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Eva Hejduková

TOLERANCE PENÁTNÍCH ROZSIVEK (BACILLARIOPHYCEAE) K EXPERIMENTÁLNÍMU ZMRAZOVÁNÍ: POROVNÁNÍ POLÁRNÍCH A TEMPERÁTNÍCH KMENŮ

TOLERANCE OF PENNATE DIATOMS (BACILLARIOPHYCEAE) TO EXPERIMENTAL FREEZING: COMPARISON OF POLAR AND TEMPERATE STRAINS

Diplomová práce

Vedoucí závěrečné práce: RNDr. Linda Nedbalová, Ph.D.

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ABSTRAKT

V polárních oblastech je prostředí charakterizováno mnoha extrémy. Nízké teploty, nedostatek kapalné vody, nepravidelný přísun živin a slunečního záření a jejich fluktuace v denních i ročních cyklech mohou vypadat pro život nevhodně. Navzdory tomu jsou rozsivky (Bacillariophyceae) jednou ze skupin mikroorganismů, které se v tomto prostředí patrně dobře adaptovaly a v široké škále polárních habitatů dominují. Strategií, jak překonat nepříznivé podmínky, je pro mnoho organismů dormance, avšak u rozsivek jsou morfologicky odlišná klidová stadia pozorována zřídka. V této studii byla experimentálně testována tolerance polárních a temperátních rozsivek k vymrzání a byl také posouzen rozdíl v přežívání vegetativních a klidových buněk.

Kmeny rozsivek pro experimenty byly izolovány v roce 2014 z přírodních vzorků z maritimní Antarktidy (ostrov Jamese Rosse, ostrov Vega) a z Arktidy (Svalbard). Další kmeny byly získány ze sbírky mikroorganismů (CCCryo a BCCM). Tvorba klidových buněk byla navozena kultivací za nedostatku dusíku a světla v nízkých teplotách. Vegetativní a klidové buňky 26 kmenů byly vystaveny šesti různým pokusům s vymrzáním (včetně těch, které simulovaly přírodní podmínky), a to do teploty –4 °C, –20 °C, –40 °C a –180 °C (tekutý dusík). Jednotlivé pokusy se také lišily v rychlosti vymrzání a tání (postupné versus náhlé).

Závěrem studie je, že rozsivky jsou citlivé vůči vymrzání. Rozdíl v přežívání mezi polárními a temperátními kmeny rozsivek nebyl signifikantní. Všechny kmeny přežily –4 °C. Většina kmenů odolala postupnému vymrzání do –20 °C a náhlému tání. Prudké vymrzání a pomalé tání nebylo letální pouze pro dva kmeny, což naznačuje, že rychlost mrznutí a tání hraje v přežívání významnou roli. Pět kmenů odolalo –40 °C, z nichž čtyři přežily také pokus s vymrzáním do –180 °C a rychlým táním. Tyto kmeny patřily do druhového komplexu *Pinnularia borealis*. Kryoprezervační pokus s použitím kryoprotektantu DMSO nebyl letální pouze pro dva polární z celkových šesti testovaných kmenů. Význam klidových buněk pro přežívání vymrzání nebyl potvrzen.

Klíčová slova: rozsivky, Bacillariophyceae, polární mikroorganismy, přežívání mrazu, stresová tolerance, dormance, kryoprezervace

ABSTRACT

Environment in Polar Regions is characterized by many extremes. Low temperatures, lack of fluid water, irregular nutrient and light supply, fluctuations in daily and annual cycles could seem unfavourable for life. In spite of this, diatoms (Bacillariophyceae) are one of groups of microorganisms that apparently well adapted to such environment and dominate in a wide range of polar habitats. For many organisms, dormancy is a strategy to overcome unfriendly conditions, but morphologically distinct resting stages are observed rarely in diatoms. In this study, the tolerance of polar and temperate diatoms to freezing was experimentally tested and the difference in survivability of vegetative and resting cells was assessed.

Diatom strains for the experiments were isolated in 2014 using natural samples from the Maritime Antarctica (James Ross Island, Vega Island) and the Arctic (Spitsbergen). Further strains were acquired from culture collections of microorganisms (CCCryo and BCCM). Resting cells were induced by incubation under nitrogen and light limitation in lower temperature. The vegetative and resting cells of 26 strains were exposed to six different freezing treatments (including those simulating natural conditions) to –4 °C, –20 °C, –40 °C and –180 °C (liquid nitrogen). Treatments differed also in the rate of freezing and thawing (continuous versus abrupt).

The study concludes that diatoms are sensitive to freezing. The difference in survival between polar and temperate diatoms was not significant. All the strains survived the –4 °C treatment. Most of the strains survived –20 °C using continuous freezing followed by fast thawing. Abrupt freezing and slow thawing was not lethal only for two strains, suggesting that the rate of freezing and melting plays an important role in survival. Five strains withstood the –40 °C freezing experiment and four of these were also resistant to the –180 °C treatment followed by fast thawing. These were strains belonging to the *Pinnularia borealis* species complex. Cryopreservation treatment using cryoprotectant DMSO was not lethal only for two polar out of six tested strains. The importance of resting cells for the freezing survival was not confirmed.

Key words: diatoms, Bacillariophyceae, polar microorganisms, freezing survival, stress tolerance, dormancy, cryopreservation

TABLE OF CONTENTS

1. INTRODUCTION

Polar Regions are characterized by extreme natural conditions. Habitats in the Arctic and Antarctic are the coldest on Earth and life there can be incredibly difficult: often rapid changes in daily and annual cycles, winter temperatures reach deeply below zero and polar night can last for months (VINCENT *et al.* 2008).

In spite of this, some groups of microorganisms were able to successfully adapt to this environment. Diatoms (Bacillariophyceae), cyanobacteria and green algae (Chlorophyta) are dominant phototrophic organisms in many Arctic and Antarctic freshwater habitats and according to recent studies a high number of Antarctic diatom species are endemic (e.g. CREMER *et al.* 2004, VAN DE VIJVER *et al.* 2014). Diatoms are one of the most abundant algal groups in Antarctica and are important component of many freshwater habitats: streams, wetlands and lakes (JONES 1996) and even moss and soil communities (VAN DE VIJVER & BEYENS 1999). Occurrence of diatoms is not limited only to Polar Regions they appear worldwide in aquatic and sub-aerial habitats as well (VANORMELINGEN *et al.* 2008). They are supposed to contribute between 40 and 45 % of oceanic primary production (MANN 1999). Due to their ecological preferences and well-preserved siliceous valves, significant importance of diatoms is in indication of environmental changes (BIRKS *et al.* 1990, DIXIT *et al.* 1992).

Extremely inhospitable polar environment with desiccation-rehydration and freeze-thaw cycles is stressful even for microorganisms. These changes in liquid water availability can cause irreversible or lethal cell damages. To avoid the negative effects of freezing, microorganisms developed various protection mechanisms, for example accumulation of cryoprotective molecules – saccharides, proteins, lipids, alcohols (WELSH 2000). One of strategies how to overcome adverse conditions is also dormancy. A lot of microalgae survive as dormant stages: resting cells, spores or cysts. But most freshwater diatom species are not known to form such stages and there are only a few studies about pennate diatoms resting stages formation (KUWATA & TAKAHASHI 1999, MCQUOID & HOBSON 1996). Their long-term survival is thus probably connected with the adaptation of vegetative cells to low temperatures and desiccation. However, vegetative cells of temperate benthic diatoms are very sensitive to desiccation, freezing and abrupt heating (SOUFFREAU *et al.* 2010) and the stress tolerance of

temperate benthic diatoms seems to depend on type of habitat (SOUFFREAU *et al.* 2013a). A study of Antarctic cyanobacteria showed that strains isolated from seepages are less tolerant to desiccation and freezing than those from other wetland habitats (ŠABACKÁ & ELSTER 2006). However, the tolerance of polar diatom species is not known.

In this thesis the response of diatoms isolated from polar and temperate freshwater habitats to freezing stress was studied. Six various freezing experiments were applied. Importance of resting cells formation for survival was assessed as well. Testing the freezing tolerance of microorganisms also became important in relation to the practical use in culture collections, which is an attractive approach for the long-term preservation of strains (DAY *et al.* 2005).

AIMS AND HYPOTHESIS

AIMS:

I. Test the ability of polar and temperate diatoms to survive different freezing temperatures.

II. Compare the tolerance of polar and temperate diatoms to experimental freezing.

III. Evaluate the importance of resting and vegetative cells for freezing survival of diatoms.

IV. Examine different freezing modes and a potential influence of freezing and thawing rate.

HYPOTHESIS:

I. The survivability of diatom strains will decrease with decreasing freezing temperature. All the strains are expected to survive –4 °C, –20 °C and –40 °C will be limiting for survival and –180 °C treatment using liquid nitrogen was established as a negative control.

II. Polar diatoms are expected to show a higher tolerance of freezing treatments when compared to temperate ones.

III. Advanced viability after treatments should appear in resting cells previously incubated under nutrient, light and temperature limitation as a preparation for a winter season (MOCK & VALENTIN 2004).

IV. Combinations of different cooling and thawing rates were chosen, since different cooling and thawing rates might have significant influences on the survival rate of cells. Slow thawing is expected to have a negative effect on viability because of the effects of recrystallization (MAZUR 1984, CAÑAVATE & LUBIAN 1997).

2. LITERATURE REVIEW

2.1 DIATOMS

Diatoms are unicellular eukaryotic photosynthetic microorganisms belonging to Chromista kingdom, class Bacillariophyceae. They are characterized by golden colour and can be also distinguished from other unicellular algae by their ornamental siliceous shell, which is unique among algae (ROUND *et al.* 1990). With species number estimated to be at least 30,000 and probably ca. 100,000 taxa (MANN & VANORMELINGEN 2013) diatoms are the most species-rich group of eukaryotic photosynthetic microorganisms (MANN 1999).

2.1.1 MORPHOLOGY AND LIFE CYCLE

Diatom cell wall is composed of silicon dioxide (SiO_2). Generally, the cell wall consists of two halves termed valves, which have slightly different size – the larger one epitheca and the smaller one hypotheca (JULIUS & THERIOT 2010). These two valves fit together and form a box known as frustule (Fig. 1). The connection is made by series of girdle bands (ROUND *et al.* 1990).

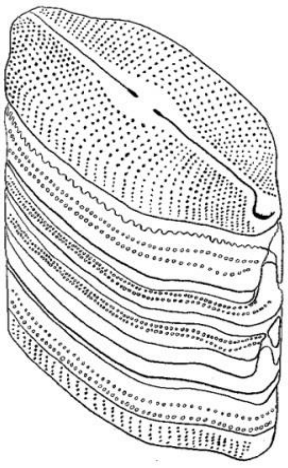


Fig. 1. Diatom frustule – valves and girdle elements (ROUND *et al.* 1990).

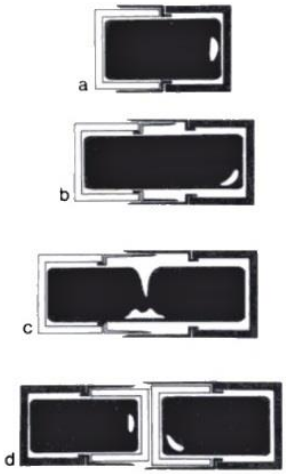


Fig. 2. Cell division: a) complete maternal cell, b) growth of the cell, c) mitosis and cytokinesis, d) daughter cells beginning to separate (ROUND *et al.* 1990).

Diatoms are able to reproduce both sexually and asexually. Sexual reproduction is relatively rare, predominantly it is vegetative via mitotic division like many other unicellular organisms (JULIUS & THERIOT 2010). When the cell divides, its epitheca and hypotheca become epitheca of both daughter cells and produce a new hypotheca. This means that one of these daughter cells is always smaller than the mother one (Fig. 2). Consequence of repeated division is a decrease of average cell size in population (ROUND *et al.* 1990, JULIUS & THERIOT 2010). Cell size is restored via sexual reproduction, when fusion of two gametes creates an auxospore that develops into a single diatom cell (LEE 2008). To overcome unfriendly periods some diatom species are known to form resting stages, which is further outlined in the chapter 2.2.3 'Freezing survival'.

2.1.2 TAXONOMY

Diatom taxonomical classification is usually based on morphology of the siliceous cell wall – key characteristics are symmetry and valve structure, presence of raphe slits for movement in the cell wall and the type, number and arrangement of tubes through the valve. Accordingly there are recognized three groups of diatoms: radially symmetrical centric diatoms, bilaterally symmetrical raphid pennate diatoms and arraphid pennate diatoms (ROUND *et al.* 1990). According to molecular studies, the classification is much more complicated. Results of modern analyses support the existence of two clades, but neither one of them correspond to the three groups or to the traditionally recognized centric or pennate diatoms (MEDLIN *et al.* 1996, MEDLIN & KACZMARSKA 2004). Furthermore, molecular analyses indicate that the cryptic diversity in diatoms (number of species that cannot be morphologically distinguished) is relatively high. For example: *Sellaphora capitata* D. G. Mann & S. M. McDonald (EVANS *et al.* 2009, VANORMELINGEN *et al.* 2013), *Pinnularia borealis* Ehrenberg complex (SOUFFREAU *et al.* 2013b), *Eunotia bilunaris* (Ehrenberg) Schaarschmidt complex (VANORMELINGEN *et al.* 2007) or *Hantzschia amphioxys* (Ehrenberg) Grunow complex (SOUFFREAU *et al.* 2013b).

2.1.3 ECOLOGY

Diatoms can be found in almost all aquatic and many sub-aerial habitats worldwide (VANORMELINGEN *et al.* 2008). They are widespread in marine, freshwater and also (semi)terrestrial habitats (MANN 1999). Diatom populations in the open oceans usually have a wide range of species but the total number of diatoms is low. In contrast the

number of different species within the population closer to shores is low, but the total number of organisms is very high (LEE 2008). In marine habitats predominantly centric diatoms occur, while pennate often dominate in freshwater, where they prosper in a wide range of habitats, including permanent water bodies like lakes and rivers but also temporary freshwater habitats such as ephemeral pools, seepages and wet to moist soils (ROUND *et al.* 1990). In these environments, they can occur as plankton or periphyton like benthic taxa with their mostly brownish-green films on substrates or aquatic plants (LEE 2008).

Diatoms found in each of these environments occur mainly solitary but sometimes there are also colonies (JULIUS & THERIOT 2010), for example: *Fragilaria crotonensis* Kitton, F. *capucina* Dezmazières var. *vaucheriae* (Kützing) Lange-Bertalot, *Melosira varians* Agardh, *Diatoma vulgaris* Bory, *Gomphoneis minuta* (Stone) Kociolek & Stoermer and *Synedra ulna* (Nitzsch) Ehrenberg (PASSY 2002).

The occurrence of diatoms is known from wide range of nutrient, salinity, pH and temperature regimes. Combinations of these factors offer the range of niches for existence of the thousands of known species (JULIUS & THERIOT 2010). Diatoms are, due to their ecological preferences and siliceous valves, well-preserved for thousands of years in sediments of lakes and used as indicators of environmental changes in both bio-monitoring and paleo-ecological reconstructions (BIRKS *et al.* 1990, DIXIT *et al.* 1992).

2.1.4 DIATOMS IN POLAR REGIONS

Polar Regions are characterized by extreme environmental conditions such as persistent low temperatures, freeze-thaw cycles, reduced amounts of solar radiation and nutrient supplies and their seasonal and inter-annual variations which could seem unfavourable for life (VINCENT *et al.* 2008). However, diatoms are apparently able to adapt or tolerate the harsh polar environment. Together with cyanobacteria and green algae (Chlorophyta), they dominate in many Arctic and Antarctic freshwater habitats.

In continental Antarctica, lakes are often characterized by extended periods of ice-cover, low nutrient input and low organic carbon production (DORAN *et al.* 2000, LAYBOURN-PARRY & PEARCE 2007), despite this diatoms are one of the most abundant and successful microbial groups in freshwater and terrestrial ecosystems in the

Antarctic and sub-Antarctic Regions (JONES 1996, VAN DE VIJVER & BEYENS 1999, VANORMELINGEN *et al.* 2008) in both number of species and number of individuals. They are an important component of many benthic algal communities in streams, wetlands, pools and lakes (JONES 1996). Diatoms are even an important part of all sub-Antarctic aquatic, moss and soil habitats (VAN DE VIJVER & BEYENS 1999).

Similarly, High Arctic diatom communities from freshwater environments have to be tolerant to various extreme conditions. During the winter season they must withstand freezing of their habitats and in the summer high levels of ultraviolet radiation and frequent desiccation of their habitats. It suggests these diatom species had to develop some specific survival strategies to cope with these environments (DOUGLAS & SMOL 1995). On the other hand many of the organisms found in polar environments appear to be cold-tolerant rather than cold-adapted (VINCENT *et al.* 2008).

Polar Regions are limnologically and biologically still poorly investigated, which is often due to their remote location. Especially in the Antarctic region, new diatom species there are still frequently found and described (SABBE *et al.* 2003, VAN DE VIJVER *et al.* 2010, KOPALOVÁ *et al.* 2011, VAN DE VIJVER *et al.* 2014) and significant part of them is or is believed to be endemic (VAN DE VIJVER *et al.* 2000, CREMER *et al.* 2004, VAN DE VIJVER *et al.* 2011). This is in contrast with a theory of ubiquity which believes a global occurrence unlimited by geographic boundaries in microbial eukaryote species with a cell size less than one millimetre (FINLAY 2002). Diatoms have also been considered as cosmopolitan, but recent studies show diatom communities are in fact controlled by the same dispersal limitation as macro-organisms and the endemism observed in isolated areas is real (VANORMELINGEN *et al.* 2008). A global freshwater diatom data set demonstrated latitudinal gradients in local and regional genus richness are present and highly asymmetric between both hemispheres (VYVERMAN *et al.* 2007).

2.2 ADAPTATION AND ACCLIMATION STRATEGIES OF MICROORGANISMS IN POLAR ENVIRONMENT WITH FOCUS ON DIATOMS

To successfully colonize low-temperature environments, photoautotrophic microorganisms have evolved a various strategies that span from molecular to whole cell and ecosystem levels. In relation to temperature regime two different environments could

be distinguished in non-marine Polar Regions: stable low-temperature habitats (permafrost, glacial ice, glacial melting surfaces, subglacial systems, temporary snowfields) and unstable low-temperature habitats (wetlands, shallow lakes, soil, lithophitic environment) (ELSTER & BENSON 2004).

According to optimal growth temperatures of microorganisms occurring in cold habitats, two groups are classified: psychrotrophs and psychrophiles. Optimal growth temperature for psychrotrophic organisms is above 15 °C and maximal could reach 40 °C. In contrast in psychrophilic organisms upper limit for growth does not exceed 20 °C and optimum is lower than 15 °C (MORITA 1975). Due to such classification is artificial, sorting of individual cold-adapted microorganisms is not that clear and may not fit the man-made definitions (RUSSELL 2006).

2.2.1 LIGHT CONDITIONS

In the polar environment the availability of photosynthetically active radiation (PAR) is highly variable. Between solar light cycles microalgae must adapt to low light levels and with the onset of winter they have to face total darkness for a period of up to five months (PALMISANO *et al.* 1985). Data about freshwater or terrestrial diatoms are limited, but an investigation of survivability of several diatom species isolated from the Southern Ocean in dark conditions was experimentally tested and showed specific survival times between polar species varied from less than four months up to nine, while temperate diatoms were found to survive 21–35 days. At the beginning of the dark period concentrations of cellular chlorophyll *a*, carbon and nitrogen decreased and remained more or less stable, which suggests a low maintenance respiration (PETERS 1996, PETERS & THOMAS 1996). Similarly, another experiment simulating polar winter conditions revealed activity in Arctic sea ice algae. During a 161 days period of darkness, composition of dominant species changed from initially pennate diatoms to small phytoflagellates (abundance of nearly all diatom species did not increased, but phytoflagellate did). Study suggests the important ways allowing microalgae to survive in the dark Arctic winter are facultative heterotrophy and energy storage (ZHANG *et al.* 1998). For instance *Chlorella* sp., strain BI isolated from a low temperature pond on the Ross Ice Shelf (Antarctica) demonstrated ability to grow under the dark condition (MORGAN-KISS *et al.* 2008).

Low photosynthetically active radiation is a fact that strongly affects the development and growth of sea ice algae. After an increase in light intensity, algal cells are able to react with fast growth within days (ZHANG *et al.* 1998). It seems algae have some universal mechanisms to overcome light limitation. One of them is high photosynthetic efficiency. Such responses were observed in High Arctic, where active photosynthesis of algae occurred at low irradiances < 0.5 $\mu E \cdot m^{-2} \cdot s^{-1}$, 0.01 % incident irradiance and increased with decreasing light intensity (COTA 1985). Spring study on sea ice microalgae from south-eastern Hudson Bay (Canadian Arctic) emphasizes importance of photoadaptation to the seasonally increasing under-ice irradiance (MICHEL *et al.* 1988). One of the processes induced under light limitation, ensuring high efficiency of photosynthesis in polar microalgae is the desaturation of chloroplast membrane lipids, which is necessary to enhance the fluidity of the thylakoid membrane and thus the flow of electrons (MOCK & KROON 2002).

On the contrary during periods of high light, polar photoautotrophs have to avoid photo-damage. Some of the mechanisms how microalgae face high ultraviolet (UV) radiation describes a study on diatom *Gyrosigma balticum* Ehrenberg. One response that occurred after enhanced UVB radiation was vertical migration into the sediment. Furthermore concentration of carotenoid pigments (ß-carotene) in diatoms was after five days of 115 % UVB treatment significantly higher. Study suggests that these two mechanisms are very important in cellular damage prevention especially in sediment associated diatoms (UNDERWOOD *et al.* 1999).

2.2.2 LOW TEMPERATURE

It is universally known, that there is a strong relationship between chemical reactions rates and temperature. Probably the major low-temperature adaptive feature in psychrophilic microorganisms to compensate for reduced reaction rates is at the level of the catalytic efficiency of cold adapted enzymes (GERDAY *et al.* 1997). Comparative graphs of two homologous enzymes from an Antarctic and a mesophilic strain of the bacterium *Pseudomonas aeruginosa* are given in the Fig. 3.

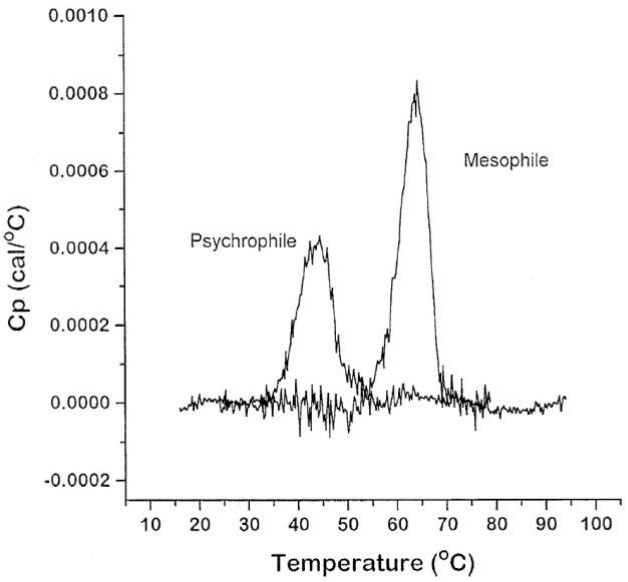


Fig. 3. Heat capacity of unfolding as a function of temperature of the alkaline Ca^{2+}–Zn^{2+} protease from the Antarctic strain *Pseudomonas aeruginosa* compared to that of the homologous enzyme from the mesophile *P. aeruginosa* IF03455. Enzyme concentration is 2 mg·ml^{-1} in 20 mM Tris, 10 mM $CaCl_2$ at pH 8. Temperature gradient 1 °C·min^{-1} (GERDAY *et al.* 1997).

Temperature dependence and thermolability of two enzymes (nitrate reductase and argininosuccinate lyase) from an Antarctic green alga belonging to the *Chloromonas* genus were investigated and compared to the corresponding enzyme activities in the mesophilic species *Chlamydomonas reinhardtii* Dangeard. Maximal activity of both psychrophilic enzymes showed in lower temperatures. Argininosuccinate lyase showed 25 % of its maximum activity at 5 °C, while the mesophilic enzyme was completely inactive. Furthermore, both psychrophilic enzymes were more sensitive to heat than the corresponding mesophilic enzymes (LOPPES *et al.* 1996). Comparable enzyme activity is supposed to occur (due to inhabiting low temperature regions) in diatoms as well.

2.2.3 FREEZING SURVIVAL

After decades of research freezing survival mechanisms are still not fully understood. However, there are some studies of universal survival strategies of microalgae from sea ice, little is known about freshwater diatoms.

THE GENERAL EFFECTS OF FREEZING

The major result of low temperatures on any system is the slowdown in molecular motion. The cooling of cells below 0 °C brings dramatic changes. At temperatures below

–0.6 °C, biological water under isotonic conditions becomes thermodynamically unstable and tent to the crystalline state (KARLSSON & TONER 1996). Intracellular freezing is almost always associated with cell injuries, often resulting in irreversible or lethal cell damages.

