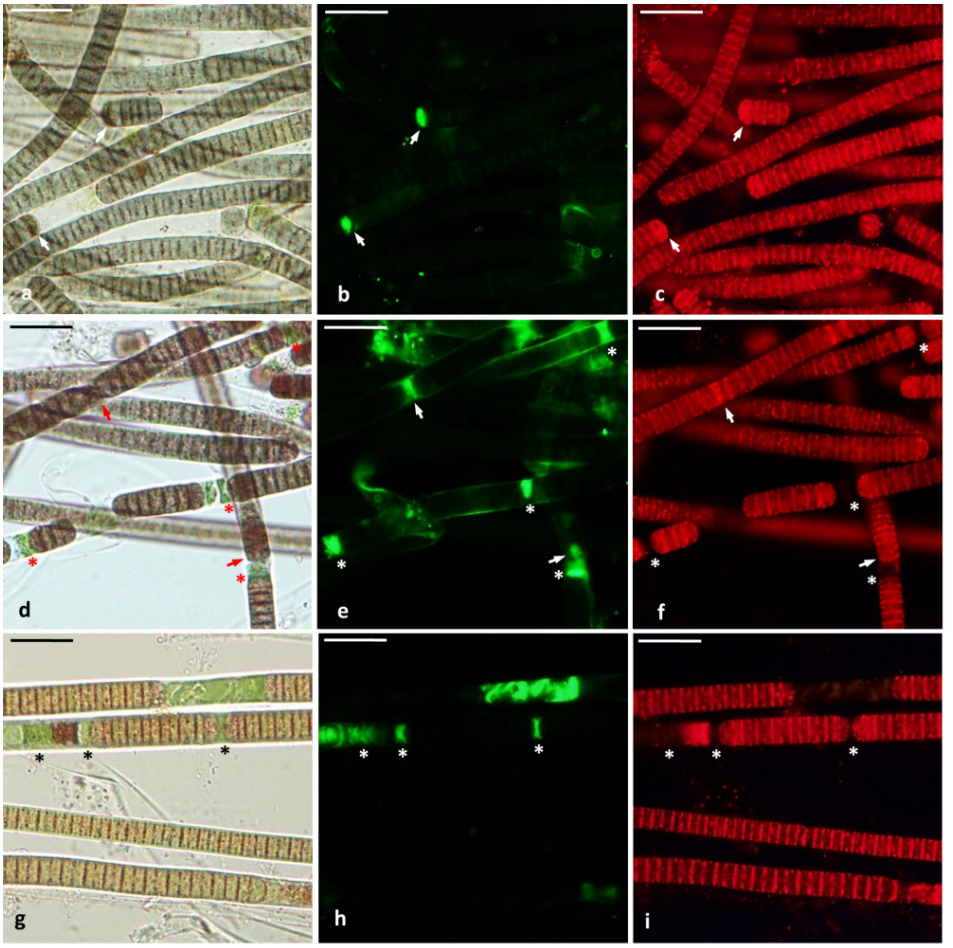

FIGURE 2. *Microcoleus vaginatus* 858 CCALA before desiccation. (**A–C**) Culture grown under optimal conditions, viewed by light microscopy (**A**), stained with SYTOX Green (**B**), and CTC (**C**) fluorescent dyes; injured cells are marked with *arrows*. (**D–F**) Culture, kept at low temperature, viewed by light microscopy (**D**), stained with SYTOX Green (**E**), and CTC (**F**); necridic (dead) cells are SYTOX Green-positive and CTC-negative (*asterisks*); the injured cells are both SYTOX Green and CTC-positive (*arrows*). (**G–I**) Nitrogen-starved culture viewed by light

microscopy (**G**), stained with SYTOX Green (**H**), and CTC (**I**); dead (in this case, decayed) cells are marked with asterisks. Scale bars are 20 μm.