Cells and surroundings could remain liquid even when temperatures fall down to –5 °C due to the process of supercooling (a state when liquids do not become solid even below their freezing point) and also because of protective solutes that are frequently present and decrease the point of freezing (MAZUR 1984). With a decreasing temperature ice begins to form in the external space from one or a small number of nucleation sites. As ice develops, the extracellular solutes are concentrated and create an osmotic gradient across the cell membrane (MERYMAN 1974) which provides a driving force for water loss from the cell. Probably the plasma membrane also blocks the growth of ice crystals into the cytoplasm and the cell contents still remain unfrozen (MAZUR 1984).

The rate of water loss is limited by the permeability of membranes. When the rate of water removal is not enough to maintain osmotic stability between the cytoplasm and the liquid part of the extracellular space, the cytoplasm is supercooled, can freeze and intracellular ice is formed (MULDREW & MCGANN 1994). According to the freezing conditions intracellular ice formation is catalysed by plasma membrane or by the internal particles. It is suggested that the occurrence of extracellular ice transforms the plasma membrane through its mechanical, thermal, electrical, ionic and chemical effect (TONER *et al.* 1990). In literature there are described many universal models for intracellular ice formation and its growth (TONER *et al.* 1990, ZHAO *et al.* 2006).

Cell damages during the freezing can occur because of a number of different reasons (MERYMAN 1974, KARLSSON & TONER 1996). For example high concentration of electrolytes and other solutes in the extracellular space induce osmotic activity of cells, which results in dehydration through the water loss (MAZUR *et al.* 1972, MULDREW & MCGANN 1990).

EXTRACELLULAR RESPONSE

Under freezing conditions it is very important for phototrophic microorganisms to maintain an aqueous external environment. Therefore microalgae are able to produce

extracellular macromolecular substances that can provide such environment. Typical effects of these substances is lowering the freezing point of a solution below the thawing point (thermal hysteresis) and the inhibition of recrystallization, i.e. of the process of grain boundary migration by which large crystals grow at the expenses of small ones (BAYER-GIRALDI *et al.* 2011).

Mechanisms such as secretion of **ice-binding proteins (IBPs)** to interfere with the growth of ice have been found in all unicellular algal populations of Antarctic and Arctic sea ice diatoms that have been studied so far. Since such molecules have not been found in non-polar diatoms and occurs in cold or icy environments, its function is probably related with protection of cells from freezing damage. For example a unicellular alga belonging to chlamydomonads isolated from an Antarctic intertidal location excreted IBPs proving ability to change ice channel structure and slow the drainage of brine from sea ice and exhibited a strong recrystallization inhibition activity. Its IBPs showed no resemblance to higher plant antifreezes (RAYMOND *et al.* 2009). An investigation of cDNA sequences of two sea ice diatom IBPs (from *Navicula glaciei* Vanheurk and *Fragilariopsis cylindrus* Grunow) also showed their different structure in comparison with fish, insect and plant IBPs. These are more closely related to fungal origin than to any other (JANECH *et al.* 2006). Furthermore IBPs sequences from a freshwater isolate of *Chlamydomonas raudensis* Ettl from Antarctica most closely matched IBPs excreted by the bacterium *Stigmatella aurantiaca*. Additionally, a mesophilic sister species, *Ch. raudensis* isolated from the Czech Republic showed no ice-binding activity. These results confirm that algal IBPs are essential for survival in icy environments and separate origins of IBPs suggest possibility of horizontal gene transport (RAYMOND & MORGAN-KISS 2013).

Antifreeze proteins (AFPs) is another group of proteins defined by their ability to bind to ice and influence its growth, they are expressed in vertebrates, invertebrates, plants, bacteria, and fungi (EWART *et al.* 1999, VENKETESH & DAYANANDA 2008). AFPs were also found in the two dominant psychrophilic diatom species *Fragilariopsis cylindrus* and *F. curta* (Van Heurck) Hustedt, who are able to grow and successfully divide below freezing temperature and above average salinity of sea water (BAYER-GIRALDI *et al.* 2010). Analyses of the multigene family of AFP genes found in *F. cylindrus* showed a decrease of a freezing point. The protein also caused strong inhibition of recrystallization. Observations of single ice crystals morphology

modifications and pitting activity suggested binding of AFPs to various appearances of the ice crystals (BAYER-GIRALDI *et al.* 2011). A rapid response to freezing stress and the antifreeze activity was also demonstrated on another Antarctic marine diatom *Chaetoceros neogracile* S. L. Van Landingham. The ice crystal shape changed to an elongated hexagonal shape in the presence of the AFP, while single ice crystal showed a circular disk shape in its absence. Moreover this analysis demonstrated a massive accumulation of protein transcripts when exposed to freezing stress (GWAK *et al.* 2010).

Similar survival mechanism related with extracellular proteins that also strongly interact with ice and modify the shape of growing ice crystals is production of **ice-active substances (IASs)**. They are also associated with many if not all, sea ice diatoms communities (RAYMOND 2000). Neither these molecules have been found in non-polar diatoms, which signifies its cryoprotective function. Some of the studies suggest a strong ability of IASs to inhibit recrystallization through a specific interaction with ice (RAYMOND & FRITSEN 2001, RAYMOND & KNIGHT 2003). Macromolecular substances causing pit modifications of growing ice crystals like IASs were also found to be linked to cyanobacterial mats, eukaryotic algae and mosses from freshwater habitats in Ross Island and the McMurdo Dry Valleys, Antarctica (RAYMOND & FRITSEN 2001).

Exopolymeric substances (EPS) are gelatinous extracellular polymeric products of phototrophic microorganisms composed of polysaccharides, amino acids and proteins (KREMBS *et al.* 2002). Many benthic pennate diatoms are able to secrete high amounts of these substances even under nutrient limitation and during absence of light (SMITH & UNDERWOOD 1998). EPSs are produced by various microalgae within sea ice. High concentrations of EPSs in the brine of such microhabitats play very important cryoprotective and buffering role in survivability of microorganisms during severe environments especially in winter conditions when the potential ice-crystal damage is high (KREMBS *et al.* 2002). EPSs interact with the ice – by clogging the brine channel network, changing the viscosity of the brine or directly associating with the ice crystals. The presence of EPSs improves survivability of sea ice algae by increasing the volume of the inhabitable liquid phase and the inner ice-liquid surface area, which has been demonstrated on sea ice diatom *Melosira arctica* var. *krembsii* I. Kaczmarska & R. Jahn. Analyses of the artificial ice containing this diatom EPSs revealed very rich variability in morphological structure of ice pores comparable with natural EPSs-rich coastal sea ice,

but EPSs-free ice (control) showed much simpler pore geometries (KREMBS *et al.* 2011). Secretion of EPSs provides effective protection to diatom cells against the severe environmental conditions of the season. Substances seem responsible for the creation and preservation of inhabitable spaces and also the positioning of the cell within the pore as it is shown in the microphotograph below (Fig. 4). EPSs effects on ice and pore microstructure improve sea ice habitability and survivability (KREMBS *et al.* 2002).

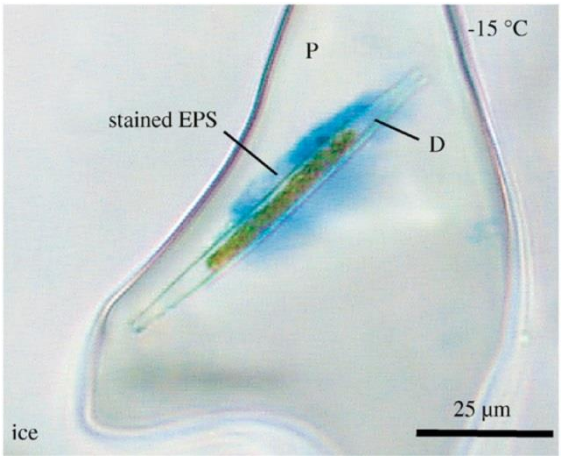


Fig. 4. Microphotograph of pennate diatom (D) residing within pore spaces (P) at a depth of 112 cm in an ice core surrounded by an EPS matrix successfully stained with Alcian blue (KREMBS *et al.* 2002).

EPS can also interact with other extracellular macromolecules, most relevantly with proteins (BAYER-GIRALDI *et al.* 2011), but such interactions under *in situ* conditions relevant to microbial life in sea ice remain largely unexplored.

INTRACELLULAR RESPONSE

One of the intracellular responses to temperature downshift is induction of **cold shock proteins (CSPs)**. They belong to a family of small, single-stranded nucleic acid binding proteins that are involved in various cellular processes including transcription, translation, protein folding and the regulation of membrane fluidity. Not only psychrophilic organisms synthesize cold shock proteins, but also mesophiles and thermophiles are able to react this way when temperature decreases (PHADTARE 2004). Studies of this type of responses in microalgae are still at their beginning, but there were observed some similarities between the CSPs and heat shock proteins that produce mesophiles (D'AMICO 2006). Some of studies suggest induction of CSPs in *F. cylindrus* a model diatom for algal adaptation to polar marine conditions (MOCK & VALENTIN 2004).

Late embryogenesis abundant (LEA) proteins are also considered multi-faceted cryoprotectants believed to work through stabilizing effects on enzymes. Two genes of Antarctic green microalga *Chlorella vulgaris* Beyerinck were described to encode products very similar to LEA proteins from plants. These two new proteins act as cryoprotectants and protect lactate dehydrogenase activity from freeze inactivation. This study suggests that different LEA proteins could protect different cellular compartments from freezing harms (LIU *et al.* 2011).

The ability to adapt to osmotic fluctuations in the cold environment is essential for survival as well. Microorganisms developed a number of strategies to deal with changes related to freezing, for instance there is a wide range of **compatible solutes.** Green algae are known to accumulate sucrose, prolin (GREENWAY & SETTER 1979), betaines (BLUNDEN *et al.* 1992), dimethylsulfide (DMS) (KELLER *et al.* 1989, BLUNDEN *et al.* 1992, LISS *et al.* 1997) et cetera. Analysis of osmoregulation network in response to hyperosmotic stress of cyanobacteria *Synechococcus* sp. showed glycine betaine as its major osmolyte. As minor osmolytes were present glucosylglycerol, glucosylglycerate, trehalose, sucrose and arginine (MAO *et al.* 2010). Diatoms are known to accumulate compatible solutes as well. The amino acid proline is abundant in many cryophilic microalgae, including the model ice diatom *F. cylindrus* (KRELL 2006). A compatible solute with important feedbacks in climate and biogeochemical cycles, dimethylsulfoniopropionate (DMSP) occurs in higher concentrations in polar marine diatom communities in contrast to low levels characteristic for temperate diatoms (KELLER *et al.* 1989).

Membrane fluidity is very important for cell transport and therefore solidification of phospholipid bilayer at freezing temperatures could be very dangerous. One of the documented cold tolerance mechanisms is increasing of **polyunsaturated fatty acids (PUFAs)** concentration. This has been detected in many low-temperature-adapted phototrophic microorganisms. It has been also shown in natural communities and isolated cultures of polar marine diatoms (e.g. NICHOLS *et al.* 1993, HENDERSON *et al.* 1998, TEOH *et al.* 2004). Comparison of strains from polar, temperate and tropical habitats revealed, that only the Antarctic strain out of the three *Chlamydomonas* strains produced predominantly PUFA. And in the Antarctic diatom *Navicula glaciei* the percentage of PUFA decreased with increasing temperature (TEOH *et al.* 2013).

Membrane fluidity maintenance is also essential to assure photosynthetic reactions. Special attention is given to lipids as structural modulators of photosystem II efficiency as well as regulators of energy flow. Desaturation of chloroplast membrane lipids appeared to be one algal universal mechanism to overcome light limitation (MOCK & KROON 2002).

RESTING STAGES FORMATION

Resting stages formation is reported in many groups of phototrophic organisms including cyanobacteria (e.g. SIMON 1977, SUTHERLAND *et al.* 1979) and green algae (e.g. REBOUD & BELL 1997, SIMONS *et al.* 1982). Resting stages are typically formed to survive unfavourable conditions. They are characterized by reduced metabolic activity and different morphological structure – changes of cellular components leading to a resting state that requires the consumption of small amounts of cellular carbon to survive (KUWATA *et al.* 1993).

Only some of diatom species are known to be able to form morphologically different resting stages – mainly reported in marine centric diatoms made to survive winter conditions, their existence in pennate diatom flora is rare (MCQUOID & HOBSON 1996). Under nutrient depletion a centric marine diatom *Chaetoceros pseudocurvisetus* Mangin is known to create two life forms: morphologically different resting spores and vegetative-looking resting cells (KUWATA & TAKAHASHI 1999). Survival experiments of natural microalgal autumn community from Arctic sea ice proved resting spore formation during the dark period in less than 4.5 % of all observed cells – for the only diatom *Chaetoceros* sp. and for some dinoflagellates. Results demonstrate the ability of algae to survive long periods of darkness as adapted vegetative cells and also ability to respond to increased light levels within days (ZHANG *et al.* 1998). Furthermore a similar study on polar and temperate diatoms isolated from the Southern Ocean showed that even sudden darkness did not induce diatom resting spore formation. All of the examined species survived treatments as their vegetative cells (PETERS 1996, PETERS & THOMAS 1996). It is considered, most of diatom species are not able to form morphologically different resting stages and survive as adapted cells morphologically identical to vegetative ones. Induction of these stages is associated with decreasing temperatures related with reductions in photosynthesis and carbon fixation genes in *F. cylindrus* in 5 °C to –1.8 °C. This is hypothesized to indicate a preparation for a winter

season (MOCK & VALENTIN 2004) and proves the importance of morphologically identical resting cells in diatom survival success.

It has been suggested that resting stages formation in terrestrial diatoms might be important for survival of freezing and desiccation and indicate specific adaptations of terrestrial diatoms to their variable habitats (SOUFFREAU *et al.* 2013a). Vegetative cells of terrestrial and benthic freshwater diatoms were shown to be highly sensitive to desiccation, freezing and abrupt heating. This may explain the widespread endemism observed in freshwater diatoms (SOUFFREAU *et al.* 2010).

3. MATERIAL AND METHODS

3.1 CULTURES AND CULTIVATION OF STRAINS

3.1.1 SAMPLE COLLECTION AND STRAIN ISOLATION

Natural samples of terrestrial habitats (soil) and benthos from different aquatic habitats (lakes, streams, seepages) were collected in plastic tubes during the 2014 summer seasons in both Polar Regions: the Arctic (Spitsbergen) and the Maritime Antarctica (James Ross Island and Vega Island). Freshwater samples were collected by scraping off epilithon from five to ten stones originating from the littoral zone of the water body. For the terrestrial samples, the upper 2 cm of soil were collected using a spoon. All material was transferred to a falcon tube and stored at cool (< 10 °C) and dark conditions during transport.

Upon arrival in the lab, small quantities of the natural material were incubated in the WC medium (GUILLARD & LORENZEN 1972) in 15 °C, a 12:12h light:dark period and a light intensity of about 20 µmol photons $m^{-2} \cdot s^{-1}$. Single diatom cells were isolated from the natural samples under an inverted microscope Nikon Diaphot 200 (Nikon Corporation, Japan) using a needle or a glass micropipette. About two hundred monoclonal cultures from 16 Antarctic and 10 Arctic localities were established at the Department of Ecology (Faculty of Science, Charles University in Prague) within the diploma project. The list of all isolated strains is presented in Appendix 1.

3.1.2 STRAIN DETERMINATION

Samples for light microscopy determination were prepared by oxidation of diatom cultures with 69% nitric acid in a 60 °C drying stove for one week. The oxidized material was then eight times washed with distilled water to remove all acid. The sedimentation of oxidized cells followed overnight between each washing procedure.

Microscopic slides with oxidized cultures embedded in Naphrax (Brunel Microscopes Ltd, UK) were examined by light microscopy using an Olympus BX51 microscope (Olympus Corporation, Japan), equipped with Nomarski or differential interference contrast (DIC). Pictures were taken using an Olympus BX43 microscope (Olympus Corporation, Japan).

Scanning electron microscopy (SEM) was also performed. Oxidized materials were poured on aluminium stubs and air-dried. When necessary to remove inorganic pollution in the cultures, samples were first filtered through a polycarbonate filter with a pore diameter of 2 μm and fixed on aluminium stubs after air-drying. The stubs were coated with a 50 nm gold layer (Bal-tec SCD 050, Lichtenstein) and studied using a JEOL JSM-6380LV scanning electron microscope (JEOL USA Inc., USA) at 20–30 kV.

Strain identification was based on publications listed in Appendix 2. A strain of chain forming *Pinnularia* (P3) was described as a new species (*P. catenaborealis*), see Appendix 3.

3.1.3 LIST OF EXPERIMENTAL STRAINS

Diatom strains for the experiments were chosen from the working collection established at the Department of Ecology (DECU, see above) and from the collection at the Laboratory of Protistology and Aquatic Ecology (PAE, Department of Biology, Faculty of Science, Ghent University, Belgium). Strains showing good performance from various genera were selected (Table 1). Additional strains were acquired from the Belgian Co-ordinated Collections of Micro-organisms (BCCM) and the Culture Collection of Cryophilic Algae (CCCryo). In total 26 polar and temperate strains representing species from both terrestrial and freshwater habitats were used for the experiments. An overview of the diatom strains used for freezing experiments, their origin and habitat is given in Table 1. Pictures of experimental strains taken using scanning electron and light microscopy are presented in Figs 5–6. The pictures are missing for three strains: MAR 1-7 (*Achnanthes coarctata*), MAR 14B-5 (*Gomphonema* sp.) and MAR 3B-2 (*Chamaepinnularia krookiformis*) because these strains died and no material was available to make a sample for scanning electron microscopy.

Table 1. Overview of the diatom strains used in the experiments. * Habitat: FP freshwater polar, FT freshwater temperate, TT terrestrial temperate, TP terrestrial polar, ** Freezing treatment: see chapter 3.2 'Experimental setup'.

Species	Strain	Collection	Habitat*	Origin of sample	GPS	Date of collection	Freezing treatment**		
							1	2	3–6
Achnanthes coarctata (Brébisson ex W.Smith) Grunow	MAR 1-7	PAE	FP	Antarctica, Vega Island, pool	63°52'56.5"S 57°31'45.6"W	February 2014	x		
Achnanthidium minutissimum (Kützing) Czarnecki	SPITS13-GAR MOSS 3	PAE	FP	Spitsbergen, Billefjorden, Garmaksla Lake	78°36'16.0"N 16°20'14.7"E	July 2013	x	x	x
Cymbopleura angustata (W.Smith) Krammer	SP14/M005-2	PAE	FP	Spitsbergen, Billefjorden, Mathiessondalen, lake 1	78°34'52.2"N 16°33'00.0"E	August 2014	x		x
Encyonema silesiacum (Bleisch) D.G.Mann	DAMLV 11	PAE	FT	Belgium, Oost-Vlaanderen, Damvallei Nature Reserve, lake	51°03'03.2"N 3°48'44.0"E	March 2015	x		x
Eucocconeis flexella (Kützing) Meister	SP14/M014-7	PAE	FP	Spitsbergen, Mimerdalen, stream	78°36'07.2"N 15°54'09.7"E	August 2014	x		x
Eunotia ambivalens Lange-Bertalot & Tagliaventi	DM 33-8	BCCM	FT	Belgium, Genk, freshwater pond	50°56'00.0"N 5°27'00.0"E	June 2003	x	x	x
Gomphonema acuminatum Ehrenberg	DAMLV 3	PAE	FT	Belgium, Oost-Vlaanderen, Damvallei Nature Reserve, lake	51°03'03.2"N 3°48'44.0"E	March 2015	x	x	x
Gomphonema sp.	G4	DECU	FP	Maritime Antarctica, James Ross Island, Black Lake	63°57'56.9"S 57°52'59.2"W	February 2014			x
Gomphonema sp.	MAR 14B-5	PAE	FP	Antarctica, Vega Island, moraine lake	63°51'45.0"S 57°37'17.6"W	February 2014	x		
Hantzschia abundans Lange-Bertalot	SPITS13 MAT 1-7	PAE	FP	Spitsbergen, Billefjorden, Mathiessondalen, lake 1	78°34'52.2"N 16°33'00.0"E	July 2013	x	x	x
Chamaepinnularia krookiformis (Krammer) Lange-Bertalot & Krammer apud H.Lange.Bertalot & S.I.Genkal	P24	DECU	FP	Antarctica, James Ross Island, Lachman Lake 1	63°47'34.9"S 57°48'13.8"W	February 2014			x
Chamaepinnularia krookiformis (Krammer) Lange-Bertalot & Krammer apud H.Lange.Bertalot & S.I.Genkal	MAR 3B-2	PAE	FP	Antarctica, Vega Island, stream	63°53'55.7"S 57°36'48.0"W	February 2014	x		

Table 1. (continued)

Species	Strain	Collection	Habitat*	Origin of sample	GPS	Date of collection	Freezing treatment**		
							1	2	3–6
Mayamaea atomus (Kützing) Lange-Bertalot	E16-5	DECU	FP	Antarctica, Vega Island, stream	63°53'55.7"S 57°36'48.0"W	February 2014	x		x
Navicula cryptocephala Kützing	DAMLV 17	PAE	FT	Belgium, Oost-Vlaanderen, Damvallei Nature Reserve, lake	51°03'03.2"N 3°48'44.0"E	March 2015	x		x
Navicula radiosa Kützing	SP14/M003-3	PAE	FP	Spitsbergen, Svenbreen, stream	78°43'47.9"N 16°24'40.4"E	August 2014	x	x	x
Nitzschia cf. *fonticola*	(CLONE A) F1-1	BCCM	FT	Spain, Ter river	41°59'00.0"N 2°48'00.0"W	July 2004	x		x
Nitzschia cf. *paleacea*	S28-5	DECU	FP	Antarctica, James Ross Island, Black Lake	63°57'56.9"S 57°52'59.2"W	February 2014	x		x
Nitzschia kleinteichiana sp. nov.	MAR 113-15	PAE	FP	Antarctica, Vega Island, lake	63°52'31.3"S 57°36'51.4"W	February 2014	x		x
Nitzschia sp.	(02) 9E	BCCM	FT	Belgium, WWTP Destelbergen, secondary clarifier	51°03'00.0"N 03°48'00.0"E	July 2005	x		x
Pinnularia australomicrostauron R.Zidarova, K.Kopalová & B.Van de Vijver	MAR 1-14	PAE	FP	Antarctica, Vega Island, pool	63°52'56.5"S 57°31'45.6"W	February 2014	x		x
Pinnularia borealis Ehrenberg complex	P3	DECU	FP	Antarctica, James Ross Island, Black Lake	63°57'56.9"S 57°52'59.2"W	February 2014	x		x
Pinnularia borealis Ehrenberg complex	SP14/T037-48A	PAE	TP	Spitsbergen, Longyearbyen, soil	78°12'56.5"N 15°37'35.0"E	August 2014	x		x
Pinnularia borealis Ehrenberg complex	RO15/1-12	PAE	TT	Italy, Apennine Moutains, soil	41°55'12.6"N 12°55'57.3"E	August 2015			x
Pinnularia borealis Ehrenberg complex	BE14/E03-3	PAE	TT	Belgium, Essen, soil	51°27'33.8"N 04°28'42.2"E	November 2014			x
Pinnularia subrostrata (A.Cleve) Cleve-Euler	PP	CCCryo	FP	Antarctica, King George Island, snow field	62°10'59.8"S 58°54'52.6"W	February 2006			x
Sellaphora pupula (Kützing) Mereschkovsky	(Pd 126xMf 03) F1-3	BCCM	FT	Progeny of the following cross: Australia, Victoria, Purrumbete Lake x Belgium, Merelbeke pond	38°17′00.0"S 143°13′00.0"E x 51°00'00.0"N 3°75'00.0"E	January 2007 x December 2005	x	x	x

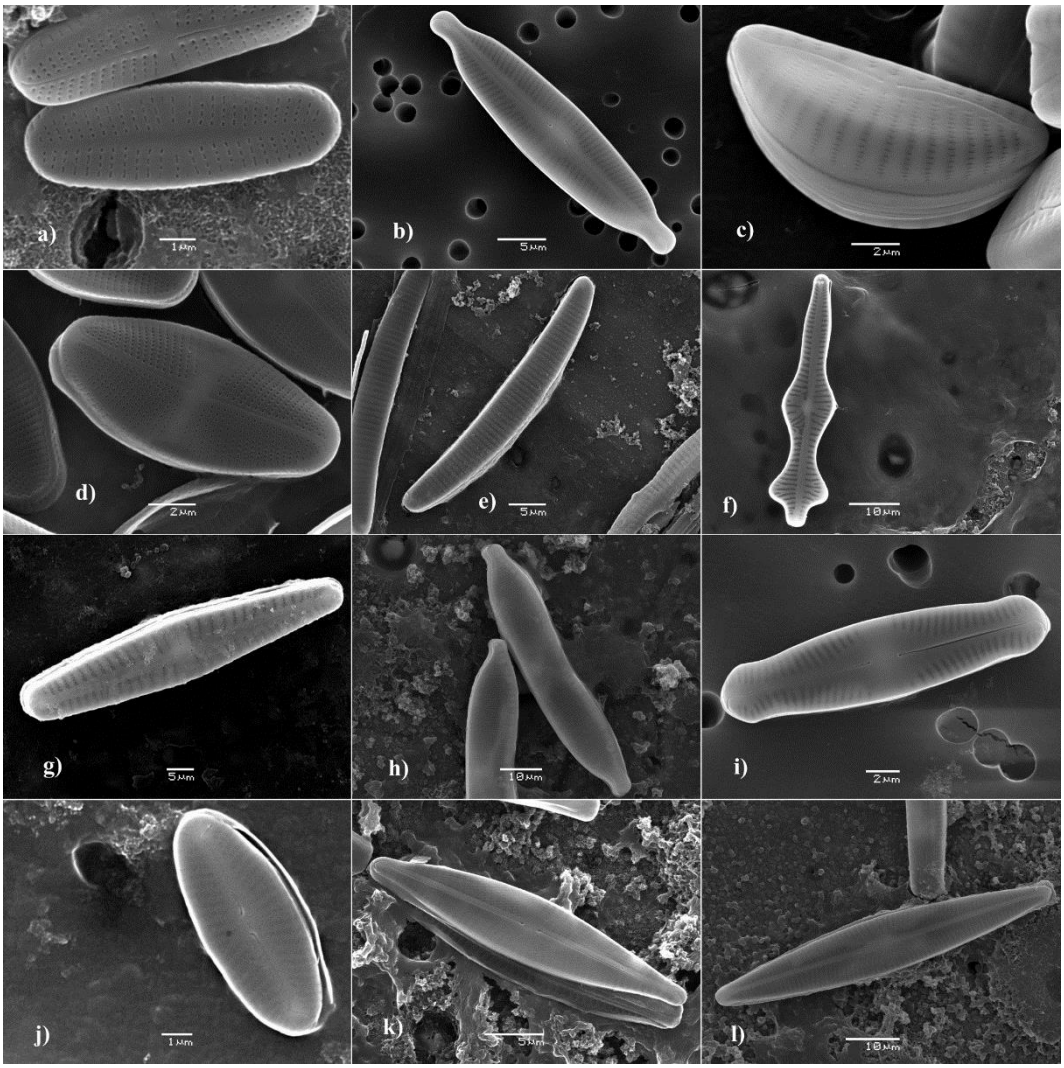


Fig. 5. SEM pictures of the selected strains used in the experiments.