Storing the cultures for 15–20 days in nitrogen-depleted medium led to a degradation of thylakoids, cell bleaching (Figure 2G), and a decomposition of phycobiliproteins, as was seen from the absence of their fluorescence in the red part of the spectrum. Despite this fact, the cultures maintained metabolic activity as was seen from the accumulation of CTC-formazan crystals in 87.4–93.5% of the cells (Figures 2G, I), of which 0.6–8.4% had permeabilized membranes (i.e., injured cells). Under nitrogen limitation, the quantity of dead cells (Figure 3A), including those with visibly deteriorated nucleoids, was the highest of all treatments (5.4–14.7%); they were scattered between filaments and across a sample without any obvious pattern (Figures 2G–I). Live, dead, and injured cells were morphologically similar, or, more often, dead cells appeared decayed (Figures 2G–I). Nucleoids were either unfolded or slightly condensed (data not shown). A proportion of cells accumulated polyphosphate deposits presumably, detected according to a shift in DAPI fluorescence from blue to yellow–green (data not shown, see Tashyreva et al., 2013). Distribution of live, injured, and dead cells were not significantly different among strains in each of the pre-desiccation treatments (see statistical comparisons at the end of results).

Cell Viability after Complete Drying (12% RH)

Samples that underwent a complete drying regime appeared dry after a few minutes under the stream of air. The samples contained 0.03 ± 0.001 g water g^{-1} dry mass (mean ± SD) after drying over silica gel for 2 weeks. No live or viable but injured cells were detected upon rehydration in any of the replicates grown under optimal (control) conditions (Figure 3B). In samples treated with low temperature and nitrogen depletion prior to drying, no viable cells were observed either (Figure 3B), despite the presence of sheaths in the nitrogen starved cultures (Figure 4C). In all treatments/replicates, the filaments started to disintegrate into single cells a short time after rehydration (usually within 1 h) followed by their quick decay (Figures 4A,B). Fluorescence staining (data not shown) revealed that all the cells were CTC-negative and SYTOX Green-positive, indicating the absence of respiration and damage to their plasma membranes. A small number of cells were both SYTOX Green and DAPI-negative, which indicated deterioration of intracellular components, including nucleoids. This staining pattern corresponded to the category of injured and inactive, or dead cells. The growth test showed consistent results – no growth was detected after 5 weeks of cultivation, and the biomass used as inoculum underwent lysis. No statistical analysis was applied to this group.

Cell Viability after Incomplete Drying (85% RH)

The samples contained 0.23 ± 0.01 g water g^{-1} dry mass (mean ± SD) after being stored over KCl solution for 2 weeks. In cultures grown under optimal conditions, only a few viable cells (5–20) per whole sample (i.e., millions of cells) were detected

in some of the replicates, whereas others lacked any viable cells (Figure 3C). The absence of viable cells also proves that the drying treatment itself did not induce development of desiccation tolerance. Those solitary cells were scattered uniformly across the sample. They were SYTOX Green-negative and accumulated CTC-formazan deposits (data not shown). However, the deposits were only few and appeared much bigger in size (Figure 5A) compared to those in non-desiccated cells grown under optimal conditions (Figures 2A, C). A similar pattern of CTC-formazan deposition was observed in cells treated with sub-lethal concentrations of formaldehyde, possibly indicating cellular damage which cannot be tracked with SYTOX Green staining (Tashyreva et al., 2013). Apparently, such cells did not propagate because there was no evidence of growth, even after 5 weeks of cultivation.

Cultures that underwent low temperature treatment prior to desiccation showed complicated patterns of their desiccation response. No viable cells were detected upon rehydration in two of three replicates of strain 858 CCALA and in one of strains 845 and 816 CCALA. The biomass in the remaining replications contained 5–15% of viable cells clustered together (Figure 3C). The viable cells contained CTC-formazan crystals, which ranged from a few big ones to numerous small ones (Figures 5B, C), and were SYTOX Green-negative.

Nitrogen-depleted cultures showed the highest rate of desiccation survival. The proportion of viable cells was 39.8–51.3% for strain 845 CCALA, 41.2 to 65.9% for 858 CCALA, and 56.8 to 62.3% for strain 816 CCALA (Figure 3C). Fluorescence staining revealed that cells that survived desiccation resumed their metabolic activity (i.e., respiration) within minutes after rehydration; their CTC-formazan deposits ranged from a few big ones to numerous small crystals (Figure 5F). Those cells remained intact according to the absence of SYTOX Green staining (Figure 5E), contained unfolded nucleoids (stained with DAPI, data not shown), and were not notably morphologically different from non-viable cells in the same sample (Figure 5D). The number of injured cells was very low after drying in all the replicates/strains, possibly because the injured cells were either not able to survive desiccation, or recovered after rehydration.

Statistical evaluation of the proportion of dead cells in the samples revealed that treatments (especially nitrogen depletion) prior to desiccation (85% RH) significantly improved desiccation survival (ANOVA for repeated measures, interaction Time × Treatment, $F = 134.61$, $p < 0.001$), i.e., the number of dead cells was the lowest after the nitrogen starvation treatment, followed by the low temperature treatment. Low temperature treatment prior to desiccation (85% RH) also significantly improved survival compared to control (ANOVA for repeated measures, interaction Time × Treatment, $F = 14.68$, $p < 0.001$) when tested separately. No significant difference was found in the response of particular strains to desiccation during the whole experiment (ANOVA for repeated measures, interaction Time × Strain, $F = 0.05$, $p = 0.95$) and no significant difference between strains was found in the effect of treatment prior to desiccation on their survival (ANOVA for repeated measures,

interaction Time × Strain × Treatment, F = 0.45, p = 0.78); this means that all the strains responded similarly to desiccation as well as to pre-desiccation treatments.

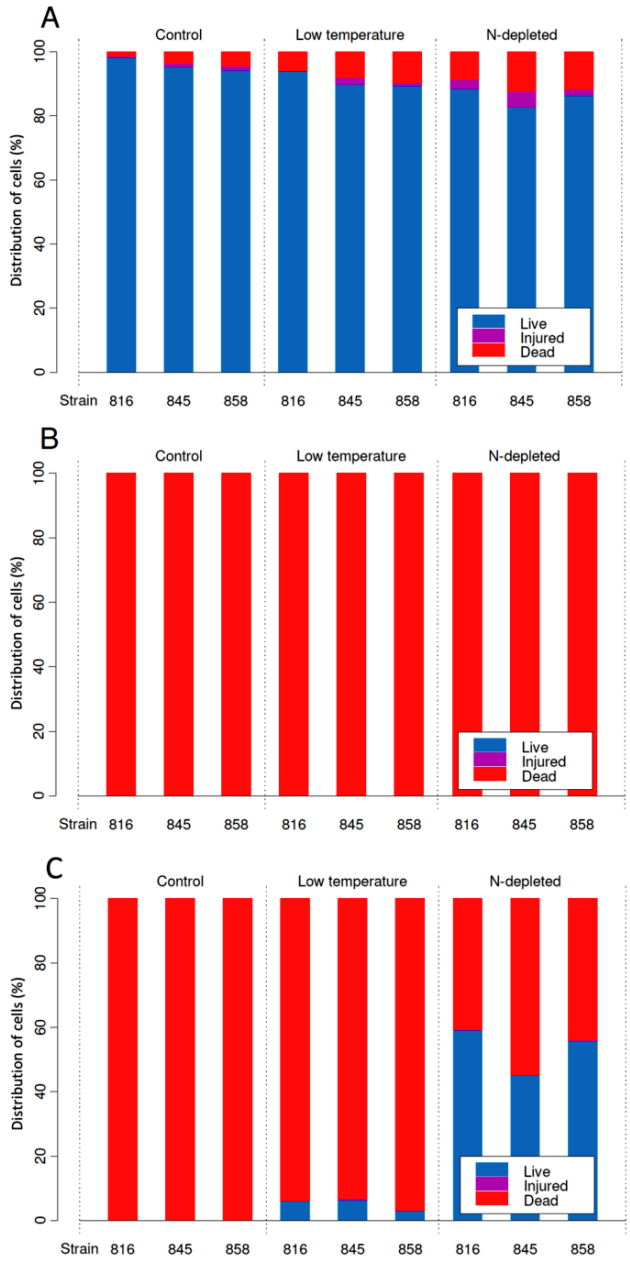

FIGURE 3. Distribution of live, injured and dead cells in cultures (means) before desiccation (**A**), and after rehydration from complete (**B**), and incomplete (**C**) desiccation regimes.