a) *Achnanthidium minutissimum* complex	SPITS13-GAR MOSS 3
b) *Cymbopleura angustata*	SP14/M005-2
c) *Encyonema silesiacum*	DAMLV 11
d) *Eucocconeis flexella*	SP14/M014-7
e) *Eunotia ambivalens*	DM 33-8
f) *Gomphonema acuminatum*	DAMLV 3
g) *Gomphonema* sp.	G4
h) *Hantzschia abundans*	SPITS13 MAT 1-7
i) *Chamaepinnularia krookiformis*	P24
j) *Mayamaea atomus*	E16-5
k) *Navicula cryptocephala*	DAMLV 17
l) *Navicula radiosa*	SP14/M003-3

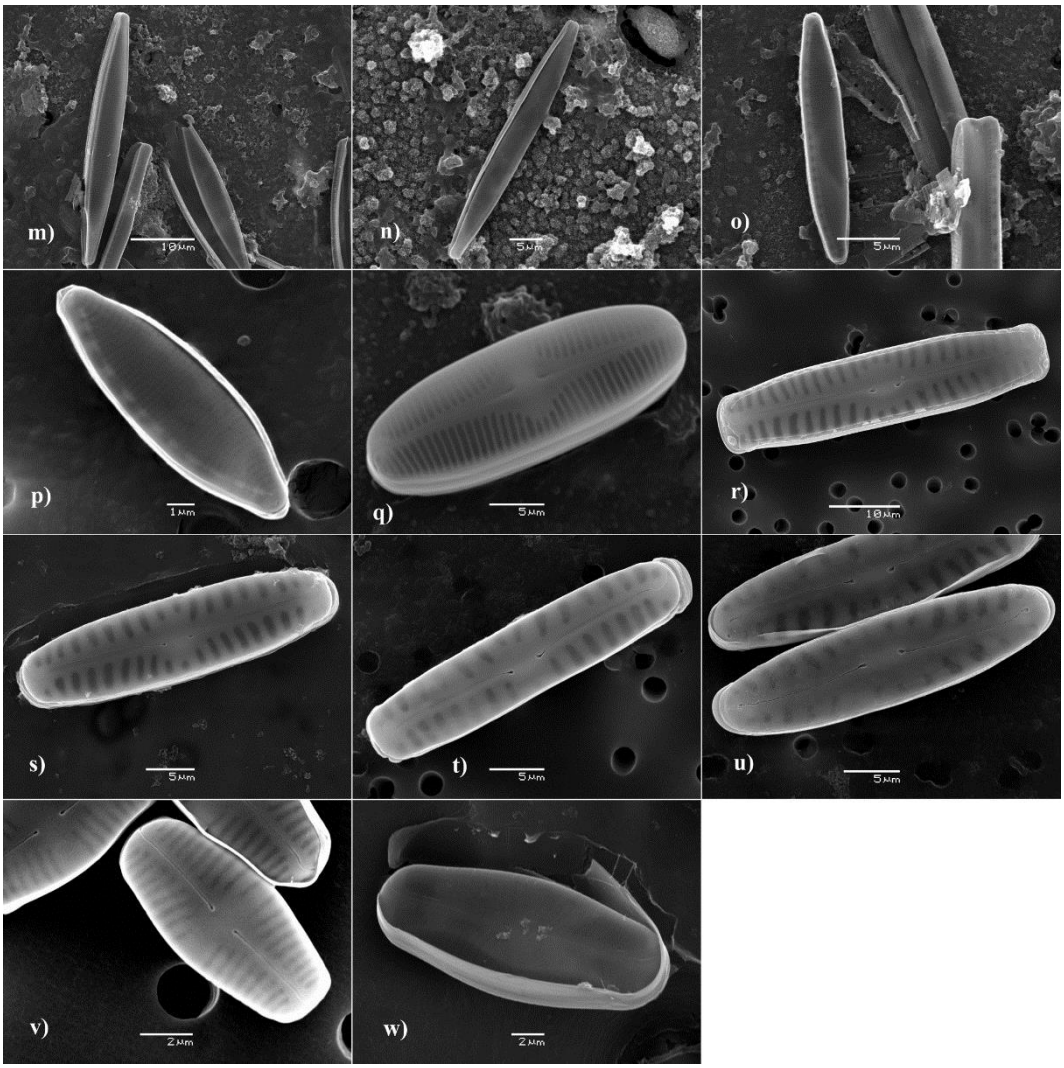


Fig. 5. (continued)

m) *Nitzschia* cf. *fonticola* (CLONE A) F1-1
n) *Nitzschia* cf. *paleacea* S28-5
o) *Nitzschia kleinteichiana* MAR 113-15
p) *Nitzschia* sp. (02) 9E
q) *Pinnularia australomicrostauron* MAR 1-14
r) *Pinnularia borealis* complex P3
s) *Pinnularia borealis* complex SP14/T037-48A
t) *Pinnularia borealis* complex RO15/1-12
u) *Pinnularia borealis* complex BE14/E03-3
v) *Pinnularia subrostrata* PP
w) *Sellaphora pupula* (Pd 126xMf 03) F1-3

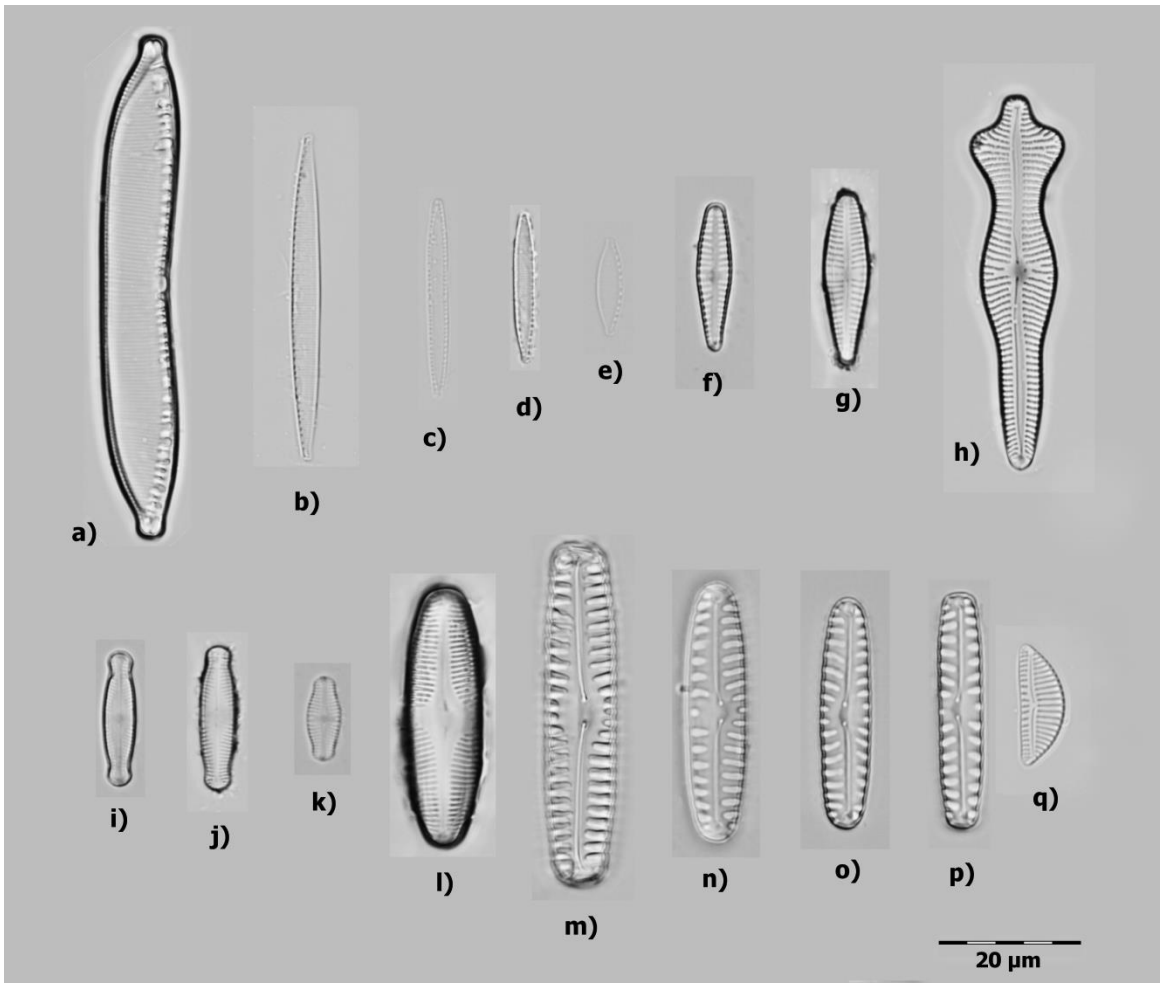


Fig. 6. Light microscopy pictures of the selected strains used in the experiments.

a)	*Hantzschia abundans*	SPITS13 MAT 1-7
b)	*Nitzschia* cf. *fonticola*	(CLONE A) F1-1
c)	*Nitzschia* cf. *paleacea*	S28-5
d)	*Nitzschia kleinteichiana*	MAR 113-15
e)	*Nitzschia* sp.	(02) 9E
f)	*Gomphonema* sp.	G4
g)	*Gomphonem*a sp.	MAR 14B-5
h)	*Gomphonema acuminatum*	DAMLV 3
i)	*Chamaepinnularia krookiformis*	P24
j)	*Chamaepinnularia krookiformis*	MAR 3B-2
k)	*Pinnularia subrostrata*	PP
l)	*Pinnularia australomicrostauron*	MAR 1-14
m)	*Pinnularia borealis* complex	P3
n)	*Pinnularia borealis* complex	SP14/T037-48A
o)	*Pinnularia borealis* complex	BE14/E03-3
p)	*Pinnularia borealis* complex	RO15/1-12
q)	*Encyonema silesiacum*	DAMLV 11

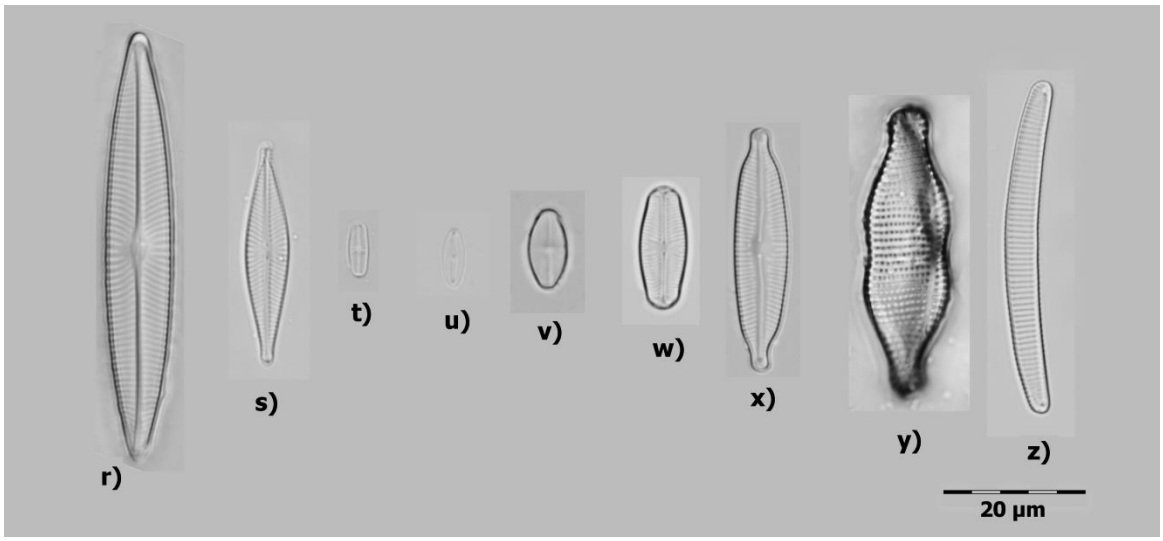


Fig. 6. (continued)

r)	*Navicula radiosa*	SP14/M003-3
s)	*Navicula cryptocephala*	DAMLV 17
t)	*Achnanthidium minutissimum* complex	SPITS13-GAR MOSS 3
u)	*Mayamaea atomus*	E16-5
v)	*Eucocconeis flexella*	SP14/M014-7
w)	*Sellaphora pupula*	(Pd 126xMf 03) F1-3
x)	*Cymbopleura angustata*	SP14/M005-2
y)	*Achnanthes coarctata*	MAR 1-7
z)	*Eunotia ambivalens*	DM 33-8

3.2 EXPERIMENTAL SETUP

Several eco-physiological experiments were performed and the survivability of polar and temperate diatoms in different freezing treatments, including those simulating natural conditions was experimentally tested. The experiments were performed at two independent institutions: Part I: Laboratory of Protistology and Aquatic Ecology, Department of Biology, Faculty of Science, Ghent University, Belgium, and Part II: Institute of Botany, Academy of Sciences of the Czech Republic, Třeboň.

The experiments consisted of six different freezing treatments to which the vegetative and resting cells of the strains were exposed:

Part I:

1. abrupt freezing to –20 °C followed by slow thawing;
2. cryopreservation – abrupt freezing to –180 °C using the cryoprotective substance DMSO followed by fast thawing;

Part II:

3. continuous freezing to –4 °C followed by fast thawing;

4. continuous freezing to –20 °C followed by fast thawing;
5. continuous freezing to –40 °C followed by fast thawing;
6. abrupt freezing to –180 °C followed by fast thawing.

3.3 EXPERIMENTAL PART I

3.3.1 STRAIN PREPARATION

All strains were grown in a special cooled room in 18 °C, a 12:12h light:dark period, using WC medium and frequently reinoculated when reaching the late exponential phase. Before the start of the experiments, all strains were inoculated in 6-well plates and moved to the to the experimental shelf (Fig. 7), where the light intensity varied between 27 and 30 μmol $m^{-2} \cdot s^{-1}$ with a light:dark period of 12:12h and a temperature of 18 °C for all experiments. During the experiments, the position of the well plates was frequently changed to ensure that position specific effects were ruled out.

Fig. 7. Experimental shelf (Part I).

GROWTH CURVES

Prior to the experiments, growth curves of the strains in experimental conditions were determined (Fig. 8). Culture densities were estimated using their basal fluorescence (F_0) determined by pulse amplitude modulated (PAM) fluorescence using a Walz MAXI Imaging-PAM, M-Series (Heinz Walz GmbH, Germany). Cell densities were measured during eight subsequent days. PAM measurements were carried out using the following settings: intensity 12, frequency 1, gain 6, damping 2, following 15 minutes dark-adaption (CONSALVEY *et al.* 2005). This allowed us to assess the F_0 value at which the cultures became stationary. Upon recalculation, F_0 value of 0.02 was chosen for reinoculation of

all cultures. A four day growth period ensured that all cultures were still in early to mid-exponential phase.

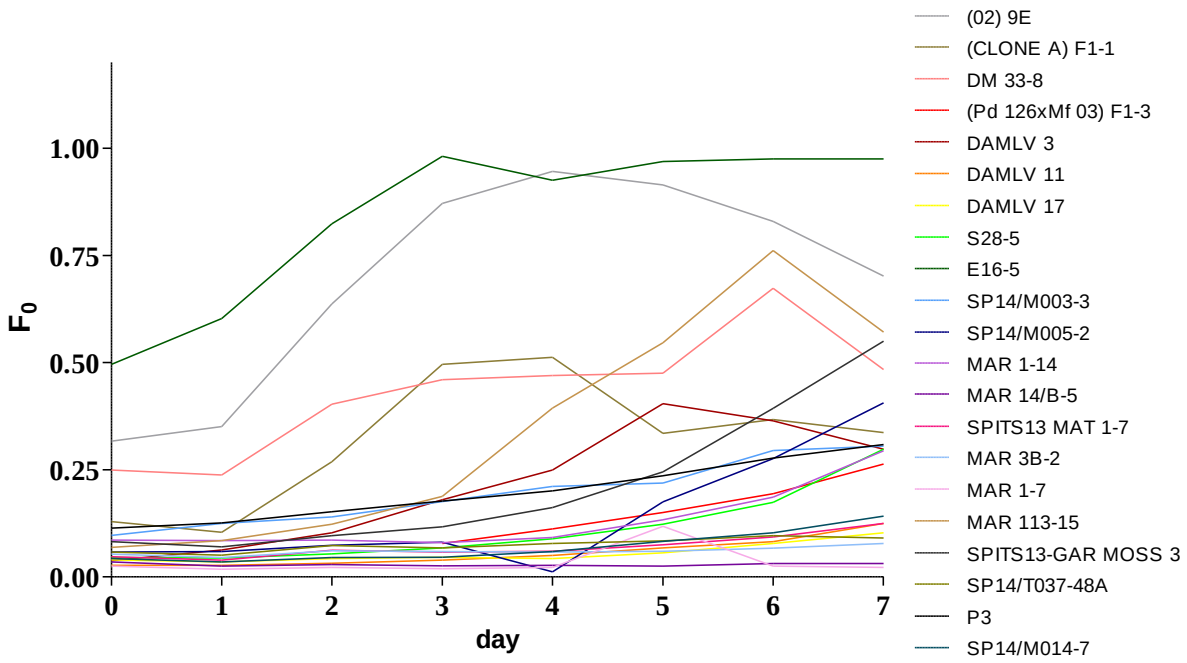


Fig. 8. Examples of growth curves of selected strains.

REINOCULATION

Prior to each reinoculation, the medium in each well was removed and replaced by fresh WC medium. Afterwards PAM measurement was performed using earlier established settings. The diatom biomass in the wells of the original plates was scratched by a micropipette and the cultures were homogenized by pipetting up and down. A certain volume of inoculum (based on the recalculations) was subsequently transferred towards a new well, previously filled with a certain volume of fresh WC medium (to have all the wells with the same volume afterwards). The amount of inoculum differed according to the recalculations for each strain and replica, which was based on the cell density established by autofluorescence measured before each reinoculation.

The 6-well plates containing the experimental strains were already present on the experimental shelf eight days prior to the experiment. Three wells per strain were used as replicates. To standardize physiological conditions, cultures were reinoculated two times prior to the experiment, each time followed by four days of exponential growth on the experimental shelf.

PREPARATION OF RESTING CELLS

Four days after the second reinoculation, the WC medium in a set of wells was replaced by nitrogen free WC medium (WC–N) without $Co(NO_3)_2 \cdot 6H_2O$ and $NaNO_3$ solutions and without pH adjustment or buffer addition. An autofluorescence measurement was performed using earlier established settings. The medium was removed and replaced with nitrogen free WC medium a second time to minimalize the volume of nitrogen. Since we did not expect significant growing rates without nitrogen, all strains were reinoculated at a density of $F_0 = 0.05$ in new plates with WC–N medium. Some of the strains were left in their former wells as their original density was too low to allow for reinoculations. These wells were washed with WC–N medium for a third time to eliminate nitrogen in the medium. All cultures were placed at standard culture conditions on the experimental shelf for seven days to allow the cells to exhaust all nitrogen and become stationary. Stationary phase cells were placed for 14 days in the dark (using two layers of aluminium foil) at 4 °C to induce dormancy (McQuoid & Hobson 1995). After that, cells of all strains had the typical characteristics of resting cells – granular cytoplasm, enlarged vacuoles or oil droplets and contracted chloroplasts (McQuoid & Hobson 1996). These resting cells were used for the experiments.

PREPARATION OF VEGETATIVE CELLS

The same wells used for the second reinoculation of resting cells were used for the preparation of vegetative cells. All the cultures were present on the experimental shelf one week prior to the first reinoculation. Three replicates for each strain were used as well. To standardize physiological conditions, cultures were reinoculated twice at $F_0 = 0.02$, each time followed by four days of exponential growth.

3.3.2 FREEZING EXPERIMENT (TREATMENT 1)

Both vegetative and resting cells were frozen on the same day. Consequently all the strains had to be in the same physiological conditions at the same time. There were 21 strains included in the experiment (Table 1, see column 'Freezing treatment' 1).

Before the start of freezing treatment, the medium of both vegetative and resting cells was changed with fresh WC medium. The experiment was conducted in 12-well plates filled with 5 ml of medium. For each strain three replicates were present in five different 12-well plates. There were two exceptions: the vegetative and resting cells of

Achnanthes coarctata (MAR 1-7) and *Gomphonema* sp. (MAR 14/B-5) were only present in one and two wells respectively, because of too low densities of the original cultures. The experimental plates were prepared as previously described (see 3.3.1 'Strain preparation').

For the abrupt freezing to –20 °C the plates were placed in an ordinary freezer for 12h after which the plates were transferred to the experimental shelf at 18 °C to thaw slowly. To avoid light damage of the cells the plates were covered with aluminium foil. Upon thawing, the medium in all wells was changed with fresh WC medium.

3.3.3 CRYOPRESERVATION EXPERIMENT USING DMSO (TREATMENT 2)

The plates for the experiment were prepared as previously described (see 3.3.1 'Strain preparation'). Because of considerable time constraints in handling time, only six diatom strains were chosen for this experiment (Table 1, see column 'Freezing treatment' 2). The experiment was conducted in 6-well plates filled with 10 ml WC medium. For each strain three replicates were present in three different well plates. The only exception were the resting cells of *Hantzschia abundans* (SPITS13 MAT 1-7), which was present only in two wells as a result of too low cell densities in the original culture. The resting cells were maintained in the dark at 4 °C for 14 days. The vegetative cells were kept in the dark at 18 °C for 24 hours prior to the cryopreservation.

DMSO TEST

Dimethyl sulphoxide (DMSO) is a toxic chemical compound that is commonly used as a cryoprotectant to avoid cell damages during cryopreservation of living material (e.g. BRANDL & DILLER 2004, DAY *et al.* 1997, PARK 2006, RHODES *et al.* 2006). To infer the sensibility of the chosen strains to DMSO, a DMSO sensibility test was performed prior to the cryopreservation experiment.

One ml of 40% DMSO was added to 5 ml well containing 4 ml of culture to reach the final 10% DMSO concentration in the wells. The cells were observed by light microscopy for 30 min and several pictures were taken. When DMSO is added to a culture, the protoplasts of the cells change their structures gradually, until there is hardly any cell content and cell is not able to recover. The influence of DMSO after 25 min of treatment is presented on *Hantzschia abundans* (SPITS13 MAT 1-7) in Fig. 9. According

to the light microscopy observations, five minutes were chosen as the incubation time for the DMSO treatment.

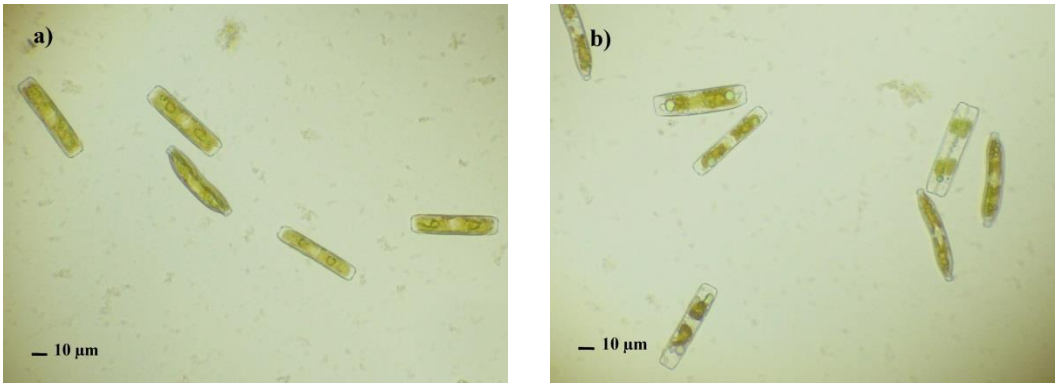


Fig. 9. Influence of DMSO: cells of *Hantzschia abundans* (SPITS13 MAT 1-7) a) in the first minute and b) after 25 minutes of the DMSO treatment.