DISCUSSION

Although *Microcoleus* species inhabit water-deficient habitats (Pentecost and Whitton, 2012), no attempts have been made to determine whether they are able to survive complete desiccation. This ability gives a great advantage to organisms inhabiting arid regions and periodically dry environments. Complete desiccation is commonly defined as water loss to a content which is below 0.1 g g^{-1} dry biomass (Alpert, 2006) This important threshold corresponds to the minimum amount of water needed to form a monolayer around cell proteins and membranes (Alpert, 2006). Upon removal of this water, cells of most organisms sustain lethal damage due to irreversible changes in the native structure of dehydrated membranes and proteins as well as chemical cross-linking between proteins, sugars, and nucleic acids (Potts, 1994). The ability to survive such an extensive water loss is termed anhydrobiosis, which is a rare property among organisms (Alpert, 2006). The upper limit for complete desiccation (i.e., 0.1 g of water g^{-1} dry biomass) is roughly equivalent to air dryness at 50% RH and 20°C (Alpert, 2005) or 30–40% RH (Sun, 2002), i.e., conditions that readily occur in terrestrial cyanobacteria habitats, especially in arid regions. Natural rates of desiccation often lead to even more extreme water loss; in hot deserts dry mass water content may drop to only 5% (ca. 0.05 g of water g^{-1} dry biomass) or less (Belnap, 2003).

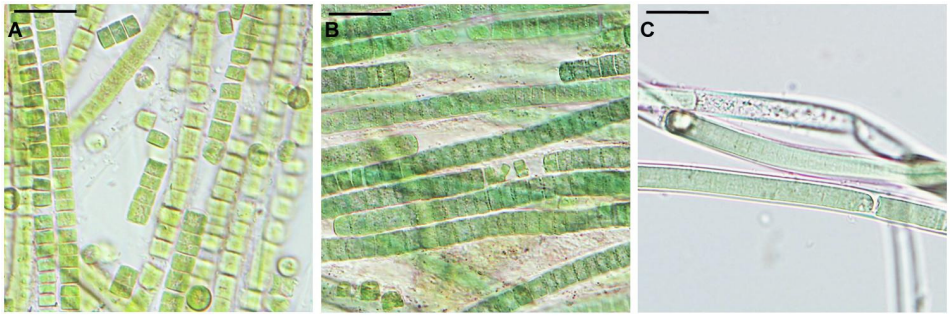

FIGURE 4. *M. vaginatus* 858 CCALA after rehydration from complete desiccation, viewed by light microscopy. Cultures grown under optimal conditions (**A**), and kept at low temperatures (**B**), both containing filaments disintegrated into single cells; nitrogen-starved culture (**C**) with filaments enclosed in sheaths. Scale bars are 20 μm.

Among cyanobacteria, only *Nostoc* and *Chroococcidiopsis* have been shown to withstand complete desiccation (Hawes et al., 1992; Billi and Potts, 2002). These species often co-exist with *Microcoleus* species in both hot and cold deserts (Hawes et al., 1992; Wynn-Williams, 2000; Jungblut and Hawes, 2005), e.g., as a part of soil crust communities (Belnap, 2003). However, we found that none of the *Microcoleus* strains were able to tolerate complete desiccation, even when exposing them to low temperature and nitrogen starvation prior to desiccation. Our results suggest that *Microcoleus* species lack the ability to tolerate complete desiccation to 0.03 g of water

g^{-1} dry mass (as tested in this study), unlike *Nostoc*, which survives in a completely dry state for decades with only 0.02 g of water g^{-1} dry mass (Billi and Potts, 2000). There are no data available whether the amount of water below the 0.1 g of water g^{-1} dry biomass threshold affects desiccation survival of cyanobacteria. However, we assume that *Microcoleus* species, which failed to survive desiccation at 10–13% RH, would also not tolerate desiccation at higher RH values, which cause the removal of a monomolecular layer of water (i.e., up to 30–50% RH). That is because either partial or extensive removal of the monomolecular water shell around biomolecules requires cells to possess fundamentally different adaptations, e.g., replacement of water with non-reducing sugars (Crowe et al., 1990, 2002; Potts, 1994). In our experiments, a consistent drying rate of the thin biofilms was a very important condition for studying the tolerance of strains to complete desiccation. Drying of thick material would result in higher hydration of the inner layers of biomass, since the outer dry layers might provide a physical barrier against evaporation. Drying of thin biofilms insured against a false interpretation of desiccation tolerance, because even a very small increase in cell water content might be critical for cell survival.

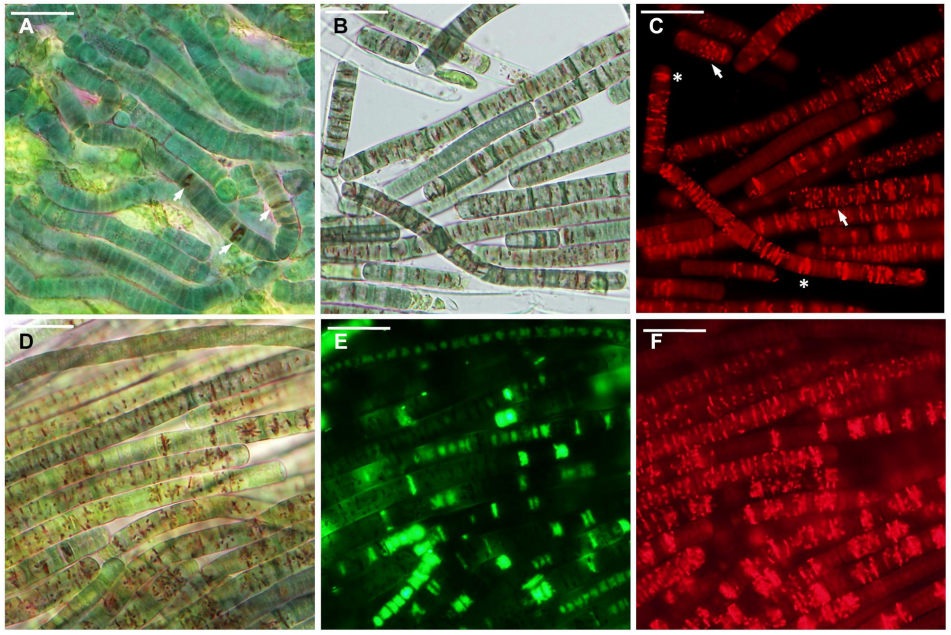

FIGURE 5. *Microcoleus vaginatus* 858 CCALA after rehydration from incomplete desiccation (85% RH). (**A**) Culture grown under optimal conditions containing cells with a few large CTC-formazan crystals (*arrows*), which are visible under transmitted light as dark-red deposits. (**B, C**) Culture, kept at low temperature, viewed by light (**B**), and fluorescence (**C**) microscopy; cells contain CTC-formazan crystals, which range from a few big ones (*asterisks*) to numerous small ones (*arrows*). (**D–F**) Nitrogen-starved culture, viewed by light microscopy (**D**), and stained with SYTOX

Green (**E**), and CTC (**F**); live cells are SYTOX Green-negative and CTC-positive; dead cells are SYTOX Green-positive and CTC-negative. Scale bars are 20 μm.