To test the mortality rate of a five min DMSO treatment all strains were exposed to 10% DMSO for five minutes. For each strain, two replicates were included. After five minutes the medium was removed and the cultures were washed twice with fresh WC medium. The cell densities were subsequently counted (at least 10 randomly chosen microscopic squares per well on average 400 cells per each well, the survival assessment is explained in the chapter 3.3.4 'Survival assessment'). A five minute interval was established as a final uniform period when most of the cells per strain survived the treatment.

CRYOPRESERVATION PROCESS

Both the vegetative and resting cells were cryopreserved on the same day. Therefore all the strains had to be in the same physiological conditions at that time.

Seven ml WC medium were removed from each well. 1.35 ml of each culture was scraped off and transferred to a labelled empty cryogenic vial. A total of 0.45 ml of a 40% DMSO solution was added to the cryogenic vials with cultures, resulting in the final DMSO concentration to equal 10 %. Cultures and DMSO were homogenized by pipetting up and down. The cryogenic vials were transferred into a prechilled Mr. Frosty™ Freezing Container (Thermo Fisher Scientific Inc., USA) and immediately placed into a –80 °C freezer (this complete procedure took less than 5 min), where the vials were left for 90 min. Subsequently the cryogenic vials with cultures were transported to the

CryoScience 10K cryostorage facility (Taylor-Wharton, Worthington Industries, USA), where the temperature varies between –180 and –186 °C.

The procedure was conducted twice in order to process all strains in less than five min. All strains and treatments were randomized in both runs. The cryovials were left in the cryostorage facility with liquid nitrogen for six days.

THAWING PROCESS (DAY 1)

The thawing process was carried out in subdued light to avoid damage of cells. The frozen cryogenic vials were transported as fast as possible to a water bath (±37 °C) prepared in advance. The vials were moved gently for two minutes to ensure continuous thawing. As soon as the ice melted the cryogenic vials were removed from the water bath and the cultures were transferred into 50 ml falcon tubes filled with fresh WC medium. Two technical replicates (A and B) were made for each biological replicates (1, 2 and 3) by subdividing the cultures in the cryovial. Prior to the transfer to the falcons, the cryogenic vials were shaken to homogenize the cultures. To avoid light stress thawed cultures were incubated in darkness in 18 °C overnight.

THAWING PROCESS (DAY 2)

47 ml of the WC medium was removed from the 50 ml falcon tubes and 3 ml of each culture were transferred towards a 24-well plate. The positions of all cultures were randomized within each well plate. In order to avoid position specific effects, biological replicates were never positioned in the same plate.

The plates were placed in an AquaLytic TC Series incubator (AquaLytic, Germany) at 18 °C and a 12:12h light:dark period. To avoid extra stress four different light filters were used to bring the cultures gradually to normal light conditions, which was lower in the incubator than on the experimental shelf (14–15 µmol photons $m^{-2} \cdot s^{-1}$). The medium in all wells was changed with fresh WC medium when all cultures were at standard light conditions.

3.3.4 SURVIVAL ASSESSMENT

When all cultures were again at standard light conditions (at least one hour after complete thawing for treatment 1 and two days which lasted thawing process in treatment 2), cell densities (t_0) were counted using a Zeiss Axiovert 40 C (Carl Zeiss

Microscopy GmbH, Germany) to evaluate the survivability of the strains. Empty silica frustules and cells with cell content were counted separately on a certain surface area. Total cell densities were determined by counting the cells in at least 10 randomly chosen microscopic squares per well (on average 200 cells per well). Dividing cells were counted as one. Representative photos of vegetative and resting cells before and after –20 °C freezing treatment are shown in Fig. 10.

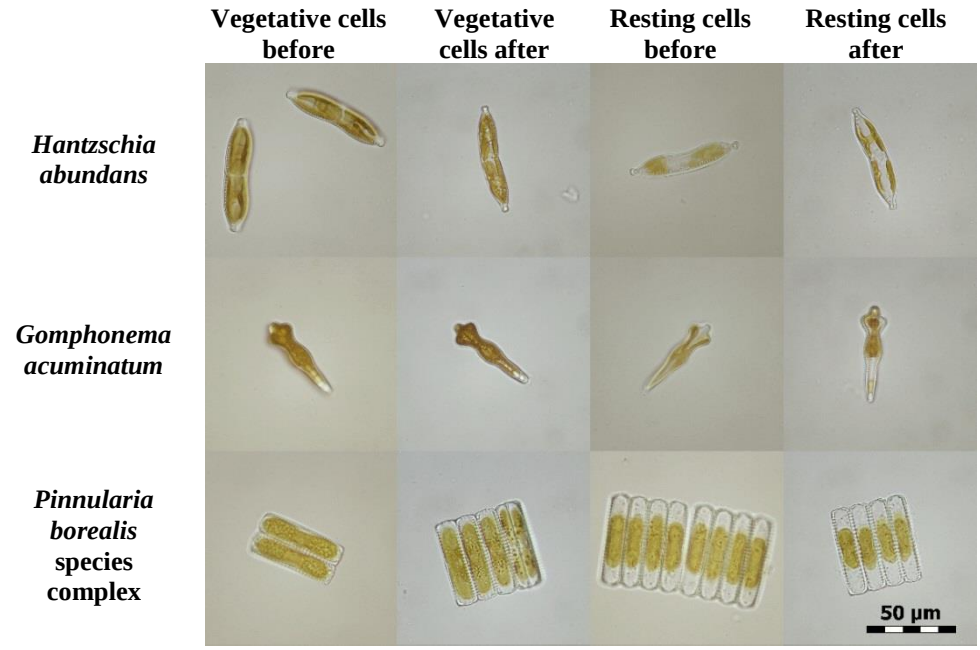


Fig. 10. Vegetative and resting cells before and after –20 °C freezing treatment.

Seven days after the freezing procedure (t_7) dead cells could be distinguished from living cells by their empty silica frustules or shrivelled and colourless cell content and densities of dead cells were determined as previously described.

The viability of cells was counted using the following formula:

$$\% \text{ viable cells} = \frac{a - (b - c)}{a} \text{ x } 100$$

a t_0 density of living cells
b t_7 density of dead cells
c t_0 density of dead cells

A strain was considered as tolerant for a treatment if at least one out of three replicates showed a percentage of viable cells higher than zero.

3.4 EXPERIMENTAL PART II

3.4.1 STRAIN PREPARATION

All strains were prepared the same way as in Experimental Part I as described in the chapter 3.3.1 ‘Strain preparation’. Plates were incubated in a Q-Cell 140 INOX cultivator (PolLab, Poland), where the light intensity varied between 10 and 27 µmol $m^{-2} \cdot s^{-1}$ (depending on the distance from fluorescent tube), the light:dark period was 12:12h and the temperature was set to 18 °C. The picture of the experimental shelf is given in Fig. 11.

Fig. 11. Experimental shelf (Part II).

The autofluorescence measurement for reinoculations was performed after 15 minutes of dark-adaption using a Varioscan™ Flash Multimode Reader (Thermo Fisher Scientific Inc., USA) with default settings: excitation wavelength 440 nm, emission wavelength 685 nm, measurement time 100 ms using average of nine measured points within a well (Fig. 12).

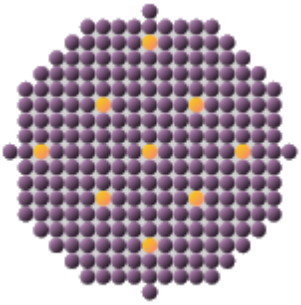


Fig. 12. Image of nine points measured using Varioscan™ Flash Multimode Reader.

Four days after the first reinoculation, the WC medium in the wells was replaced by nitrogen free WC medium without pH adjustment or buffer addition, in which $Co(NO_3)_2 \cdot 6H_2O$ was replaced with $CoCl_2 \cdot 6\ H_2O$ and $NaNO_3$ with NaCl. Cultures were placed in the experimental shelf for five days to allow the cells to exhaust all nitrogen and become stationary and afterward placed for 10 days in the dark to induce resting cells. Vegetative cells were prepared completely in the same way as previously described in the chapter 3.3.1 'Strain preparation'.

For the reinoculation schedule and freezing treatments for both Experimental Parts see Table 2.

Table 2. Schedule of reinoculations and freezing treatments in the experimental Part I and II.

Experimental Part I		**Experimental Part II**	
day 1	Initial inoculation	day 1	Initial inoculation
day 8	Resting cells, 1st reinoculation	day 8	Resting cells, 1st reinoculation
day 12	Resting cells, 2nd reinoculation	day 12	Resting cells, 2nd reinoculation (WC–N medium)
day 16	Resting cells, 3rd reinoculation (WC–N medium)		
day 23	Resting cells, incubation in 4 °C in the dark	day 19	Resting cells, incubation in 4 °C in the dark
day 29	Vegetative cells, 1st reinoculation	day 20	Vegetative cells, 1st reinoculation
day 33	Vegetative cells, 2nd reinoculation	day 24	Vegetative cells, 2nd reinoculation
day 37	Freezing of both the vegetative and resting cells (overnight)	day 28	Freezing of both the vegetative and resting cells (during the day) First counting (t_0)
day 38	First counting (t_0)		
day 45	Second counting (t_7)	day 35	Second counting (t_7)

3.4.2 FREEZING EXPERIMENTS (TREATMENTS 3–6)

There were 23 strains included in the experiments (Table 1, see columns 'Freezing treatments' 3–6) present in three replicates in six different 12-well plates. Before the start of each treatment, the medium of both vegetative cells and resting cells was changed with fresh WC medium.

The continuous freezing treatments (3–5) were carried on in a programmable freezer Planer Kryo 10, Series III (Planer PLC, United Kingdom) (Fig. 13). The plates were exposed to a continuous freezing program simulating natural process, where the starting temperature was 15 °C and the rate of cooling was 0.5 °C·min^{-1} until the required temperature (–4 °C, –20 °C and –40 °C) was reached. In the final temperature

experimental plates were kept in a chamber at the same conditions for an hour. Subsequently the plates were rapidly thawed.

Fig. 13. A programmable freezer Planer Kryo 10, Series III.

For the abrupt freezing to –180 °C (treatment 6), only one ml of fresh WC medium was left in each well during the last washing. Biomass of each well was scratched by a micropipette and transferred to labelled empty 2 ml cryogenic vials. The well was subsequently washed with one ml of fresh WC medium to transport as much biomass as possible. The vials were placed into a MVE XLC 230 liquid nitrogen freezer (Chart MVE Inc., Luxembourg) for 14 days. Subsequently they were thawed fast.

A fast thawing process (treatments 3–6) was carried out in subdued light to avoid cell damages. Immediately after the end of the freezing program (treatments 3–5) or after the removal from liquid nitrogen storage facility (treatment 6), the experimental plates / cryogenic vials were transported to a water bath (±39 °C) prepared in advance. They were moved gently in the water bath during two minutes to ensure continuous thawing. Additionally, small holes were made in the bottom of the plates to ensure equal thawing rates for each well (Fig. 14). To make sure that none of the water from a bath entered the plates and prevent contamination of wells between themselves, there was a double layer of sterilised laboratory parafilm between the lid and the plate itself.

After thawing, the plates (cryogenic vials) were covered with paper for one hour to avoid light stress. Cultures from cryogenic vials were transferred to new 12-well plates. The vials were washed with two ml of fresh WC medium to transport as much biomass as possible.

Fig. 14. A modified 12-well plate with holes in the bottom.

3.4.3 SURVIVAL ASSESSMENT

When all cultures were at standard light conditions again (at least one hour after thawing procedure), cell densities were counted using an inverted microscope Olympus IX51 (Olympus Corporation, Japan) in at least 10 randomly chosen microscopic squares per well (on average 100 cells per well). Cell densities and percentages of viable cells were established as previously described in the 3.3.4 'Survival assessment' chapter. A strain was considered tolerant for a treatment if at least one out of three replicates showed a percentage of viable cells higher than zero.

Additionally, autofluorescence (F_0) measurements were performed eight subsequent days in the Experimental Part II, starting the day of thawing. To minimize the effect of non-random distribution of cells, growth curves were made using the average of 31 measured points within a well (Fig. 15).

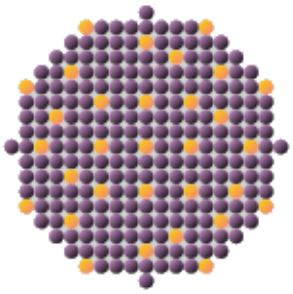


Fig. 15. Image of 31 points measured using Varioscan™ Flash Multimode Reader.

3.4.4 STATISTICAL ANALYSES

Statistical analyses were performed on two variables: percentages of viable cells calculated as a ratio between cell densities counted on day 0 and day 7, and ratios (in %)

between basal fluorescence (F_0) values measured eight days and immediately after the freezing treatments. Due to counting and measurement errors and to comply with the assumptions of the models the original data had to be modified. Square roots of cell viabilities were used and autofluorescence increases were logarithmically transformed (some of the values measured the eighth day were below zero, which caused negative final ratios, to analyse positive ratios, F_0 values for all replicates were increased by 0.016). To test the influence of temperature (–4 °C, –20 °C, –40 °C, –180 °C), cell type (resting / vegetative) and habitat (polar / temperate) on survivability a split-plot ANOVA was used. Strain was used as a whole plot, temperature as a linear predictor and the rest as a factor. Statistical analyses were performed with R: a language and environment for statistical computing (R Core Team, Austria) and the significance level was set at $P = 0.05$.

To analyse influence of freezing and thawing rate a Fisher's exact test was performed using software GraphPad Prism 5 (GraphPad Software, USA). All the graphs were created in the same software.

4. RESULTS

In total, 26 strains of 21 species belonging to 14 genera were used for the Experimental Parts I and II. Strains originated from four types of habitat: freshwater temperate (7), terrestrial temperate (2), freshwater polar (16) and terrestrial polar (1). The vegetative and resting cells were exposed to six different freezing treatments. Summary of survivability for all the strains and treatments is given in Table 3. Percentages of tolerant strains in each treatment are compared for polar and temperate strains separately in a graph below (Fig. 16). It is evident that freezing treatment down to –4 °C was the only treatment, where all the strains survived. Only some polar strains survived experiments from the Part I, with a higher percentage of tolerant strains included in cryopreservation treatment. There was a gradual decrease in survivability in Part II experiments depending on the treatment temperature.

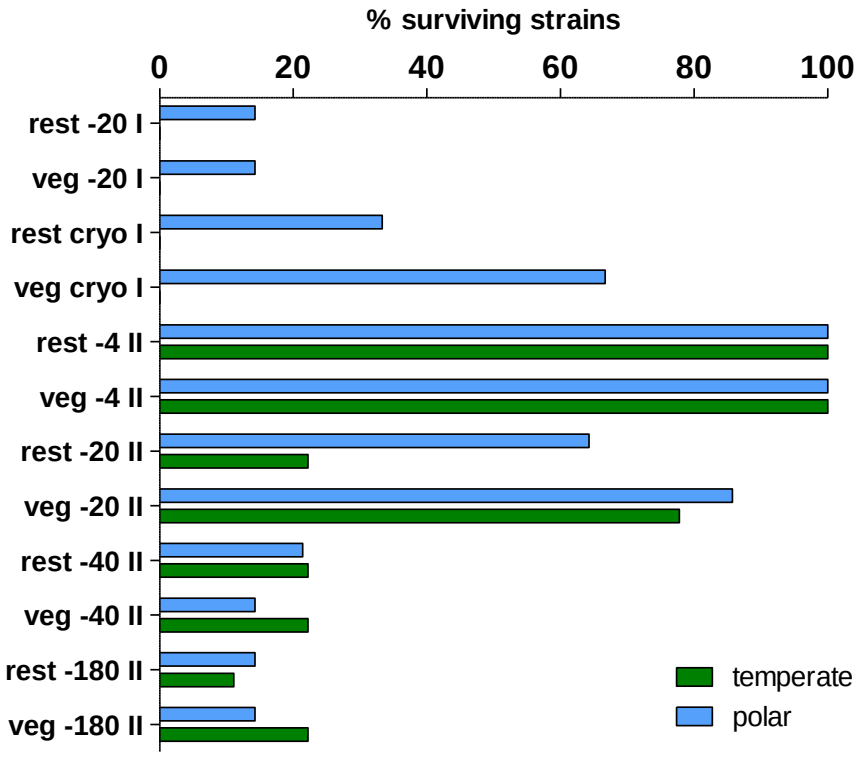


Fig. 16. Percentages of tolerant strains (representing growth in at least one out of three replicates per strain) from temperate (green bars) and polar habitats (blue bar) for each treatment. Resting cells (rest), vegetative cells (veg). Experimental Part I (I): –20 °C treatment (number of temperate strains *N = 7*, polar *N = 14*), cryopreservation (temperate *N = 3*, polar *N = 3*). Experimental Part II (II) (temperate *N = 9*, polar *N = 14*).

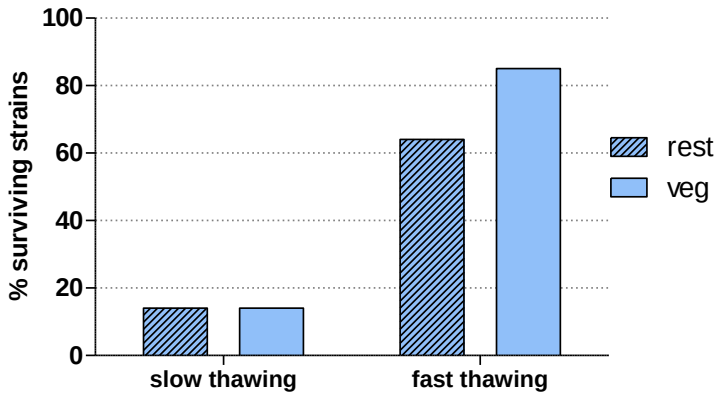
Table 3. Overview of the survivability of all strains used in the experiments; [-] non viable, [x] viable, [ ] not included.

Species	Strain	Habitat	Experimental Part I												Experimental Part II																							
			Abrupt freezing, slow thawing						Cryopreservation						Continuous freezing, fast thawing																		Abrupt freezing, fast thawing					
			–20 rest			–20 veg			–180 rest			–180 veg			–4 rest			–4 veg			–20 rest			–20 veg			–40 rest			–40 veg			–180 rest			–180 veg		
Encyonema silesiacum	DAMLV 11	FT	-	-	-	-	-	-							x	x	x	x	x	x	-	-	-	-	-	x	-	-	-	-	-	-	-	-	-	-	-	-
Eunotia ambivalens	DM 33-8	FT	-	-	-	-	-	-	-	-	-	-	-	-	x	x	x	x	x	x	-	-	-	-	-	x	-	-	-	-	-	-	-	-	-	-	-	-
Gomphonema acuminatum	DAMLV 3	FT	-	-	-	-	-	-	-	-	-	-	-	-	x	x	x	x	x	x	-	-	-	-	x	x	-	-	-	-	-	-	-	-	-	-	-	-
Navicula cryptocephala	DAMLV 17	FT	-	-	-	-	-	-							x	x	x	x	x	x	-	-	-	-	x	x	-	-	-	-	-	-	-	-	-	-	-	-
Nitzschia cf. *fonticola*	(CLONE A) F1-1	FT	-	-	-	-	-	-							x	x	x	x	x	x	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitzschia sp.	(02) 9E	FT	-	-	-	-	-	-							x	x	x	x	x	x	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pinnularia borealis	BE14/E03-3	TT													x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	-	-	x	-	-	-	x	-	x
Pinnularia borealis	RO15/1-12	TT													x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Sellaphora pupula	(Pd 126xMf 03) F1-3	FT	-	-	-	-	-	-	-	-	-	-	-	-	x	x	x	x	x	x	-	-	-	-	-	x	-	-	-	-	-	-	-	-	-	-	-	-
Achnanthes coarctata	MAR 1-7	FP	-																																			
Achnanthidium minutissimum	SPITS13-GAR MOSS 3	FP	-	-	-	-	-	-	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	-	-	x	-	-	-	-	-	-	-	-	-	-	-	-
Cymbopleura angustata	SP14/M005-2	FP	-	-	-	-	-	-							x	x	x	x	x	x	-	-	x	-	-	x	-	-	-	-	-	-	-	-	-	-	-	-
Eucocconeis flexella	SP14/M014-7	FP	-	-	-	-	-	-							x	x	x	x	x	x	x	-	x	-	-	x	-	-	-	-	-	-	-	-	-	-	-	-
Gomphonema sp.	G4	FP													x	x	x	x	x	x	-	-	x	-	-	x	-	-	-	-	-	-	-	-	-	-	-	-
Gomphonema sp.	MAR 14B-5	FP	-	-		-																																
Hantzschia abundans	SPITS13 MAT 1-7	FP	-	-	-	-	-	-	-	-		x	x	x	x	x	x	x	x	x	-	x	x	-	-	x	-	x	-	-	-	-	-	-	-	-	-	-
Chamaepinnularia krookiformis	MAR 3B-2	FP	-	-	-	-	-	-																														
Chamaepinnularia krookiformis	P24	FP													x	x	x	x	x	x	-	-	x	-	-	x	-	-	-	-	-	-	-	-	-	-	-	-
Mayamaea atomus	E16-5	FP	-	-	-	-	-	-							x	x	x	x	x	x	-	-	-	-	-	x	-	-	-	-	-	-	-	-	-	-	-	-
Navicula radiosa	SP14/M003-3	FP	-	-	-	-	-	-	-	-	-	-	-	-	x	x	x	x	x	x	-	-	-	-	-	x	-	-	-	-	-	-	-	-	-	-	-	-
Nitzschia cf. *paleacea*	S28-5	FP	-	-	-	-	-	-							-	x	-	x	x	x	-	-	x	-	-	x	-	-	-	-	-	-	-	-	-	-	-	-
Nitzschia kleinteichiana	MAR 113-15	FP	-	-	-	-	-	-							x	x	x	x	x	x	-	-	-	-	x	x	-	-	-	-	-	-	-	-	-	-	-	-
Pinnularia australomicrostauron	MAR 1-14	FP	-	-	-	-	-	-							x	x	x	x	x	x	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pinnularia borealis	P3	FP	x	x	x	x	x	x							x	-	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Pinnularia borealis	SP14/T037-48A	TP	x	x	x	x	x	x							x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	-	-	x	x	-
Pinnularia subrostrata	PP	FP													x	x	x	x	x	x	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

INFLUENCE OF FREEZING AND THAWING RATE

The results of –20 °C freezing treatments from Part I and II showed a high difference in strain survivability depending on the rate of freezing / thawing (Fisher's exact test $P < 0.001$). In the abrupt cooling and slow thawing treatment from Part I a lower survival (only two strains out of 21) was observed in comparison with continuous cooling and fast thawing treatment from Part I (19 out of 23 survived). Graphs bellow (Fig. 17) show percentages of surviving strains using the data for strains common for both Parts.

a)



b)

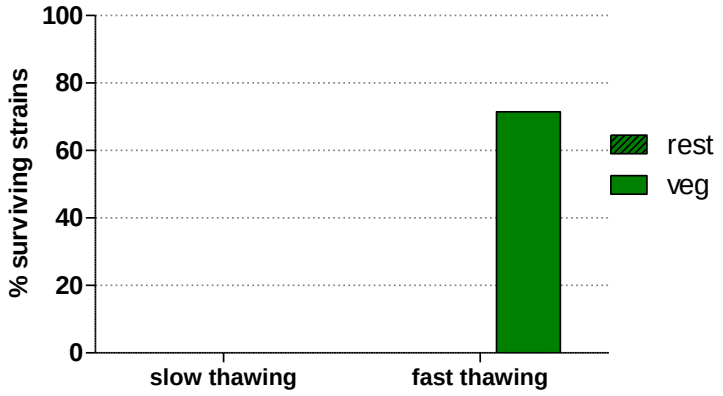


Fig. 17. Percentages of surviving strains for a) polar and b) temperate strains for two –20 °C treatments: abrupt freezing to –20 °C followed by slow thawing (Part I) and continuous freezing to –20 °C followed by fast thawing (Part II). Only the data for strains common for both Part I and II were used, so the results are not entirely consistent with Fig. 16.

4.1 EXPERIMENTAL PART I

4.1 MICROSCOPIC EVALUATION

Graphs concluding the average percentages of viable resting and vegetative cells per strain calculated as a ratio between cell densities counted on day 0 and day 7 for the treatments are shown in Appendix 4.

ABRUPT FREEZING TO –20 °C FOLLOWED BY SLOW THAWING (TREATMENT 1)

Only two strains out of 21 included in this freezing treatment (Table 3) were able to survive abrupt freezing and slow melting. These two polar strains (P3 and SP14/T037-48A) belonged to the *Pinnularia borealis* species complex. The average percentages of viable cells varied between 50 % and 90 %, but only few living vegetative cells were observed in all three replicates of the strain SP14/T037-48A. None of the temperate species survived.

CRYOPRESERVATION – ABRUPT FREEZING TO –180 °C USING CRYOPROTECTIVE SUBSTANCE DMSO FOLLOWED BY FAST THAWING (TREATMENT 2)

The liquid nitrogen treatment (–180 °C) was also lethal for four out of six tested strains, even when using the cryoprotective substance DMSO. Due to time constraints in handling time, only six strains were chosen for the experiment. Both the resting (34 % viable cells) and vegetative cells (72 % viable cells) of an Arctic strain belonging to the *Achnanthidium minutissimum* complex (SPITS13-GAR MOSS 3) survived the treatment. The vegetative cells of an Arctic strain assigned to *Hantzschia abundans* (SPITS13 MAT 1-7) survived in very low density. None of temperate species was tolerant for the cryopreservation treatment (Appendix 4).

DMSO TEST

To estimate the toxicity of cryoprotective substance DMSO a survival test was conducted. The percentage of viable cells for six tested strains after five minutes of exposure to 10% DMSO is shown in Fig. 18. It is evident that the toxicity of DMSO highly varied between strains. Living cells were observed in all the tested wells. Average percentage of viable cells higher than 50 % was observed for *Achnanthidium minutissimum* complex (SPITS13-GAR MOSS 3) and *Gomphonema acuminatum* (DAMLV 3). Strains *Eunotia ambivalens* (DM 33-8) and *Navicula radiosa* (SP14/M003-

3) showed around 20 % viable cells. And a survivability lower than 10 % was observed in the strain *Sellaphora pupula* ((Pd 126xMf 03) F1-3) and *Hantzschia abundans* (SPITS13 MAT 1-7).

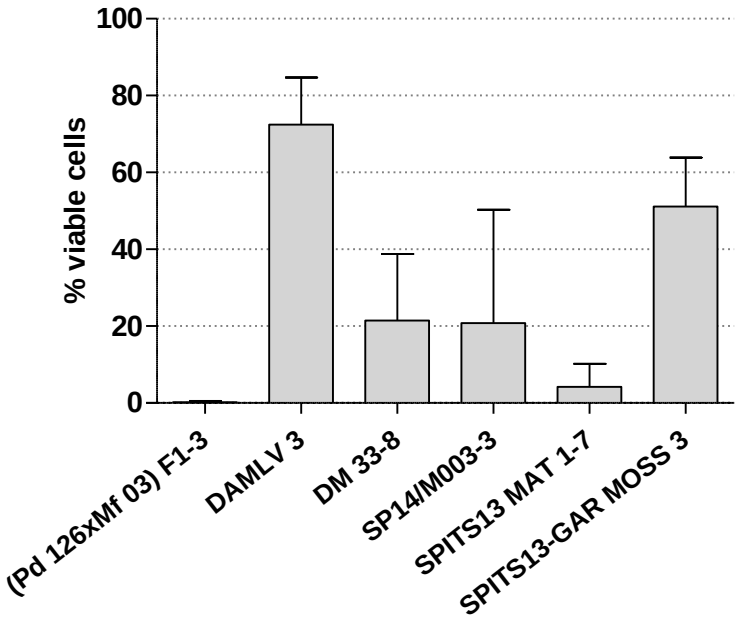


Fig. 18. Average percentage of viable cells per strain after five minutes of exposure to 10% DMSO.

4.2 EXPERIMENTAL PART II

4.2.1 MICROSCOPIC EVALUATION

Graphs concluding the average percentages of viable resting and vegetative cells per strain calculated as a ratio between cell densities counted on day 0 and day 7 for the treatments 3–6 are shown in Appendix 4.

CONTINUOUS FREEZING TO –4 °C FOLLOWED BY FAST THAWING (TREATMENT 3)

Both vegetative and resting cells of all 23 strains included in the Part II experiments survived the –4 °C continuous freezing treatment, most of them reached in average 100 % viable cells. However, one out of three resting replicates of *Pinnularia borealis* (strain P3) and two replicates of *Nitzschia* cf. *paleacea* (S28-5) did not survive (see Table 3). These strains were probably in poor health at the time of freezing, because their fluorescence values were low. All the three vegetative replicates of both these strains were viable.

CONTINUOUS FREEZING TO –20 °C FOLLOWED BY FAST THAWING (TREATMENT 4)

Nearly half of the resting cells and most of the vegetative cells included in the Part II survived the –20 °C treatment using continuous freezing followed by fast thawing. Average percentages of counted viable cell densities varied widely between strains (Appendix 4). Eleven out of 23 strains of the resting cells survived the treatment: for five strains all three replicates survived, two strains were viable in two replicates and four strains had only one viable replicate (Table 3). None of the freshwater temperate strains survived as resting cells. Vegetative cells of 19 strains were viable for at least one out of three replicates: for four strains all three replicates survived, three strains were viable in two replicates and twelve strains had only one viable replicate (Table 3) showing viability 22–99 % which caused low average percentages of viable cells and long error bars. Vegetative cells of two strains of temperate *P. borealis* species complex (BE14/E03-3 and RO15/1-12) and polar one *Navicula radiosa* (SP14/M003-3) survived in very low densities (Appendix 4).

CONTINUOUS FREEZING TO –40 °C FOLLOWED BY FAST THAWING (TREATMENT 5)

Freezing at –40 °C was lethal for most of the tested strains (Table 3). Only five out of 23 strains tolerated the treatment: one replicate of the resting cells of *Hantzschia abundans* (SPITS13 MAT 1-7) showing average viability of cells lower than 20 % and both resting and vegetative cells of four strains belonging to the *P. borealis* species complex, represented by two terrestrial temperate (BE14/E03-3 and RO15/1-12), one freshwater polar (P3) and one terrestrial polar (SP14/T037-48A) strain(s).

ABRUPT FREEZING TO –180 °C FOLLOWED BY FAST THAWING (TREATMENT 6)

Of the 23 tested strains, all four strains of *P. borealis* species complex included in experiments survived also the liquid nitrogen treatment (–180 °C) as vegetative and resting cells with only one exception – resting cells of the terrestrial temperate strain BE14/E03-3 for which was the treatment lethal. These four surviving strains were the same strains which survived the –40 °C treatment (Table 3). The average cell viability observed was lower than 20 %. And in polar strains P3 and SP14/T037-48 there were only few living cells present.

EFFECT OF FREEZING TEMPERATURE, HABITAT AND CELL TYPE ON CELL VIABILITY

The results of statistical analyses based on percentages of viable cells (calculated as a ratio between cell densities counted on day 0 and day 7) are shown below (Table 4)**.** Freezing temperature had a statistically significant influence on strain viability ($P < 0.001$) and explained the highest amount of variability (26 %).

Table 4. Results of statistical analyses (split-plot ANOVA) based on cell viabilities. Bold: significant values, type (type of cells): resting or vegetative cells, habitat: temperate or polar, temperature: –4, –20, –40, –180 °C.

	Df	Sum Sq	*P*	variability
temperature	1	2197.0	**< 0.001**	**26%**
habitat	1	31.7	0.174	0%
type	1	1.0	0.727	0%
habitat : type	1	2.0	0.670	0%
habitat : temperature	1	16.0	0.235	0%
type : temperature	1	7.0	0.425	0%
habitat : type : temperature	1	4.0	0.549	0%

A possible latitudinal effect on strain survivability was tested. Influence of habitat type on the cell viability for the resting and vegetative cells is shown in Fig. 19. No statistically significant difference appeared between polar and temperate strains ($P = 0.174$), neither any of the interactions (Table 4). However, when looking at the results of the –20 °C treatment, temperate strains showed much lower values (median 10 %) in comparison with polar strains (median 34 %).

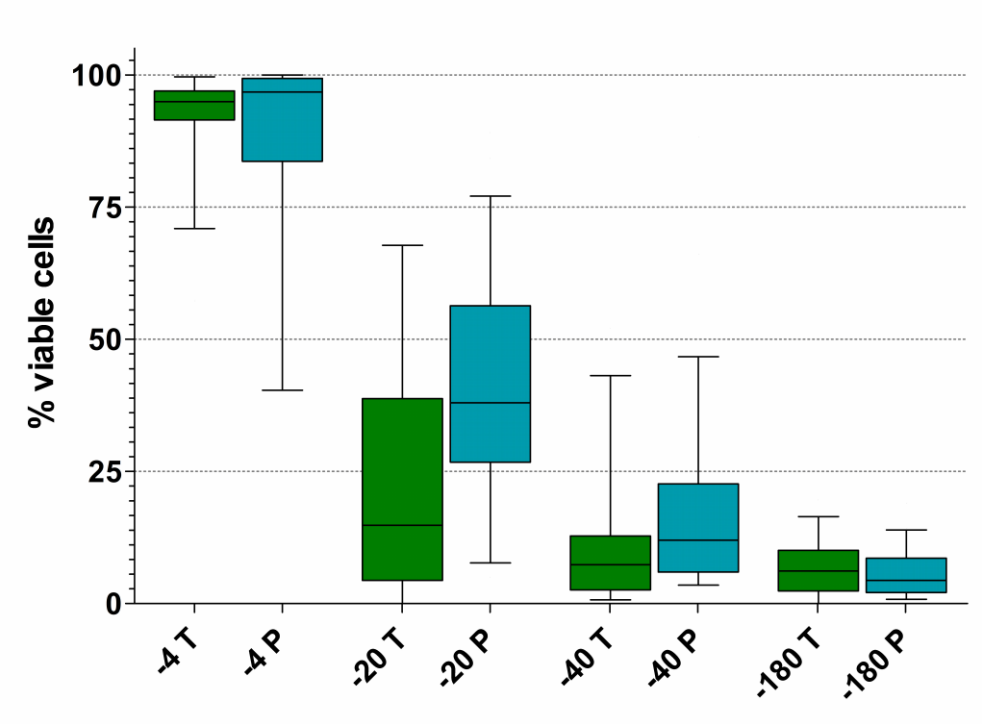


Fig. 19. Box plots of viable cells percentages for strains from temperate (T, green bars) or polar (P, blue bars) habitats for freezing treatments 3–6 (line: median, box: first and third quartiles, whiskers: 10–90 percentile).

Percentages of viable cells for resting and vegetative cells for each treatment are demonstrated in Fig. 20. No significant difference in cell viability appeared between the two cell types (resting / vegetative), $P = 0.727$ (Table 4).

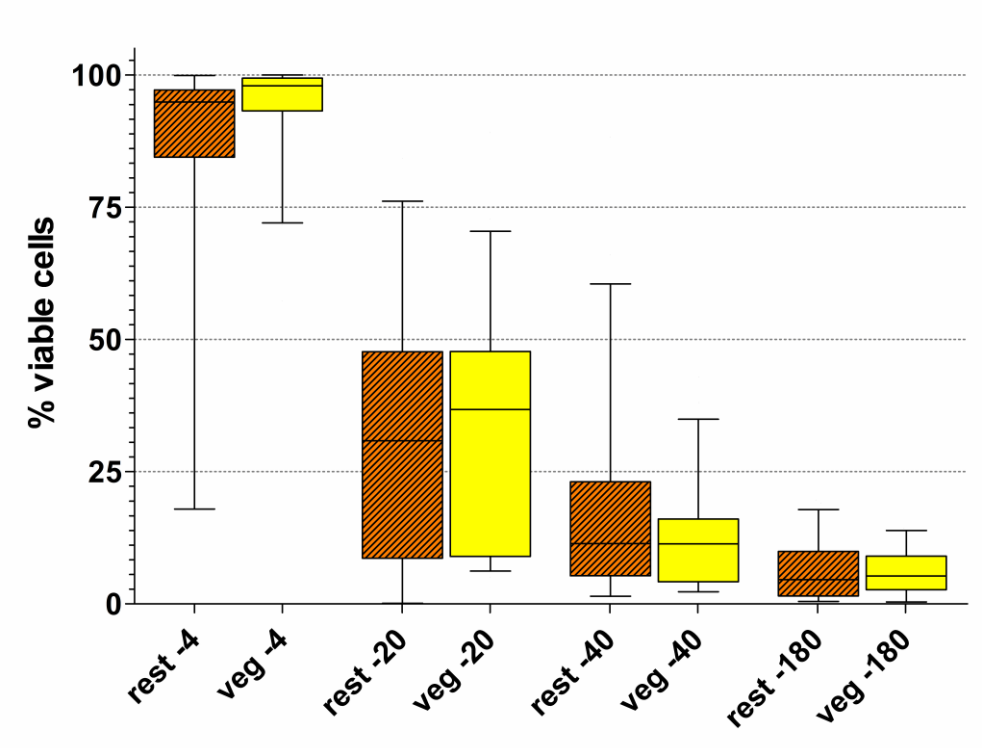


Fig. 20. Box plots of viable cells percentages for resting (rest) and vegetative (veg) cells for freezing treatments 3–6 (line: median, box: first and third quartiles, whiskers: 10–90 percentile).

4.2.2 AUTOFLUORESCENCE EVALUATION

Appendix 5 shows growth curves of vegetative and resting cells measured for each replicate as their basal fluorescence (F_0) during eight subsequent days after freezing treatments 3–6.

EFFECT OF FREEZING TEMPERATURE, HABITAT AND CELL TYPE ON GROWTH

An overview of the results of statistical analyses based on the increase of basal fluorescence (F_0) eight days after the treatments is given in Table 5. Similarly to the analysis based on cell counts, freezing temperature had a statistically significant influence on strain growth ($P < 0.001$) and explained the highest amount of variability (37 %).

Table 5. Results of the statistical analyses (split-plot ANOVA) based on autofluorescence measurements. Bold: significant values, type (type of cells): resting or vegetative cells, habitat: temperate or polar, temperature: –4, –20, –40, –180 °C.

	Df	Sum Sq	*P*	variability
temperature	1	379.10	**< 0.001**	**37%**
habitat	1	0.01	0.958	0%
type	1	5.60	**0.024**	**1%**
habitat : type	1	0.80	0.404	0%
habitat : temperature	1	0.00	0.851	0%
type : temperature	1	12.80	**0.001**	**1%**
habitat : type : temperature	1	1.80	0.194	0%

Influence of habitat type on the survivability of the strains is demonstrated in Fig. 21. No significant differences were detected between polar and temperate strains, $P = 0.958$ (Table 5).

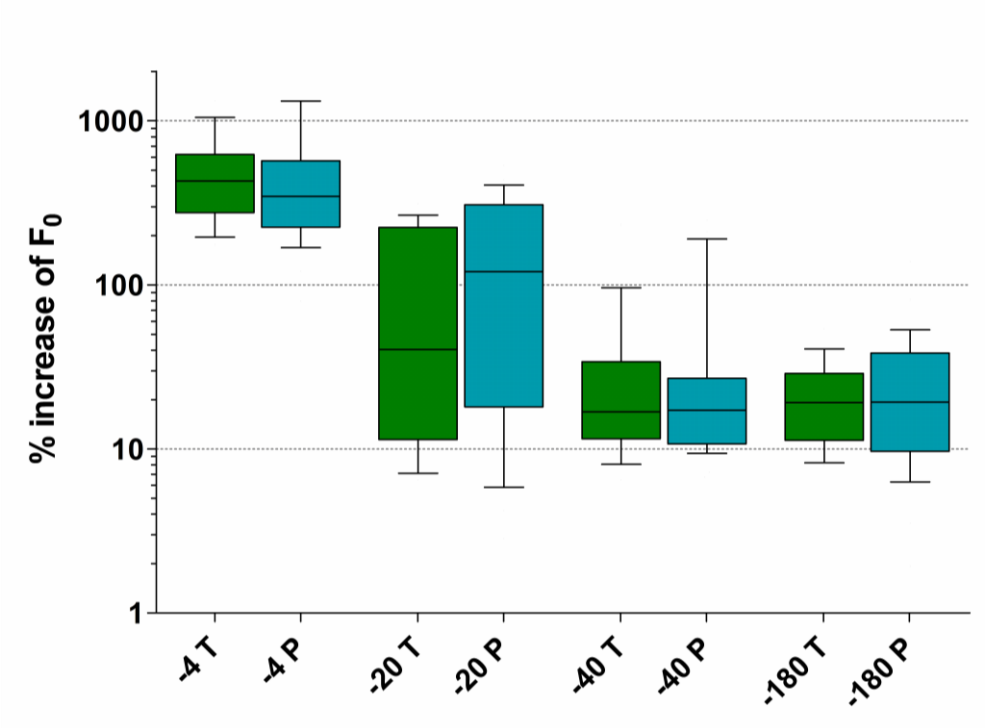


Fig. 21. Box plots of average increase of basal fluorescence (F_0) eight days after treatments 3–6 for strains from temperate (T, green bars) and polar (P, blue bars) habitats (line: median, box: first and third quartiles, whiskers: 10–90 percentile).

In contrast to the evaluation based on counted cell viabilities, there was a significant difference in survivability between resting and vegetative cells ($P = 0.024$, Table 5, Fig. 22). Significant interaction appeared between temperature (–4, –20, –40, –180 °C) and cell type, $P = 0.001$. In "higher" temperatures (–4 °C) resting cells showed higher increase of autofluorescence when compared to the vegetative ones. An opposite trend was observed in the liquid nitrogen treatment (–180 °C), where resting cells showed lower increase in fluorescence in comparison with vegetative ones. No other statistically significant interactions were detected (Table 5).

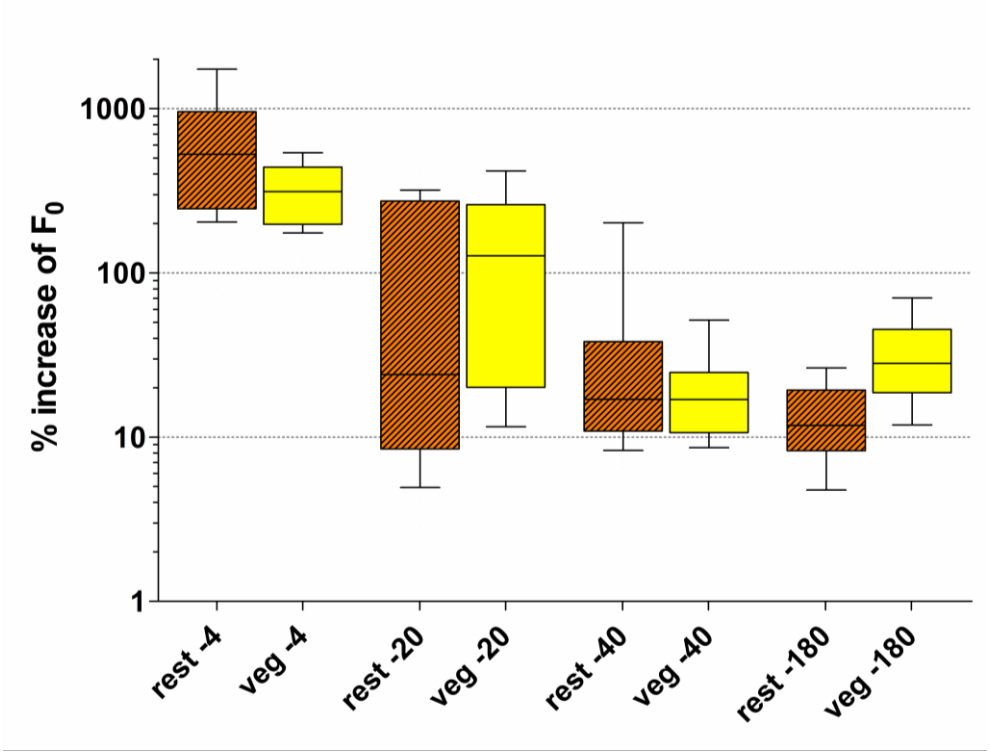


Fig. 22. Box plots of average increase of basal fluorescence (F_0) per strain eight days after freezing treatments 3–6 for resting (rest) and vegetative (veg) cells (line: median, box: first and third quartiles, whiskers: 10–90 percentile).

SUPERHERO *PINNULARIA BOREALIS*

A relatively high increase of basal fluorescence (F_0) was observed in all the four strains of *Pinnularia borealis* species complex included in experiments in Part II. Average increase of autofluorescence for each strain, cell type and treatment and their divergence from average increase calculated for all other strains is given in Fig. 23. Especially polar *Pinnularia* strains showed higher increase in freezing treatments lower than –20 °C when compared to the average increase per treatment. The results of the freezing experiments suggest that *P. borealis* is an extremely resistant species complex.

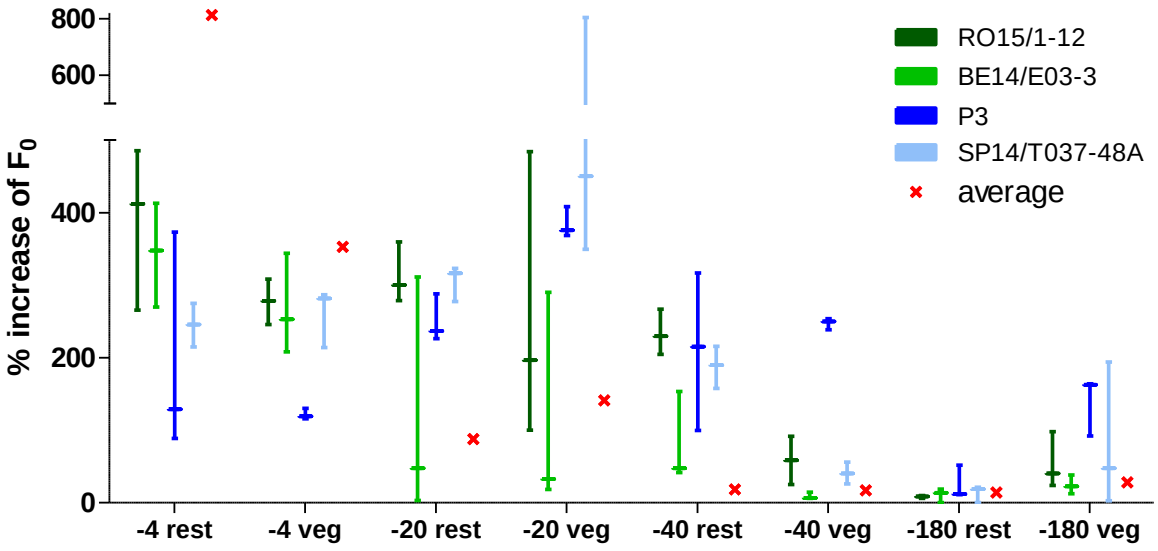


Fig. 23. Increase of basal fluorescence (F_0) for each replica of *Pinnularia borealis* species complex strains from temperate (green bars) or polar (blue bars) habitats and their divergence from average increase calculated for all other strains (red cross) eight days after the treatments 3–6.

5. DISCUSSION

5.1 EFFECT OF TEMPERATURE ON FREEZING SURVIVAL

Polar Regions are characterized by extreme climate conditions, which are likely to play a main role in activity of organismal communities. One of the most important factors affecting the composition of these communities is the temperature stress (VINCENT *et al.* 2008). Some of the freezing treatments conducted in this study simulated conditions found in polar environments. Overall, the results confirmed a high sensitivity of freshwater benthic diatoms to freezing stress. This study demonstrates the differences in tolerance to six different freezing treatments (including those relevant for cryopreservation of microalgal strains), which were tested under controlled laboratory conditions.

Four freezing temperatures were chosen for the treatments. The –4 °C temperature was established as a common temperature that can occur in both the polar and temperate regions. Freezing temperatures from –15 °C to –60 °C constitute more risk as they often result in damages of cells caused by ice formation, water migration or ion concentration (MAZUR 1984). Thus –20 °C and –40 °C were chosen for the experiments to simulate the more stressful conditions of the polar winter. The temperature of –180 °C representing liquid nitrogen (–196 °C) was established as a negative control and also an interesting limit for freezing survival.

In this study, freezing down to –4 °C did not influence the survival of diatom strains. This temperature commonly occurs during autumn and spring in temperate regions and in polar during the summer periods (REYNOLDS 1981). A big difference in survivability of diatoms was observed in the two –20 °C treatments, which differed in the rate of temperature changes, while more injurious was the rapid freezing followed by slow thawing. This is in agreement with previously published data (MAZUR 1984, CAÑAVATE & LUBIAN 1995, 1997) and further discussed in more detail. It is interesting to note is that some strains included in –20 °C continuous freezing and slow thawing treatment, survived in a single replicate. The reason for this occasional survival is not clear and should be further investigated, but one of possible explanation is that temperatures around –20 °C could constitute some limit in survivability of diatoms.

Freshwater polar strains included in the experiments originated from Arctic or Maritime Antarctic water bodies, where the winter temperatures do not reach extremely low values. Bottom temperature of two shallow lakes from James Ross Island was recorded during 2011 (Fig. 24). In Lake Dulanek winter minimal temperature reached –17.5 °C and annual mean was –5.2 °C. In Lachman Lake 1 (this is the original locality of one experimental strain), annual mean temperature reached –4.6 °C and winter minima were below –20 °C (VÁCZI *et al.* 2011). These bottom temperatures could explain poor survival of diatoms in freezing treatments lower than –20 °C.