Nevertheless, the results show that *Microcoleus* strains are able to survive extensive though incomplete dehydration to only 0.23 g of water g^{-1} dry biomass. Their resistance is not a constitutive property since cultures in their active phase of growth under optimal conditions failed to survive this drying treatment. We found that resistance to dehydration is inducible by prior exposure to suboptimal conditions, to some extent by low temperature, and to a greater extent by nitrogen starvation.

It is known that both freezing at natural rates (<10°C min^{-1}) and desiccation stresses result in loss of intracellular water, therefore protective mechanisms to these stresses frequently overlap (Mazur, 1984; Holmstrup et al., 2002; Tashyreva and Elster, 2012). Thus, this may explain why transfer of the cultures to low temperature might promote their acclimation not only to freezing, but also to drying, and act as a direct inducer of desiccation resistance. The observed patchiness of viable biomass after the drying treatment, and the absence of viable cells in some of the replicates, may be explained by partial acclimation to dehydration, which allowed for the survival of cells only in denser parts of biofilms, which presumably contained slightly more water.

It has been previously widely discussed that cultures of heterotrophic bacteria and yeasts that entered a stationary phase of growth and starved cultures display enhanced resistance to heat shock and osmotic stress, and better survive freezing and desiccation (Gilbert et al., 1990; Jenkins et al., 1990; Morgan et al., 2006; Welch et al., 2013). The stationary phase is associated with a complex of stress factors, depending on the specific conditions. However, it is commonly accepted that nutrient starvation is one of the main inducers for transition of bacterial cultures into a stationary phase of growth (Siegele and Kolter, 1992; Gefen et al., 2014). The lack of nutrients triggers not only a response directed to cope with starvation, but also a general stress response that provides cross-protection against different kinds of environmental insults (McCann et al., 1991; Siegele and Kolter, 1992). Moreover, starved cells often have a higher rate of stress tolerance than cells pre-adapted to a particular stress by exposing cells to non-lethal levels of the stress factor. For example, a short starvation episode provided *Escherichia coli* with stronger osmotic resistance than treatment with hyperosmotic solutions (Jenkins et al., 1990). Apparently, nitrogen starvation, apart from other stationary phase stresses, plays a key role in the acquisition of desiccation tolerance by cyanobacteria.

Microorganisms are able to survive desiccation conditions through avoidance of water loss (including forming spores that retain water), or true desiccation tolerance by surviving extensive water loss (Potts, 1994; Holzinger and Karsten, 2013). Desiccation tolerance is generally defined as the ability to survive severe water loss; however, there is no clear threshold between desiccation tolerant and sensitive organisms (Walters et al., 2002). Some authors suggest that desiccation tolerant organisms are able to survive dehydration below critical points of 0.25 and 0.3 g of water g^{-1} dry mass, at which point the hydration shell of molecules is gradually lost (Hoekstra et al., 2001), or loss of up to 95% of their initial water (Toldi et al., 2009). Since the studied strains failed to survive

complete desiccation, it seems that *Microcoleus* species are not truly anhydrobiotic, but evidently tolerant to milder desiccation rates. Therefore, we consider that their survival strategy is attributed to tolerance of extensive dehydration, which is induced by suboptimal conditions, but avoidance of complete desiccation. *Microcoleus* avoids complete desiccation through active migration of organisms to more hydrated conditions (Garcia-Pichel and Pringault, 2001), retention of water by mucilage EPS sheaths (Chen et al., 2012) as well as by formation of thick multilayered mats that decreases the surface-to-volume ratio. In addition, such population structures generate heterogeneous conditions, under which different parts may be limited with light and nutrients. It may stimulate higher stress resistance in some of the cells. Hence, those cells might also be responsible for survival of even sudden desiccation episodes.

The three studied *Microcoleus* strains isolated from terrestrial habitats of the Arctic showed strikingly similar patterns of their response to drying. However, the results of this study do not rule out the possibility that other *Microcoleus* species/strains from extreme habitats (e.g., hot or cold deserts) or aquatic environments would respond to desiccation differently. Stronger desiccation tolerance might be induced by a stress factor other than nitrogen limitation, e.g., lack of another nutrient(s), changes in the light regime, slow dehydration, or by a combination of all the mentioned factors. Nevertheless, the results of this study provide an important background for further research on *Microcoleus* desiccation tolerance.