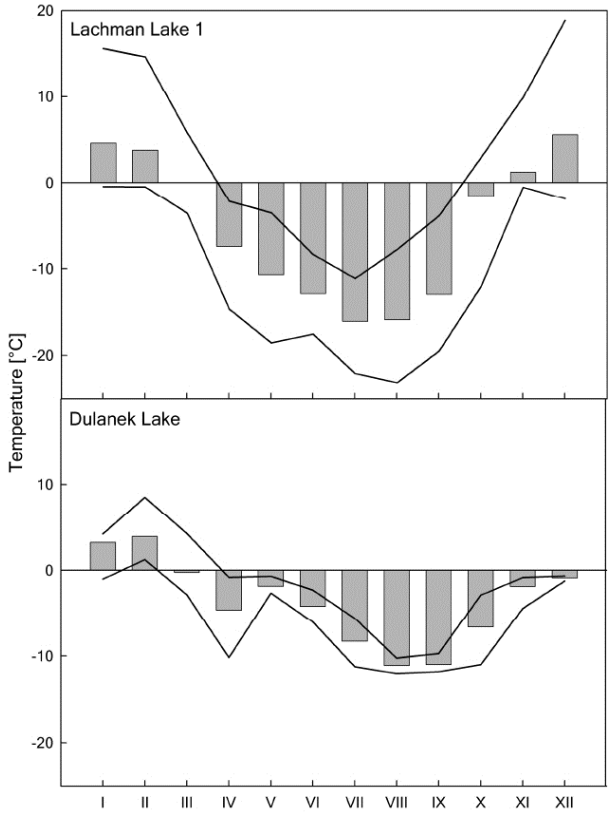


Fig. 24. Monthly mean water temperature (columns), monthly maxima (upper line) and minima (lower line) for Lachman Lake 1 and Lake Dulanek (James Ross Island, Antarctica) (VÁCZI *et al.* 2011).

Freezing experiment to –40 °C was not harmful only for five strains. This low diatom survivability is in contrast with published data about a high survivability of green algae. Most of the tested strains of filamentous green alga *Klebsormidium* P. C. Silva, Mattox & Blackwell, isolated from distinct geographical regions (Antarctic, Arctic and Central Europe), survived a very similar –40 °C freezing experiment (ELSTER *et al.* 2008). So did other Antarctic green algae and also cyanobacteria, which showed even higher tolerance in comparison with green algae (ŠABACKÁ & ELSTER 2006). In this

study four diatom strains surviving the –40 °C treatment tolerated liquid nitrogen (–180 °C) as well. The survivability was lower, but it could be explained by biomass loss during transfer to cryogenic vials necessary for the storage in the nitrogen freezer and back to new plates after the treatment. If no loss occurred, the survivability of –40 °C and –180 °C treatments could be comparable, due to almost the same tolerating species. This also corresponds with tolerance of cyanobacteria and green algae which showed approximately same survivability for –40 °C, –100 °C and liquid nitrogen treatments (ŠABACKÁ & ELSTER 2006).

Even though some phototrophic microorganisms, mainly cyanobacteria, are able to withstand freezing in the absence of cryoprotective substance by plunging directly into liquid nitrogen without any harm, green algae are poorly able to survive such conditions (DAY *et al.* 1997, PARK 2006, NÚÑEZ-VÁZQUEZ *et al.* 2011). To enhance the survival various cryoprotectants are used (MORRIS 1978, BEN-AMOTZ & GILBOA 1980, NAKANISHI *et al.* 2012). Very little work has been done on diatoms (e.g. REDEKAR & WAGH 2000, RHODES *et al.* 2006). Effects of cryopreservation on a diatom *Chaetoceros calcitrans* (Paulsen) H. Takano indicated that to ensure the viability after cryopreservation it is necessary to use some cryoprotectant (SALAS-LEIVA & DUPRÉ 2011). Results of this study partially confirm these data. Only four strains of *Pinnularia borealis* species complex out of all the tested strains were able to survive uncontrolled direct freezing in liquid nitrogen without cryoprotection. Concerning the cryopreservation treatment with the usage of cryoprotectant, only two polar (*Achnanthidium minutissimum* and *Hantzschia abundans*) out of six tested strains survived. Unfortunately most of the tested diatoms were not able to survive such conditions. Moreover, the results of DMSO test in this study showed a significant toxicity of the cryoprotectant itself. Toxicity of DMSO is known at higher concentrations (40%) from a clinical study (ROWLEY & ANDERSON 1993) and a recent study reports its toxicity at low concentrations in a retinal neuronal cell line as well (GALVAO *et al.* 2014).

5.2 EFFECT OF HABITAT TYPE ON FREEZING SURVIVAL

One of the aims of this study was to compare the survival of polar and temperate strains. To test this theory, a statistical analysis on the whole dataset was performed and

no significant relation was found. This is in agreement with a comparison of *Klebsormidium* strains from polar (Antarctic, Arctic) and temperate areas (Central Europe), which indicated no statistically significant differences between strains originating from different regions (ELSTER *et al.* 2008). On the other hand, temperature below zero occurs during winter season in temperate habitats as well.

However, obvious trends were found in the –20 °C treatments – polar strains showed higher percentage of viable cells and also the average increase of basal fluorescence (F_0) was higher, when compared to strains from temperate regions. Additional statistical test for the –20 °C continuous freezing and fast thawing treatment showed significant relationship for data from microscopic evaluation (Mann Whitney test $P = 0.003$) but not for autofluorescence evaluation. According to the –40 °C and –180 °C treatments it is likely that differences among algal strains are determined by the specific characteristics of each strain (species) rather than the habitat or original locality as demonstrated on the ability of both polar and temperate strains of *Pinnularia borealis* to survive all the freezing treatments in this study.

In literature, a habitat dependence related to temperature extremes in the freezing tolerances was described among filamentous cyanobacteria isolated from maritime and continental Antarctica, and also among strains originating from various habitat types (ŠABACKÁ & ELSTER 2006). Likewise a higher tolerance of terrestrial diatoms to temperature extremes was indicated. In a comparison focused on temperate benthic diatoms, only terrestrial species persisted experimental freezing and aquatic diatoms exhibited high sensitivity to gradual heating up to 40 °C. This is in agreement with the high population differentiation observed in a freshwater benthic diatoms and may explain their dispersal capacities and widespread endemism (SOUFFREAU *et al.* 2010). Living cells of aquatic diatoms are also generally absent from air traps, which might be related to a higher sensitivity to desiccation and a low chance of being picked up by wind. This could explain for example the large population differentiation within the benthic freshwater diatom *Sellaphora capitata* (e.g. EVANS *et al.* 2009, VANORMELINGEN *et al.* 2013).

In this study primarily temperature-stress tolerance for polar and temperate strains was compared. Three terrestrial strains were included more or less as a control since it is known from the results of study described above (SOUFFREAU *et al.* 2010, 2013a),

that they are able to survive freezing. From the results of this study we can only note that the strains isolated from terrestrial habitats (all of them belonging to *Pinnularia borealis* species complex) tolerated all five treatments in which they were included. They also survived as the only strains the liquid nitrogen treatment without adding any cryoprotective substance, with only one exception: an aquatic strain of the same species complex, which survived as well. It is also interesting to note, that freshwater polar strain of *Mayamaea atomus* from this study did not survive –20 °C abrupt freezing and slow thawing treatment, whereas terrestrial temperate *M. atomus* survived comparable freezing experiment (abrupt freezing and slow thawing) relatively well (SOUFFREAU *et al.* 2010, 2013a). The same occurred in *Achnanthes coarctata* – but our freshwater polar strain was in a poor health before the treatments started.

In contrast to freshwater diatoms, terrestrial ones probably possess some protection mechanisms to survive in soils and temporary water bodies where they are exposed to strong fluctuations in humidity and temperature. It is probable that diatoms inhabiting permanent water bodies, which are more buffered against abrupt fluctuations in temperature, do not invest energy for protection mechanisms, resulting in sensitivity to abrupt freezing and desiccation (SOUFFREAU *et al.* 2013a).

5.3 INFLUENCE OF CELL TYPE (RESTING OR VEGETATIVE)

Enhanced tolerance levels to freezing and desiccation stress were observed in resting cells of some terrestrial diatoms, even though exact mechanisms that give such stress tolerance are not known (SOUFFREAU *et al.* 2013a). In planktonic diatoms the importance of resting stages formation was indicated not only as a survival mechanism but it also may contribute to patterns of seasonal phytoplankton succession (LUND 1954, MCQUOID & HOBSON 1996). Acclimatization increases the tolerance of unfavourable conditions in various organisms. It should be noted that resting cells in experiments in this study were induced at 4 °C, while vegetative cells were kept at 18 °C, which was hypothesized to signalize a winter season and initiate changes in the cells (MOCK & VALENTIN 2004). Moreover this might have caused a positive acclimatization unrelated to the resting stage itself. Nevertheless, the results did not emphasize the importance of resting cells for freezing tolerance.

Resting cells showed higher average increase of basal autofluorescence (F_0) in "higher" temperatures (–4 °C treatment) and lower increase in more extreme "lower" temperatures when compared to vegetative ones. This could be explained by the fact, that resting cells were incubated in WC medium without nitrogen prior to the freezing, which limited their normal growth rate, so their low densities allowed them to grow more after nitrogen replenishment. In addition resting spores under nitrogen limitation probably require a certain lag period to initiate growth, which is supported by a study of resting life-forms of the planktonic diatom *Chaetoceros pseudocurvisetus* Mangin (KUWATA & TAKAHASHI 1999). This fact together with a low level of viable cells surviving treatments could explain a low average increase of basal autofluorescence after liquid nitrogen treatment.

A consequence of freezing and desiccation is dehydration of cells because both are osmotic stresses and perhaps require comparable (but not necessarily identical) protection mechanisms (WELSH 2000). Diatom *Stauroneis anceps* Ehrenberg provided evidence that desiccated cells are much more tolerant to temperature extremes than vegetative cells growing in an aqueous medium. These were able to successfully withstand temperatures between –15 °C and –20 °C (HOSTETTER & HOSHAW 1970).

Additionally, the formation of stress tolerant resting cells, in which state diatoms may be present on the bottom during periods unfavourable to their growth, is an important mechanism which allow for diatom populations to persist locally over time and act as "seed banks" for the potential recolonization or metapopulation formation in both aquatic and terrestrial habitats, as reported on planktonic diatom species *Melosira italica* subspecies *subarctica* Otto Müller (LUND 1954) or other various algal species (POULÍČKOVÁ *et al.* 2008). Moreover, anoxic conditions usual in sediments of deep lakes are known to enhance the preservation of resting stages of dinoflagellates (RENGEFORS & ANDERSON 1998) and some of diatoms, who can even persist in the dark and anaerobic conditions for three years (LUND 1954). Diatom strains in this study are mainly isolated from shallow lakes, where are no anoxic conditions, and potential role of resting cells for population persistence remain unknown.

5.4 IMPACT OF FREEZING AND THAWING RATE

It is interesting to note that the freezing and thawing rates of treatments influenced the survivability of the observed strains. If both the –20 °C treatments (Part I and II) are compared, abrupt cooling and slow thawing caused much lower survival (only two strains out of 21) in comparison with continuous cooling and fast thawing, which is in agreement with published studies about tolerance of benthic diatoms from temperate habitats to experimental freezing, where a similar –20 °C abrupt cooling and slow thawing treatment showed low diatom survivals (SOUFFREAU *et al.* 2010, 2013a). Explanation for the differences in survival in this study gives previously published data suggesting the physical events in the cell in freezing temperatures depend on cooling velocity. If cooling is appropriately slow (below 1 °C·min^{-1}, in our experiments the rate was 0.5 °C·min^{-1}), the cell is able to lose water and maintain the chemical potential of intracellular water in equilibrium with extracellular. The result is that the cell dehydrates and intracellular ice does not form but small numbers of ice crystals grow to a larger size in extracellular space (Fig. 25 upper right). Rapid cooling rates, which does not occur in nature, tend to encourage the formation of intracellular ice crystals with mechanically destructive consequences (Fig. 25 centre right). If the cell is cooled too rapidly (Fig. 25 bottom right), it is not able to lose water fast enough to maintain equilibrium and becomes supercooled and reaches equilibrium by intracellular freezing resulting in numerous small size ice crystals (MAZUR 1963, 1988).

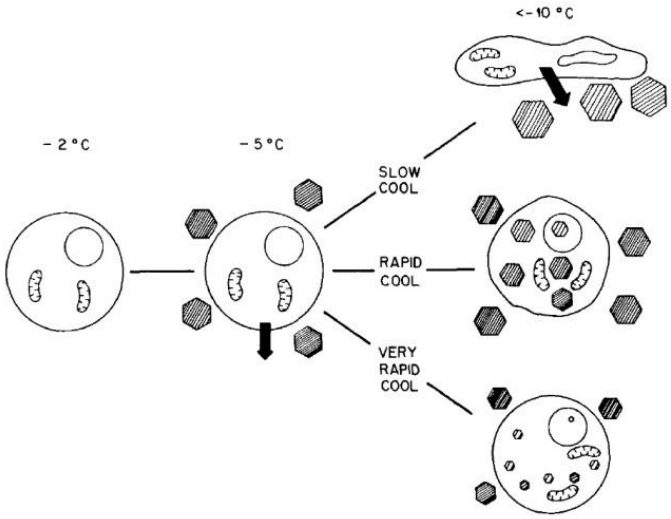


Fig. 25. Physical events in the cell during freezing process. The cross-hatched hexagons represent ice crystals (MAZUR 1984).

On the other hand, the process of thawing might represent more risk, because ice crystal growth can still occur during a warming process. Small intracellular ice particles that may have formed during freezing can fuse into larger and damaging crystals in order to decrease the surface energy during warming if the rate of warming is low. The process is known as recrystallization (MAZUR 1984). It was suggested that viability in marine microalgae improved when the algae were thawed rapidly – between 0.25 and 16 °C·min^{-1} (CAÑAVATE & LUBIAN 1995). And further published data report significantly higher viabilities observed in algae thawed rapidly (CAÑAVATE & LUBIAN 1997), which correspond with the results of this study.

5.5 SUPERHERO *PINNULARIA BOREALIS*

Surprisingly all the four strains of *Pinnularia borealis* species complex included in the experiments persisted all the freezing treatments used, even the liquid nitrogen treatment. Though *P. borealis* strains were not included in the cryopreservation treatment using cryoprotective substance DMSO in this study, this species is also able to survive it (PINSEEL, unpublished data). The results indicate that *P. borealis* is an extremely resistant species complex. Observed resistance could be related to success of the species in nature and its worldwide dispersal.

What is important to note is, that none of the examined strains of *P. borealis* actually does belong to the same species (PINSEEL, unpublished data). Molecular analyses revealed this taxon consists of multiple lineages and together with *Hantzschia amphioxys* and many other presumably cosmopolitan Antarctic diatoms are more likely species complexes possibly including endemics with low temperature preferences (SOUFFREAU *et al.* 2013b). There were also other representatives of the genera *Pinnularia* included in the experiments: *P. australomicrostauron* and *P. subrostrata*. Both of them were isolated from freshwater habitats in Antarctic region and for both only the –4 °C treatment was not lethal.

Since three of *P. borealis* strains were the only terrestrial strains included in experiments it is not possible to generally judge the influence of habitat type (terrestrial / freshwater) on freezing tolerance. The fact that one *P. borealis* freshwater strain was able to survive the treatments as well, does not confirm the theory and generally *P. borealis* is

more regarded as terrestrial species. Nevertheless, this is the evidence within only one, but probably very resistant species.

Also a polar strain of *Hantzschia abundans* (SPITS13 MAT 1-7) did survive the –40 °C freezing treatment (Part II) as a single replicate of resting cells. The percentages of viable cells were very low, which could be because of the combination of a low probability of survival and a low number of tested cells per well. This means that some cells of these species are capable of surviving freezing. So does suggest the fact that all three replicates survived liquid nitrogen treatment using cryoprotectant DMSO. Despite not being psychrophilic, the Antarctic lineages of *P. borealis* and *H. amphioxys* have a relatively low optimal growth temperature and upper lethal temperature limit for growth than most lineages from more temperate areas, indicating niche differentiation (SOUFFREAU *et al.* 2013b).

5.6 OTHER FACTORS INFLUENCING CELL SURVIVAL IN FREEZING EXPERIMENTS

Also the growth phase of cultures could play an important role in survival. Microbial cells in early stationary or late logarithmical phase of growth exhibit greater tolerance to the freezing process, when compared to younger or older cells (SIMIONE 1992). Increased survival could be caused by the accumulation of metabolites and their secretion in the medium, which suggest results of stress tolerance experiments in diatom populations of *Stauroneis anceps* (HOSTETTER & HOSHAW 1970). Cell densities could influence survivability as well, for example in relation to biofilm formation, when residual biofilm of benthic algae improve re-colonization known from temporary dry rocky streams in south-eastern Australia (ROBSON 2000). But according to the results of this study the cell densities established by basal autofluorescence measured for each strain and each replica cannot confirm this potential survival mechanism. Due to clustering of cells, mechanisms involving intercellular communication and a subsequent response of the individual cells could be hypothesised in successful survival in stressful conditions such as desiccation or freezing (SOUFFREAU *et al.* 2010).

Mechanisms of vertical migration into the sediment are known in the context of enhanced radiation in benthic diatoms (UNDERWOOD *et al.* 1999, PERKINS *et al.* 2010). But possibility of a migration deeper in the sediment where there is a higher

temperature as a survival strategy could be also considered. Another aspect is acclimatization. In this study, resting cells were induced under nitrogen limitation, low temperature and dark conditions. It would be interesting to test, if for example a gradual temperature decrease results in a higher survivability in diatoms. It would be also interesting to assess, whether there is an influence of other nutrient limitations (e.g. phosphorus), changes in light conditions (shortening of sunlight period) or other shifts in the environment common in nature.

In some of freshwater diatoms was also found an ability to metabolize organic compounds and grow heterotrophically with carbohydrates as their only carbon source. Only glucose, glycerol, and fructose were evidenced to enhance primary production. Glucose uptake was proven in the absence of carbon dioxide in both dark and light. Also photoinhibition was found (LEWIN 1953, SAKS 1983). These findings suggest that such alternative nutrient uptake could be effective in improving diatom survival during long polar winter conditions.

As concerns the methods from this study, the experiments were performed at two independent institutions, but due to different characters of the two Experimental Parts the influence could be ruled out. In microscopic evaluation in some replicas a high error occurred during the determination of cell densities (Appendix 4). Therefore some negative values were determined, however, living cells were observed and vice versa. In autofluorescence evaluation some negative values occurred because of very low values measured the eighth day (Appendix 5). This might have caused slightly distorted some graphs in the results. And important to note is that the graph demonstrating percentages of tolerant strains in each treatment for polar and temperate strains separately (Fig. 16) is calculated on data where the number of polar does not equal the number of terrestrial strains. Percentage expression was chosen for easier comparability. The results are based on a limited number of strains (species) chosen for each treatment, which is necessary to bear in mind.

5.7 IMPLICATIONS FOR DIATOM CRYOPRESERVATION IN CULTURE COLLECTIONS

Freezing is a practical, not expensive and safe way how to preserve microorganisms. Experimental testing of the freezing tolerance of cyanobacteria and algae

became important in relation to application of various cryopreservation methods in culture collections (DAY *et al.* 2005). Effective methods of cryopreservation provide long-term conserving of large culture collections and can also reduce the risks of losing particular strains or species through contamination or genetic drift up to many years of storage. Cryopreservation was also successfully applied in the area of medical biology e.g. reproductive medicine (PEGG 2005), in biological research (MAZUR *et al.* 2008) and agricultural industry as well in both animal and plant areas (ENGELMANN 2004, BAILEY *et al.* 2008).

According to published data about four green algae, cryopreservation viability persisted during a storage period of 15 years and chlorophyll content was not significantly changed (NAKANISHI *et al.* 2012). But many of tested protocols using various cryoprotectants (penetrating: dimethyl sulphoxide, glycerol, methanol, ethylene glycol and non-penetrating: trehalose and polyvinylpyrrolidone) and their combinations at different concentration, time and temperature of exposure rarely ensure 100 % satisfactory results in cryopreservation of diatoms. Rate of success differs across species and could be explained by peculiarities in the cell wall composition with higher content of silicon and fewer organic components (REDEKAR & WAGH 2000, RHODES *et al.* 2006, SALAS-LEIVA & DUPRÉ 2011, BORODA *et al.* 2013).

6. CONCLUSION

The tolerance of diatoms from polar and temperate habitats to experimental freezing was compared. Their ability to survive different freezing modes (including those simulating natural conditions) and the importance of resting cells was experimentally tested.

Diatom strains for the experiments were isolated from natural samples collected during 2014 in Antarctica and Spitsbergen. Further strains were acquired from culture collections of microorganisms (CCCryo and BCCM). In total 26 strains representing species from freshwater and terrestrial habitats were used for the experiments. For resting cells induction diatoms were incubated under nitrogen limitation in conditions of dark and low temperature (4 °C). The experiments consisted of six different freezing treatments to which the vegetative and resting cells were exposed: 1. abrupt freezing to –20 °C followed by slow thawing, 2. cryopreservation – abrupt freezing to –180 °C using the cryoprotective substance DMSO followed by fast thawing, 3. continuous freezing to –4 °C followed by fast thawing, 4. continuous freezing to –20 °C followed by fast thawing, 5. continuous freezing to –40 °C followed by fast thawing, 6. abrupt freezing to –180 °C followed by fast thawing. Rate of continuous cooling selected for natural freezing simulation was 0.5 °C·min^{-1}.

The study showed that cells of tested diatom strains are sensitive to experimental freezing. Whereas the freezing temperature had a significant effect on strain survival, the results did not prove significant difference in survival between polar and temperate strains of diatoms, neither the importance of resting cells for the survival of freezing treatments. Survivability was assessed using percentages of viable cells (established by cell densities determination) and growth assessment (as percentages of basal fluorescence increases). As expected both vegetative and resting cells of all the strains survived the –4 °C treatment which commonly occurs in both polar and temperate habitats. Most of the strains survived –20 °C using continuous freezing followed by fast thawing. In this treatment, polar strains showed higher viability in comparison with temperate ones suggesting that they are better adapted to the temperature that is habitual in their natural environment. Only two terrestrial strains survived the –20 °C treatment using abrupt freezing and slow melting, which suggests that the speed of freezing and melting plays a role in survival. Five strains survived the –40 °C freezing experiment and four of these also survived the liquid

nitrogen treatment (–180 °C) followed by fast thawing. These were strains belonging to the *Pinnularia borealis* species complex, represented by two terrestrial temperate, one freshwater polar and one terrestrial polar strain. This indicates that *P. borealis* is an extremely resistant species complex. Cryopreservation treatment using DMSO was not lethal only for two polar out of six tested strains.

To our knowledge, this is the first study about freezing tolerance limits of diatoms comparing strains from polar and temperate habitats. How do polar diatoms cope with their natural environment remains an open question, which could be solved based on detailed field studies that will include sampling throughout the year. Even though the conditions of the experiments were artificial, the study brought valuable data that could be useful for the introduction of diatom cryopreservation in culture collections.

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8. APPENDICES

APPENDIX 1. LIST OF STRAINS ISOLATED DURING THIS STUDY.

code	species	region	island	location	GPS
A1	*Amphora* sp.	Antarctica	James Ross	Black Lake	63°57'56.9"S 57°52'59.2"W
A2	*Amphora* sp.	Antarctica	James Ross	Black Lake	63°57'56.9"S 57°52'59.2"W
A3	*Amphora* sp.	Antarctica	James Ross	Black Lake	63°57'56.9"S 57°52'59.2"W
E1	not determined	Antarctica	Vega Island	Esmeralda Lake	63°52'21.1"S 57°36'24.0"W
E2	not determined	Antarctica	Vega Island	Esmeralda Lake	63°52'21.1"S 57°36'24.0"W
E3	not determined	Antarctica	Vega Island	Esmeralda Lake	63°52'21.1"S 57°36'24.0"W
E4	not determined	Antarctica	James Ross	Lachman Lake 1	63°47'59.5"S 57°48'31.4"W
E5	*Psammothidium* sp.	Antarctica	James Ross	Lachman Lake 1	63°47'59.5"S 57°48'31.4"W
E6	not determined	Antarctica	Vega Island	pool 164	63°52'38.8"S 57°31'56.4"W
E7	not determined	Antarctica	Vega Island	pool 164	63°52'38.8"S 57°31'56.4"W
E8	not determined	Antarctica	Vega Island	pool 164	63°52'38.8"S 57°31'56.4"W
E9	not determined	Antarctica	Vega Island	pool 164	63°52'38.8"S 57°31'56.4"W
E10	not determined	Antarctica	James Ross	Black Lake	63°57'56.9"S 57°52'59.2"W
E11	not determined	Antarctica	Vega Island	Pan Negro Lake	63°52'02.8"S 57°37'06.7"W
E12	not determined	Antarctica	Vega Island	Esmeralda Lake	63°52'21.1"S 57°36'24.0"W
E13	not determined	Antarctica	Vega Island	Esmeralda Lake	63°52'21.1"S 57°36'24.0"W
E14	not determined	Antarctica	Vega Island	Pan Negro Lake	63°52'02.8"S 57°37'06.7"W
E15	not determined	Antarctica	Vega Island	Pan Negro Lake	63°52'02.8"S 57°37'06.7"W
E16	*Mayamaea atomus*	Antarctica	Vega Island	stream 168	63°53'55.7"S 57°36'48.0"W
E17	*Mayamaea* sp.	Antarctica	Vega Island	stream 2	63°51'55.7"S 57°37'22.6"W
E18	*Microcostatus* sp.	Antarctica	Vega Island	moraine lake 4	63°51'45.8"S 57°37'13.0"W
E19	*Microcostatus naumannii*	Antarctica	James Ross	Lachman Lake 1	63°47'59.5"S 57°48'31.4"W
E20	*Mayamaea* sp.	Antarctica	James Ross	Dulanek Lake	63°49'01.3"S 57°50'45.1"W
E21	not determined	Antarctica	James Ross	Black Lake	63°57'56.9"S 57°52'59.2"W
E22	*Mayamaea* sp.	Antarctica	Vega Island	stream 2	63°51'55.7"S 57°37'22.6"W
E23	*Psammothidium papilio*	Antarctica	Vega Island	Pan Negro Lake	63°52'02.8"S 57°37'06.7"W
G1	*Gomphonema* sp.	Antarctica	Vega Island	moraine lake 1	63°51'46.1"S 57°37'22.8"W
G2	*Gomphonema* sp.	Antarctica	Vega Island	moraine lake 1	63°51'46.1"S 57°37'22.8"W
G3	*Gomphonema* sp.	Antarctica	Vega Island	moraine lake 1	63°51'46.1"S 57°37'22.8"W
G4	*Gomphonema* sp.	Antarctica	James Ross	Black Lake	63°57'56.9"S 57°52'59.2"W
G5	*Gomphonema* sp.	Antarctica	Vega Island	stream 2	63°51'55.7"S 57°37'22.6"W
G6	*Gomphonema* sp.	Antarctica	Vega Island	moraine lake 5	63°51'42.1"S 57°37'40.3"W
G7	*Gomphonema* sp.	Antarctica	Vega Island	moraine lake 5	63°51'42.1"S 57°37'40.3"W
G8	*Gomphonema* sp.	Antarctica	James Ross	Black Lake	63°57'56.9"S 57°52'59.2"W
G9	*Gomphonema* sp.	Antarctica	Vega Island	pool 164	63°52'38.8"S 57°31'56.4"W
G10	*Gomphonema* sp.	Antarctica	Vega Island	pool 164	63°52'38.8"S 57°31'56.4"W
G11	*Gomphonema* sp.	Antarctica	James Ross	Black Lake	63°57'56.9"S 57°52'59.2"W
G12	*Gomphonema* sp.	Antarctica	Vega Island	Pan Negro Lake	63°52'02.8"S 57°37'06.7"W
G13	*Gomphonema* sp.	Antarctica	Vega Island	Pan Negro Lake	63°52'02.8"S 57°37'06.7"W
H1	*Hantzschia* sp.	Antarctica	James Ross	Black Lake	63°57'56.9"S 57°52'59.2"W
H2	*Hantzschia* sp.	Antarctica	James Ross	Black Lake	63°57'56.9"S 57°52'59.2"W
H3	*Hantzschia* sp.	Antarctica	James Ross	Lachman Lake 1	63°47'59.5"S 57°48'31.4"W
H4	*Hantzschia* sp.	Antarctica	James Ross	Lachman Lake 1	63°47'59.5"S 57°48'31.4"W
H5	*Hantzschia* sp.	Antarctica	Vega Island	stream 168	63°53'55.7"S 57°36'48.0"W
H6	*Hantzschia* sp.	Antarctica	Vega Island	moraine lake 2	63°51'45.4"S 57°37'18.1"W
H7	*Hantzschia* sp.	Antarctica	Vega Island	stream 168	63°53'55.7"S 57°36'48.0"W
H8	*Hantzschia* sp.	Antarctica	James Ross	Black Lake	63°57'56.9"S 57°52'59.2"W
L1	*Luticola permuticopsis*	Antarctica	Vega Island	Esmeralda Lake	63°52'21.1"S 57°36'24.0"W
L2	*Chamaepinnularia krookiformis*	Antarctica	Vega Island	Pan Negro Lake	63°52'02.8"S 57°37'06.7"W
L3	*Diadesmis* sp.	Antarctica	Vega Island	Pan Negro Lake	63°52'02.8"S 57°37'06.7"W
L4	*Planothidium capitatum*	Antarctica	Vega Island	moraine lake 1	63°51'46.1"S 57°37'22.8"W
L5	not determined	Antarctica	Vega Island	seepage pool 1	63°51'51.6"S 57°37'00.7"W
L6	*Humidophila australis*	Antarctica	James Ross	Black Lake	63°57'56.9"S 57°52'59.2"W
L7	*Planothidium capitatum*	Antarctica	Vega Island	moraine lake 1	63°51'46.1"S 57°37'22.8"W
N1	*Navicula* sp.	Antarctica	Vega Island	stream 168	63°53'55.7"S 57°36'48.0"W
N2	*Navicula* sp.	Antarctica	James Ross	Black Lake	63°57'56.9"S 57°52'59.2"W
N3	*Navicula* sp.	Antarctica	Vega Island	Pan Negro Lake	63°52'02.8"S 57°37'06.7"W
N7	*Nitzschia* sp.	Antarctica	Vega Island	Esmeralda Lake	63°52'21.1"S 57°36'24.0"W
N12	*Navicula australoshetlandica*	Antarctica	Vega Island	Pan Negro Lake	63°52'02.8"S 57°37'06.7"W

code	species	region	island	location	GPS
N13	*Navicula australoshetlandica*	Antarctica	Vega Island	Pan Negro Lake	63°52'02.8"S 57°37'06.7"W
N14	*Navicula* sp.	Antarctica	Vega Island	stream 168	63°53'55.7"S 57°36'48.0"W
N15	*Nitzschia* sp.	Antarctica	James Ross	Lachman Lake 1	63°47'59.5"S 57°48'31.4"W
N16	*Navicula* sp.	Antarctica	James Ross	Lachman Lake 1	63°47'59.5"S 57°48'31.4"W
N17	*Navicula* sp.	Antarctica	Vega Island	moraine lake 2	63°51'45.4"S 57°37'18.1"W
N18	*Navicula* sp.	Antarctica	Vega Island	moraine lake 2	63°51'45.4"S 57°37'18.1"W
N19	*Navicula gregaria*	Antarctica	Vega Island	moraine lake 2	63°51'45.4"S 57°37'18.1"W
N20	*Navicula* sp.	Antarctica	Vega Island	moraine lake 2	63°51'45.4"S 57°37'18.1"W
N21	*Navicula* sp.	Antarctica	Vega Island	pool 164	63°52'38.8"S 57°31'56.4"W
N22	*Navicula* sp.	Antarctica	Vega Island	pool 164	63°52'38.8"S 57°31'56.4"W
N23	*Navicula* sp.	Antarctica	Vega Island	pool 164	63°52'38.8"S 57°31'56.4"W
N25	*Stauroneis* sp.	Antarctica	James Ross	Black Lake	63°57'56.9"S 57°52'59.2"W
N26	*Navicula cremeri*	Antarctica	Vega Island	Pan Negro Lake	63°52'02.8"S 57°37'06.7"W
N27	*Navicula* sp.	Antarctica	Vega Island	Pan Negro Lake	63°52'02.8"S 57°37'06.7"W
N28	*Navicula australoshetlandica*	Antarctica	Vega Island	moraine lake 1	63°51'46.1"S 57°37'22.8"W
N29	*Navicula* sp.	Antarctica	Vega Island	Esmeralda Lake	63°52'21.1"S 57°36'24.0"W
P1	*Pinnularia borealis*	Antarctica	James Ross	Black Lake	63°57'56.9"S 57°52'59.2"W
P2	*Pinnularia borealis*	Antarctica	James Ross	Black Lake	63°57'56.9"S 57°52'59.2"W
P3	*Pinnularia borealis*	Antarctica	James Ross	Black Lake	63°57'56.9"S 57°52'59.2"W
P4	*Pinnularia* sp.	Antarctica	James Ross	Black Lake	63°57'56.9"S 57°52'59.2"W
P5	*Pinnularia* sp.	Antarctica	James Ross	Black Lake	63°57'56.9"S 57°52'59.2"W
P6	*Chamaepinnularia* sp.	Antarctica	Vega Island	Pan Negro Lake	63°52'02.8"S 57°37'06.7"W
P7	*Pinnularia* sp.	Antarctica	Vega Island	stream 168	63°53'55.7"S 57°36'48.0"W
P8	*Chamaepinnularia* sp.	Antarctica	Vega Island	stream 168	63°53'55.7"S 57°36'48.0"W
P9	*Pinnularia* sp.	Antarctica	Vega Island	stream 168	63°53'55.7"S 57°36'48.0"W
P10	*Chamaepinnularia* sp.	Antarctica	Vega Island	stream 2	63°51'55.7"S 57°37'22.6"W
P11	*Pinnularia* sp.	Antarctica	Vega Island	moraine lake 4	63°51'45.8"S 57°37'13.0"W
P12	*Pinnularia* sp.	Antarctica	James Ross	Black Lake	63°57'56.9"S 57°52'59.2"W
P13	*Chamaepinnularia* sp.	Antarctica	Vega Island	Pan Negro Lake	63°52'02.8"S 57°37'06.7"W
P14	*Pinnularia borealis*	Antarctica	James Ross	Black Lake	63°57'56.9"S 57°52'59.2"W
P15	*Chamaepinnularia krookiformis*	Antarctica	Vega Island	Esmeralda Lake	63°52'21.1"S 57°36'24.0"W
P16	*Chamaepinnularia* sp.	Antarctica	Vega Island	Esmeralda Lake	63°52'21.1"S 57°36'24.0"W
P17	*Chamaepinnularia krookiformis*	Antarctica	James Ross	Black Lake	63°57'56.9"S 57°52'59.2"W
P18	*Chamaepinnularia krookiformis*	Antarctica	Vega Island	moraine lake 4	63°51'45.8"S 57°37'13.0"W
P19	*Chamaepinnularia* sp.	Antarctica	Vega Island	Esmeralda Lake	63°52'21.1"S 57°36'24.0"W
P20	*Pinnularia* sp.	Antarctica	Vega Island	moraine lake 5	63°51'42.1"S 57°37'40.3"W
P21	*Pinnularia* sp.	Antarctica	Vega Island	Esmeralda Lake	63°52'21.1"S 57°36'24.0"W
P22	*Chamaepinnularia* sp.	Antarctica	Vega Island	Esmeralda Lake	63°52'21.1"S 57°36'24.0"W
P23	*Denticula* sp.	Antarctica	Vega Island	Esmeralda Lake	63°52'21.1"S 57°36'24.0"W
P24	*Chamaepinnularia* sp.	Antarctica	James Ross	Lachman Lake 1	63°47'59.5"S 57°48'31.4"W
P25	*Pinnularia* sp.	Antarctica	James Ross	Lachman Lake 1	63°47'59.5"S 57°48'31.4"W
P26	*Pinnularia* sp.	Antarctica	James Ross	Black Lake	63°57'56.9"S 57°52'59.2"W
P27	*Pinnularia* sp.	Antarctica	James Ross	Black Lake	63°57'56.9"S 57°52'59.2"W
P28	*Pinnularia* sp.	Antarctica	James Ross	Black Lake	63°57'56.9"S 57°52'59.2"W
P29	*Pinnularia* sp.	Antarctica	Vega Island	Pan Negro Lake	63°52'02.8"S 57°37'06.7"W
P30	*Chamaepinnularia krookiformis*	Antarctica	Vega Island	Pan Negro Lake	63°52'02.8"S 57°37'06.7"W
P31	*Chamaepinnularia* sp.	Antarctica	Vega Island	Pan Negro Lake	63°52'02.8"S 57°37'06.7"W
R1	not determined	Antarctica	Vega Island	moraine lake 1	63°51'46.1"S 57°37'22.8"W
R2	*Nitzschia* sp.	Antarctica	Vega Island	seepage pool 1	63°51'51.6"S 57°37'00.7"W
R3	not determined	Antarctica	James Ross	Black Lake	63°57'56.9"S 57°52'59.2"W
R4	not determined	Antarctica	Vega Island	moraine lake 1	63°51'46.1"S 57°37'22.8"W
S1	*Nitzschia* sp.	Antarctica	James Ross	Black Lake	63°57'56.9"S 57°52'59.2"W
S2	*Nitzschia* sp.	Antarctica	James Ross	Black Lake	63°57'56.9"S 57°52'59.2"W
S3	*Nitzschia* sp.	Antarctica	Vega Island	Pan Negro Lake	63°52'02.8"S 57°37'06.7"W
S4	*Nitzschia* sp.	Antarctica	Vega Island	moraine lake 5	63°51'42.1"S 57°37'40.3"W
S5	*Nitzschia* sp.	Antarctica	James Ross	Black Lake	63°57'56.9"S 57°52'59.2"W
S6	*Nitzschia* sp.	Antarctica	James Ross	Black Lake	63°57'56.9"S 57°52'59.2"W
S7	*Nitzschia* sp.	Antarctica	Vega Island	seepage pool 1	63°51'51.6"S 57°37'00.7"W
S8	*Nitzschia* sp.	Antarctica	Vega Island	Esmeralda Lake	63°52'21.1"S 57°36'24.0"W
S9	*Nitzschia* sp.	Antarctica	Vega Island	moraine lake 1	63°51'46.1"S 57°37'22.8"W
S10	*Nitzschia* sp.	Antarctica	Vega Island	Esmeralda Lake	63°52'21.1"S 57°36'24.0"W
S11	*Nitzschia* sp.	Antarctica	Vega Island	seepage pool 1	63°51'51.6"S 57°37'00.7"W
S12	*Nitzschia* sp.	Antarctica	James Ross	Black Lake	63°57'56.9"S 57°52'59.2"W
S13	*Nitzschia* sp.	Antarctica	Vega Island	moraine lake 5	63°51'42.1"S 57°37'40.3"W
S14	*Nitzschia* sp.	Antarctica	Vega Island	Pan Negro Lake	63°52'02.8"S 57°37'06.7"W
S15	*Nitzschia* sp.	Antarctica	Vega Island	stream 168	63°53'55.7"S 57°36'48.0"W

code	species	region	island	location	GPS
S16	*Nitzschia perminuta*	Antarctica	Vega Island	stream 168	63°53'55.7"S 57°36'48.0"W
S17	*Nitzschia* sp.	Antarctica	Vega Island	moraine lake 4	63°51'45.8"S 57°37'13.0"W
S18	*Nitzschia* sp.	Antarctica	James Ross	Lachman Lake 1	63°47'59.5"S 57°48'31.4"W
S19	*Nitzschia* sp.	Antarctica	James Ross	Black Lake	63°57'56.9"S 57°52'59.2"W
S20	*Nitzschia* sp.	Antarctica	Vega Island	Esmeralda Lake	63°52'21.1"S 57°36'24.0"W
S21	*Nitzschia* sp.	Antarctica	James Ross	Lachman Lake 1	63°47'59.5"S 57°48'31.4"W
S22	*Nitzschia* sp.	Antarctica	James Ross	Black Lake	63°57'56.9"S 57°52'59.2"W
S23	*Nitzschia* sp.	Antarctica	Vega Island	moraine lake 5	63°51'42.1"S 57°37'40.3"W
S24	*Nitzschia paleacea*	Antarctica	Vega Island	moraine lake 5	63°51'42.1"S 57°37'40.3"W
S25	*Nitzschia* sp.	Antarctica	Vega Island	Pan Negro Lake	63°52'02.8"S 57°37'06.7"W
S26	*Nitzschia* sp.	Antarctica	Vega Island	seepage pool 1	63°51'51.6"S 57°37'00.7"W
S27	*Nitzschia* sp.	Antarctica	James Ross	Black Lake	63°57'56.9"S 57°52'59.2"W
S28	*Nitzschia* cf. *paleacea*	Antarctica	James Ross	Black Lake	63°57'56.9"S 57°52'59.2"W
S29	*Nitzschia* sp.	Antarctica	Vega Island	moraine lake 2	63°51'45.4"S 57°37'18.1"W
S30	*Nitzschia* sp.	Antarctica	Vega Island	moraine lake 2	63°51'45.4"S 57°37'18.1"W
S31	*Nitzschia* sp.	Antarctica	Vega Island	pool 164	63°52'38.8"S 57°31'56.4"W
T1	*Navicula gregaria*	Antarctica	Vega Island	moraine lake 1	63°51'46.1"S 57°37'22.8"W
T2	*Achnanthes muelleri*	Antarctica	Vega Island	stream 168	63°53'55.7"S 57°36'48.0"W
T3	not determined	Antarctica	Vega Island	Pan Negro Lake	63°52'02.8"S 57°37'06.7"W
T4	not determined	Antarctica	Vega Island	Pan Negro Lake	63°52'02.8"S 57°37'06.7"W
T5	*Stauroneis jarensis*	Antarctica	James Ross	Black Lake	63°57'56.9"S 57°52'59.2"W
AM1	*Amphora* sp.	Arctic	Spitsbergen	Ragnarbreen, lake 2	78°44'21.4"N 16°38'53.5"E
AM2	*Amphora* sp.	Arctic	Spitsbergen	Mathiesondalen, lake 1	78°34'52.2"N 16°33'00.0"E
AM3	*Euocconeis* sp.	Arctic	Spitsbergen	Garmaksla Lake	78°36'16.0"N 16°20'14.7"E
AM4	*Amphora* sp.	Arctic	Spitsbergen	Garmaksla Lake	78°36'16.0"N 16°20'14.7"E
AM5	*Cymbella* sp.	Arctic	Spitsbergen	Garmaksla Lake	78°36'16.0"N 16°20'14.7"E
AM6	*Amphora* sp.	Arctic	Spitsbergen	Mimardalen, lake 1	78°37'27.8"N 15°58'02.7"E
AM7	*Encyonema* sp.	Arctic	Spitsbergen	Mimardalen, stream	78°36'07.2"N 15°54'09.7"E
AM8	*Encyonema* sp.	Arctic	Spitsbergen	Mathiesondalen, lake 1	78°34'52.2"N 16°33'00.0"E
AM9	*Cymbella* sp.	Arctic	Spitsbergen	Mathiesondalen, lake 2	78°33'47.4"N 16°35'14.1"E
EU1	*Navicula* cf. *bjoernoeyaensis*	Arctic	Spitsbergen	Ragnarbreen, lake 3	78°44'21.4"N 16°38'53.5"E
EU2	*Navicula* sp.	Arctic	Spitsbergen	Ragnarbreen, lake 2	78°44'21.4"N 16°38'53.5"E
EU3	*Achnanthidium* sp.	Arctic	Spitsbergen	Ragnarbreen, lake 2	78°44'21.4"N 16°38'53.5"E
EU4	*Achnanthidium* sp.	Arctic	Spitsbergen	Nordenskiöldbreen, lake	78°38'08.5"N 16°51'52.6"E
EU5	*Achnanthidium* sp.	Arctic	Spitsbergen	Nordenskiöldbreen, lake	78°38'08.5"N 16°51'52.6"E
EU6	*Achnanthidium* sp.	Arctic	Spitsbergen	Nordenskiöldbreen, lake	78°38'08.5"N 16°51'52.6"E
EU7	*Achnanthidium* sp.	Arctic	Spitsbergen	Nordenskiöldbreen, lake	78°38'08.5"N 16°51'52.6"E
EU8	*Achnanthidium* sp.	Arctic	Spitsbergen	Nordenskiöldbreen, lake	78°38'08.5"N 16°51'52.6"E
EU8	*Achnanthidium* sp.	Arctic	Spitsbergen	Nordenskiöldbreen, lake	78°38'08.5"N 16°51'52.6"E
EU9	*Achnanthidium* sp.	Arctic	Spitsbergen	Nordenskiöldbreen, lake	78°38'08.5"N 16°51'52.6"E
EU9	*Achnanthidium* sp.	Arctic	Spitsbergen	Nordenskiöldbreen, lake	78°38'08.5"N 16°51'52.6"E
EU10	*Achnanthidium* sp.	Arctic	Spitsbergen	Mathiesondalen, lake 3	78°33'33.4"N 16°36'54.2"E
EU12	*Achnanthidium* sp.	Arctic	Spitsbergen	Garmaksla Lake	78°36'16.0"N 16°20'14.7"E
EU13	*Achnanthidium* sp.	Arctic	Spitsbergen	Garmaksla Lake	78°36'16.0"N 16°20'14.7"E
EU14	*Achnanthidium* sp.	Arctic	Spitsbergen	Mimardalen, lake 1	78°37'27.8"N 15°58'02.7"E
EU15	*Achnanthidium* sp.	Arctic	Spitsbergen	Mimardalen, lake 1	78°37'27.8"N 15°58'02.7"E
EU16	*Achnanthidium* sp.	Arctic	Spitsbergen	Mimardalen, lake 1	78°37'27.8"N 15°58'02.7"E
EU17	*Achnanthidium* sp.	Arctic	Spitsbergen	Mimardalen, lake 1	78°37'27.8"N 15°58'02.7"E
EU18	*Achnanthidium* sp.	Arctic	Spitsbergen	Ragnarbreen, lake 1	78°44'21.4"N 16°38'53.5"E
EU19	*Achnanthidium* sp.	Arctic	Spitsbergen	Ragnarbreen, lake 1	78°44'21.4"N 16°38'53.5"E
EU20	*Achnanthidium* sp.	Arctic	Spitsbergen	Ragnarbreen, lake 1	78°44'21.4"N 16°38'53.5"E
EU21	*Achnanthidium* sp.	Arctic	Spitsbergen	Mimardalen, stream	78°36'07.2"N 15°54'09.7"E
EU22	*Achnanthidium* sp.	Arctic	Spitsbergen	Mimardalen, stream	78°36'07.2"N 15°54'09.7"E
EU23	*Achnanthidium* sp.	Arctic	Spitsbergen	Mimardalen, stream	78°36'07.2"N 15°54'09.7"E
EU24	*Achnanthidium* sp.	Arctic	Spitsbergen	Mimardalen, stream	78°36'07.2"N 15°54'09.7"E
EU25	*Achnanthidium* sp.	Arctic	Spitsbergen	Mimardalen, stream	78°36'07.2"N 15°54'09.7"E
EU27	*Achnanthidium* sp.	Arctic	Spitsbergen	Mathiesondalen, lake 2	78°33'47.4"N 16°35'14.1"E
EU28	*Achnanthidium* sp.	Arctic	Spitsbergen	Mathiesondalen, lake 2	78°33'47.4"N 16°35'14.1"E
EU29	*Achnanthidium* sp.	Arctic	Spitsbergen	Ragnarbreen, lake 3	78°44'21.4"N 16°38'53.5"E
FR1	*Staurosira* sp.	Arctic	Spitsbergen	Garmaksla Lake	78°36'16.0"N 16°20'14.7"E
FR2	*Fragillaria* sp.	Arctic	Spitsbergen	Garmaksla Lake	78°36'16.0"N 16°20'14.7"E
FR3	*Fragillaria* sp.	Arctic	Spitsbergen	Mimardalen, lake 1	78°37'27.8"N 15°58'02.7"E
FR4	*Fragillaria* sp.	Arctic	Spitsbergen	Mimardalen, lake 1	78°37'27.8"N 15°58'02.7"E
FR5	*Nitzschia* sp.	Arctic	Spitsbergen	Mathiesondalen, lake 1	78°34'52.2"N 16°33'00.0"E
FR6	*Fragillaria* sp.	Arctic	Spitsbergen	Mathiesondalen, lake 2	78°33'47.4"N 16°35'14.1"E
MM1	not determined	Arctic	Spitsbergen	Nordenskiöldbreen, lake	78°38'08.5"N 16°51'52.6"E