SUPPLEMENTARY MATERIAL

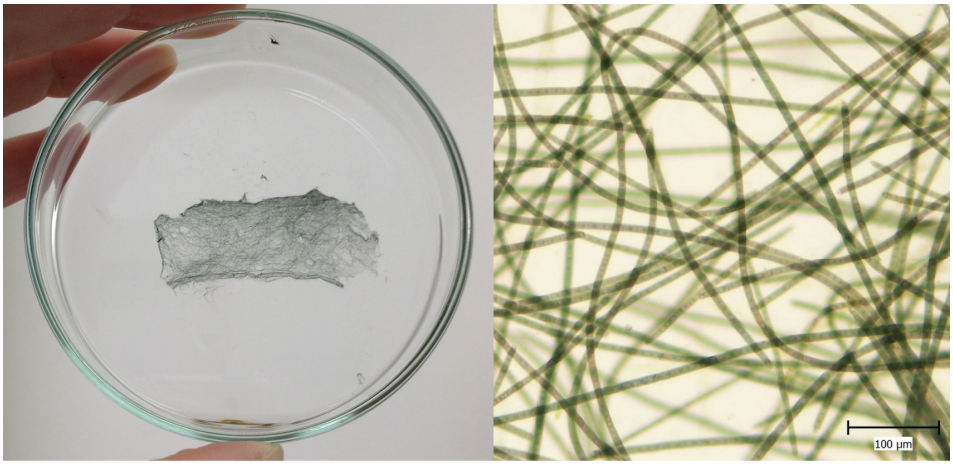

Figure S1. Example of a thin biofilm grown on a glass slide.

CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The Review Editor Daniela Billi declares that, despite having collaborated

with author Daria Tashyreva, the review process was handled objectively and no conflict of interest exists.

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ANNUAL CYCLES OF TWO CYANOBACTERIAL MAT POPULATIONS IN HYDRO-TERRESTRIAL HABITATS OF THE HIGH ARCTIC

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ABSTRACT

Cyanobacteria form extensive macroscopic mats in shallow freshwater environments in the High Arctic and Antarctic. In these habitats, the communities are exposed to seasonal freezing and desiccation as well as to freeze-thawing and drying-rewetting cycles. Here, we characterized the annual cycles of two *Phormidium* populations in very shallow seepages located in central Svalbard. We observed the structure of the populations, and the morphology, ultrastructure, metabolic activity and viability of filaments and single cells.

The communities overwintered as frozen mats, which were formed by long filaments enclosed in thick multilayered polysaccharide sheaths. No morphologically and/or ultrastructurally distinct spore-like cells were produced for surviving the winter, and the winter survival of the populations was not provided by a few resistant cells, which did not undergo visible morphological and ultrastructural transformations. Instead, a high proportion of cells in samples (85%) remained viable after prolonged freezing. The sheaths were the only morphological adaption, which seemed to protect the trichomes from damage due to freezing and freeze-associated dehydration. The cells in the overwintering communities were not dormant, as all viable cells rapidly resumed respiration after thawing and their nucleoids were not condensed. During the whole vegetative season, defined by the presence of water in a liquid state, the populations were constantly metabolically active and contained <1% of dead and injured cells. The morphology and ultrastructure of the cells remained unaltered during observations throughout the year, except for light-induced changes in thylakoids. The dissemination events are likely to occur in spring as most of the trichomes were split into short fragments (hormogonia), a substantial proportion of which were released into the environment by gliding out of their sheaths, as well as by cracking and dissolving their sheaths. The short fragments subsequently grew longer and gradually produced new polysaccharide sheaths.

Key words: *Phormidium*, life cycle, overwintering strategy, Polar Regions, viability, hormogonia.

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