code	species	region	island	location	GPS
MM2	not determined	Arctic	Spitsbergen	Nordenskiöldbreen, lake	78°38'08.5"N 16°51'52.6"E
MM3	not determined	Arctic	Spitsbergen	Nordenskiöldbreen, lake	78°38'08.5"N 16°51'52.6"E
MM4	*Navicula* sp.	Arctic	Spitsbergen	Mathiesondalen, lake 3	78°33'33.4"N 16°36'54.2"E
MM5	not determined	Arctic	Spitsbergen	Mathiesondalen, lake 3	78°33'33.4"N 16°36'54.2"E
MM6	not determined	Arctic	Spitsbergen	Mathiesondalen, lake 3	78°33'33.4"N 16°36'54.2"E
MM7	not determined	Arctic	Spitsbergen	Mathiesondalen, lake 3	78°33'33.4"N 16°36'54.2"E
MM8	not determined	Arctic	Spitsbergen	Mathiesondalen, lake 3	78°33'33.4"N 16°36'54.2"E
MM9	*Diploneis ovalis*	Arctic	Spitsbergen	Mathiesondalen, lake 3	78°33'33.4"N 16°36'54.2"E
MM10	not determined	Arctic	Spitsbergen	Garmaksla Lake	78°36'16.0"N 16°20'14.7"E
MM11	not determined	Arctic	Spitsbergen	Garmaksla Lake	78°36'16.0"N 16°20'14.7"E
MM12	not determined	Arctic	Spitsbergen	Mimardalen, lake 1	78°37'27.8"N 15°58'02.7"E
MM13	not determined	Arctic	Spitsbergen	Mimardalen, stream	78°36'07.2"N 15°54'09.7"E
MM14	not determined	Arctic	Spitsbergen	Mathiesondalen, lake 1	78°34'52.2"N 16°33'00.0"E
MM15	*Psammothidium* sp.	Arctic	Spitsbergen	Mathiesondalen, lake 1	78°34'52.2"N 16°33'00.0"E
MM16	not determined	Arctic	Spitsbergen	Mathiesondalen, lake 1	78°34'52.2"N 16°33'00.0"E
MM17	not determined	Arctic	Spitsbergen	Mathiesondalen, lake 2	78°33'47.4"N 16°35'14.1"E
MM18	not determined	Arctic	Spitsbergen	Ragnarbreen, lake 2	78°44'21.4"N 16°38'53.5"E
MM19	not determined	Arctic	Spitsbergen	Ragnarbreen, lake 3	78°44'21.4"N 16°38'53.5"E
MM21	not determined	Arctic	Spitsbergen	Ragnarbreen, lake 3	78°44'21.4"N 16°38'53.5"E
MM22	not determined	Arctic	Spitsbergen	Mathiesondalen, lake 1	78°34'52.2"N 16°33'00.0"E
NA1	*Nitzschia* sp.	Arctic	Spitsbergen	Ragnarbreen, lake 2	78°44'21.4"N 16°38'53.5"E
NA2	*Navicula* sp.	Arctic	Spitsbergen	Ragnarbreen, lake 2	78°44'21.4"N 16°38'53.5"E
NA3	*Navicula* sp.	Arctic	Spitsbergen	Mathiesondalen, lake 3	78°33'33.4"N 16°36'54.2"E
NA4	*Navicula* sp.	Arctic	Spitsbergen	Mathiesondalen, lake 3	78°33'33.4"N 16°36'54.2"E
NA5	*Nitzschia* sp.	Arctic	Spitsbergen	Ragnarbreen, lake 1	78°44'21.4"N 16°38'53.5"E
NA6	*Navicula* sp.	Arctic	Spitsbergen	Ragnarbreen, lake 1	78°44'21.4"N 16°38'53.5"E
NA7	*Navicula* sp.	Arctic	Spitsbergen	Ragnarbreen, lake 1	78°44'21.4"N 16°38'53.5"E
NA8	*Navicula* sp.	Arctic	Spitsbergen	Ragnarbreen, lake 1	78°44'21.4"N 16°38'53.5"E
NA9	*Navicula* sp.	Arctic	Spitsbergen	Ragnarbreen, lake 1	78°44'21.4"N 16°38'53.5"E
NA10	*Achnanthidium* sp.	Arctic	Spitsbergen	Mimardalen, stream	78°36'07.2"N 15°54'09.7"E
NA11	*Navicula* sp.	Arctic	Spitsbergen	Mathiesondalen, lake 2	78°33'47.4"N 16°35'14.1"E
NA12	*Nitzschia* sp.	Arctic	Spitsbergen	Mathiesondalen, lake 2	78°33'47.4"N 16°35'14.1"E
NA13	*Navicula* sp.	Arctic	Spitsbergen	Ragnarbreen, lake 2	78°44'21.4"N 16°38'53.5"E
NA14	*Navicula* sp.	Arctic	Spitsbergen	Ragnarbreen, lake 2	78°44'21.4"N 16°38'53.5"E
PI1	*Pinnularia borealis*	Arctic	Spitsbergen	Mathiesondalen, lake 3	78°33'33.4"N 16°36'54.2"E
PI2	*Pinnularia* sp.	Arctic	Spitsbergen	Mathiesondalen, lake 3	78°33'33.4"N 16°36'54.2"E
PI3	*Pinnularia* sp.	Arctic	Spitsbergen	Mathiesondalen, lake 3	78°33'33.4"N 16°36'54.2"E
PI4	*Caloneis* sp.	Arctic	Spitsbergen	Garmaksla Lake	78°36'16.0"N 16°20'14.7"E
PI5	*Pinnularia* sp.	Arctic	Spitsbergen	Garmaksla Lake	78°36'16.0"N 16°20'14.7"E
PI6	*Pinnularia* sp.	Arctic	Spitsbergen	Ragnarbreen, lake 1	78°44'21.4"N 16°38'53.5"E
PI7	*Caloneis* sp.	Arctic	Spitsbergen	Ragnarbreen, lake 1	78°44'21.4"N 16°38'53.5"E
PI8	*Pinnularia borealis*	Arctic	Spitsbergen	Ragnarbreen, lake 2	78°44'21.4"N 16°38'53.5"E
PI9	*Pinnularia* sp.	Arctic	Spitsbergen	Mathiesondalen, lake 3	78°33'33.4"N 16°36'54.2"E
SP1	*Asterionella* sp.	Arctic	Spitsbergen	Nordenskiöldbreen, lake	78°38'08.5"N 16°51'52.6"E
SP2	*Navicula* sp.	Arctic	Spitsbergen	Mathiesondalen, lake 3	78°33'33.4"N 16°36'54.2"E
SP3	not determined	Arctic	Spitsbergen	Mathiesondalen, lake 3	78°33'33.4"N 16°36'54.2"E
SP4	not determined	Arctic	Spitsbergen	Mathiesondalen, lake 3	78°33'33.4"N 16°36'54.2"E
SP5	not determined	Arctic	Spitsbergen	Mathiesondalen, lake 3	78°33'33.4"N 16°36'54.2"E
SP6	*Fragillaria* sp.	Arctic	Spitsbergen	Garmaksla Lake	78°36'16.0"N 16°20'14.7"E
SP7	*Fragillaria* sp.	Arctic	Spitsbergen	Garmaksla Lake	78°36'16.0"N 16°20'14.7"E
SP8	*Hantzschia* sp.	Arctic	Spitsbergen	Mimardalen, stream	78°36'07.2"N 15°54'09.7"E
SP9	*Hantzschia* sp.	Arctic	Spitsbergen	Mimardalen, stream	78°36'07.2"N 15°54'09.7"E
SP10	*Denticula* sp.	Arctic	Spitsbergen	Mimardalen, stream	78°36'07.2"N 15°54'09.7"E
SP11	not determined	Arctic	Spitsbergen	Mathiesondalen, lake 1	78°34'52.2"N 16°33'00.0"E
SP12	*Hantzschia* sp.	Arctic	Spitsbergen	Mathiesondalen, lake 1	78°34'52.2"N 16°33'00.0"E
SP13	*Nitzschia* sp.	Arctic	Spitsbergen	Mathiesondalen, lake 1	78°34'52.2"N 16°33'00.0"E

APPENDIX 2. LIST OF PUBLICATIONS USED FOR THE DETERMINATION OF EXPERIMENTAL STRAINS.

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APPENDIX 3.

Pinnularia catenaborealis sp. nov. (Bacillariophyceae), a unique chain-forming diatom species from James Ross Island and Vega Island (Maritime Antarctica)

Eveline Pinseel[1,2,3*], Eva Hejduková[4], Pieter Vanormelingen[1], Kateřina Kopalová[4], Wim Vyverman[1] & Bart Van de Vijver[2,3]

(accepted to Phycologia)

[1] Protistology & Aquatic Ecology (PAE), Department of Biology, Faculty of Science, Ghent University, Krijgslaan 281-S8, B–9000 Ghent, Belgium
[2] Department of Bryophyta & Thallophyta, Botanic Garden Meise, Nieuwelaan 38, B–1860 Meise, Belgium
[3] Ecosystem Management Research Group (ECOBE), Department of Biology, Faculty of Science, University of Antwerp, Universiteitsplein 1, B–2610 Wilrijk, Antwerpen, Belgium
[4] Department of Ecology, Faculty of Science, Charles University in Prague, Viničná 7, CZ–12844 Prague 2, Czech Republic
*Corresponding author (eveline.pinseel@ugent.be)

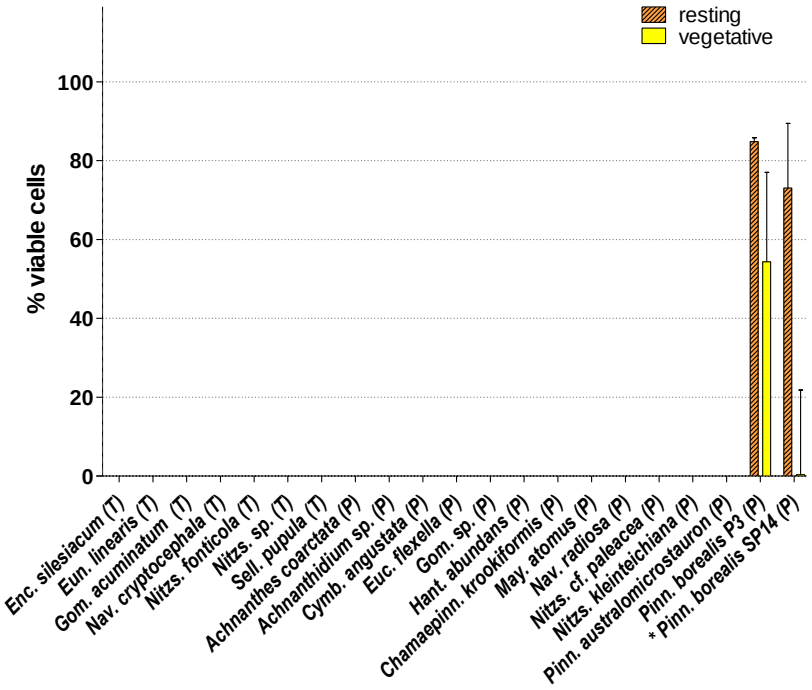
Abstract

Recently, a detailed survey of the Maritime Antarctic diatom flora using a fine-grained taxonomy resulted in the description of many new species belonging to *Pinnularia* in general and the section Distantes, including the *P. borealis* species complex, in particular. Moreover, DNA-based studies of *P. borealis* revealed that many more species need to be discovered and described within this complex. During a survey of the freshwater littoral diatom flora of Maritime Antarctic James Ross Island (Ulu Peninsula) and Vega Island an unknown chain-forming Distantes taxon belonging to the *P. borealis* species complex was cultured from three different localities. Molecular phylogenies based on the nuclear encoded D1–D3 LSU rDNA and plastid *rbc*L genes revealed that all cultures belong to a distinct highly supported lineage within the *P. borealis* species complex. *Pinnularia catenaborealis* sp. nov. is morphologically characterized by the presence of small spines located on a raised, thin silica ridge that almost entirely surrounds the valve face near the valve face/mantle junction and the presence of small silica plates near the apices. In culture, *P. catenaborealis* forms chains of several tens of cells and in oxidised natural material, chains up to seven frustules have been observed. *Pinnularia catenaborealis* is described from the littoral zone of freshwater Black Lake (Ulu Peninsula, James Ross Island) and has also been observed on nearby Vega Island. Although *P. borealis* is generally regarded as a (semi-)terrestrial diatom complex mainly occurring in (moist) soils and mosses, *P. catenaborealis* was found in freshwater habitats with an alkaline pH and low conductivity.

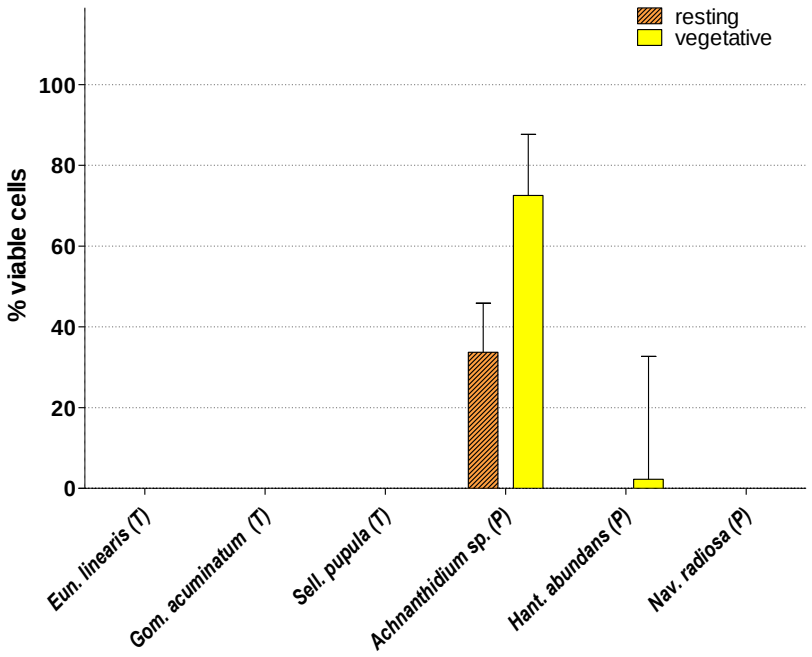
Key words: colony-formation, cultures, molecular phylogeny, nuclear encoded LSU rDNA, *Pinnularia borealis*, *rbc*L

APPENDIX 4. AVERAGE SURVIVABILITY OF THE STRAINS FOR ALL TREATMENTS (WHISKERS: STANDARD DEVIATION).

1. Abrupt freezing to –20 °C followed by slow thawing (Part I).

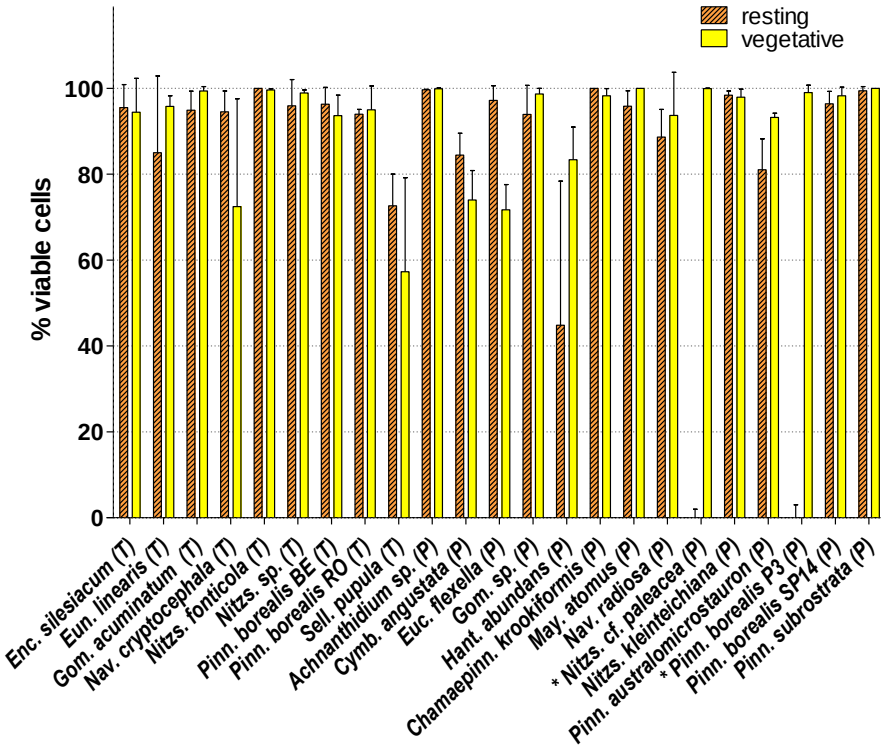


2. Cryopreservation – abrupt freezing to –180 °C using cryoprotective substance DMSO followed by fast thawing (Part I).

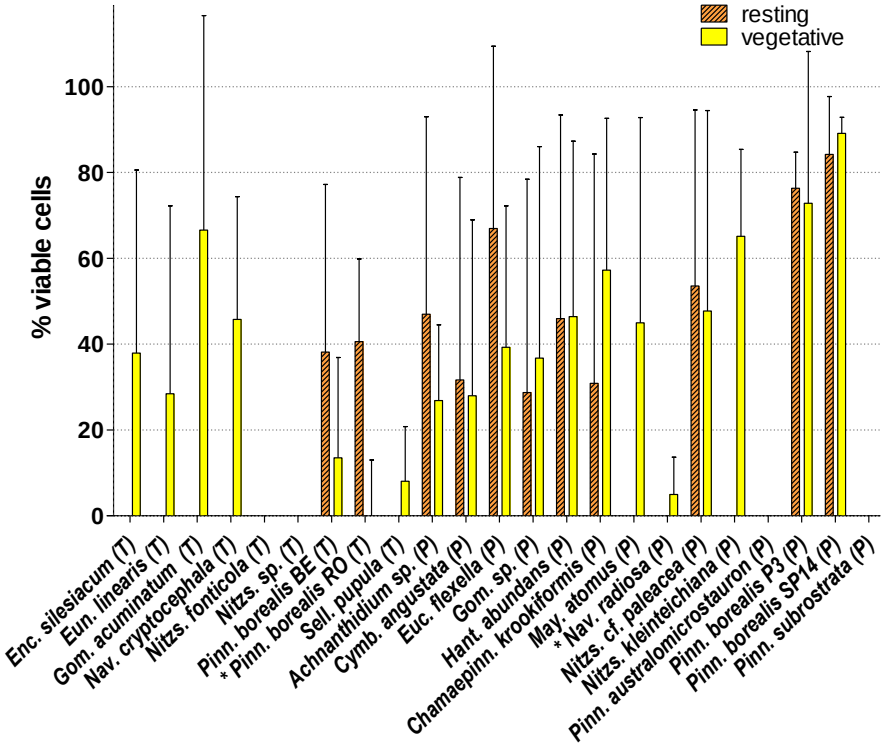


* very low densities of living cells were observed in at least one out of three replicates

3. Continuous freezing to –4 °C followed by fast thawing (Part II).

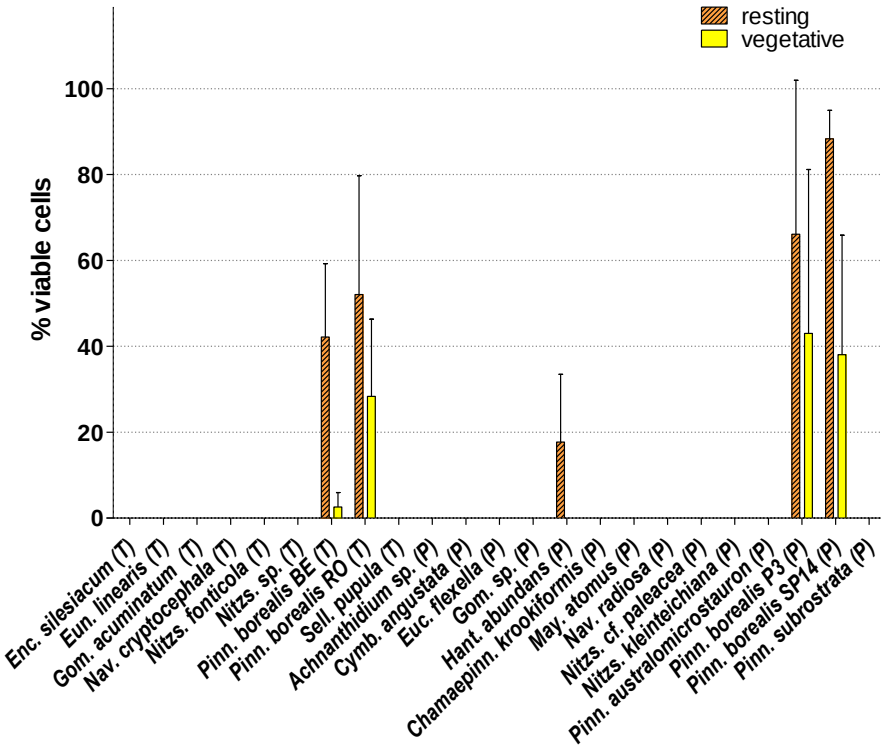


4. Continuous freezing to –20 °C followed by fast thawing (Part II).

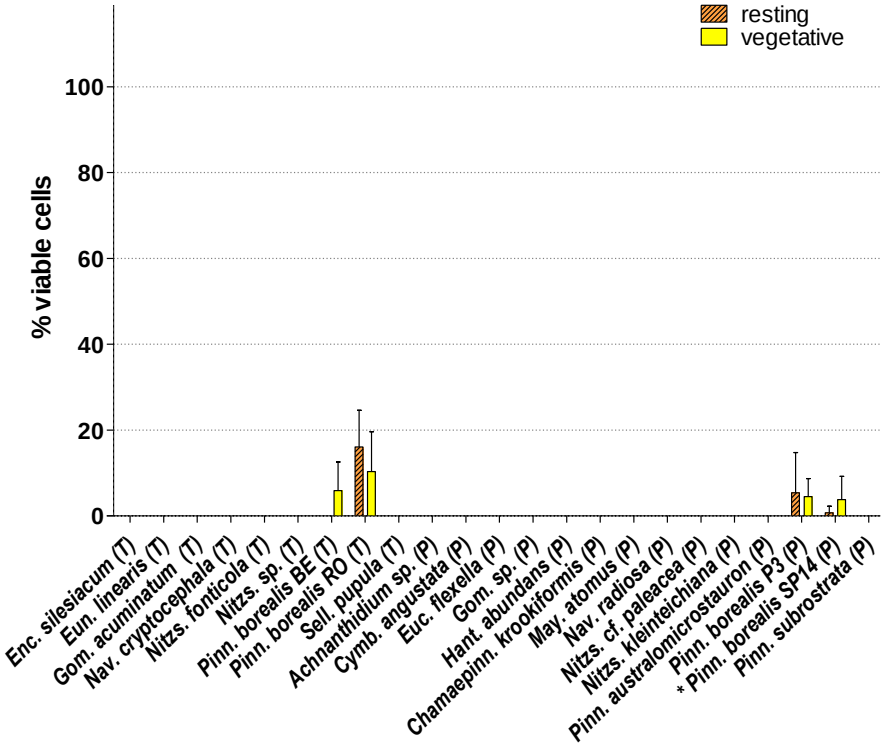


* very low densities of living cells were observed in at least one out of three replicates

5. Continuous freezing to –40 °C followed by fast thawing (Part II).



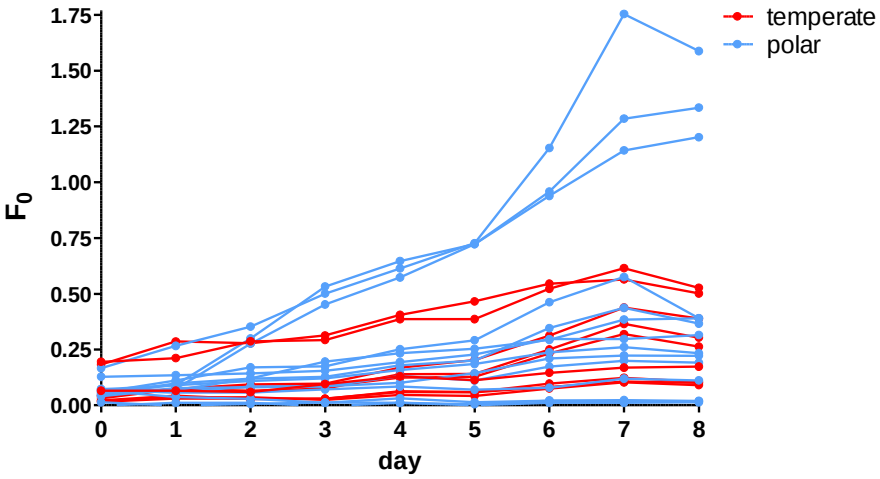
6. Abrupt freezing to –180 °C followed by fast thawing (Part II).

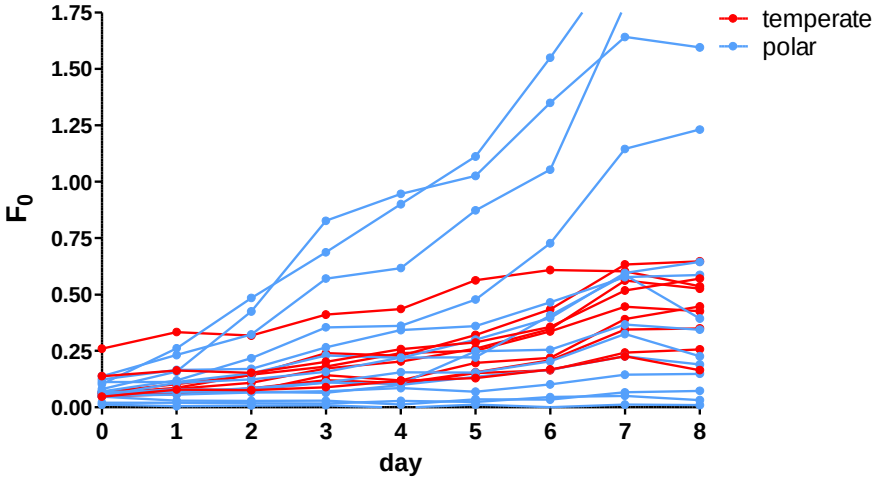


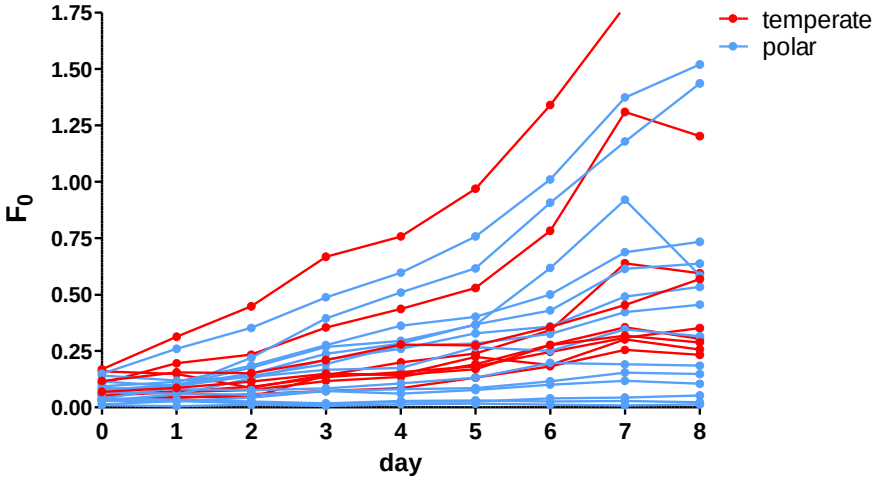
* very low densities of living cells were observed in at least one out of three replicates

APPENDIX 5. GROWTH CURVES OF THE STRAINS BASED ON FLUORESCENCE MEASUREMENTS FOR THE THREE REPLICATES.

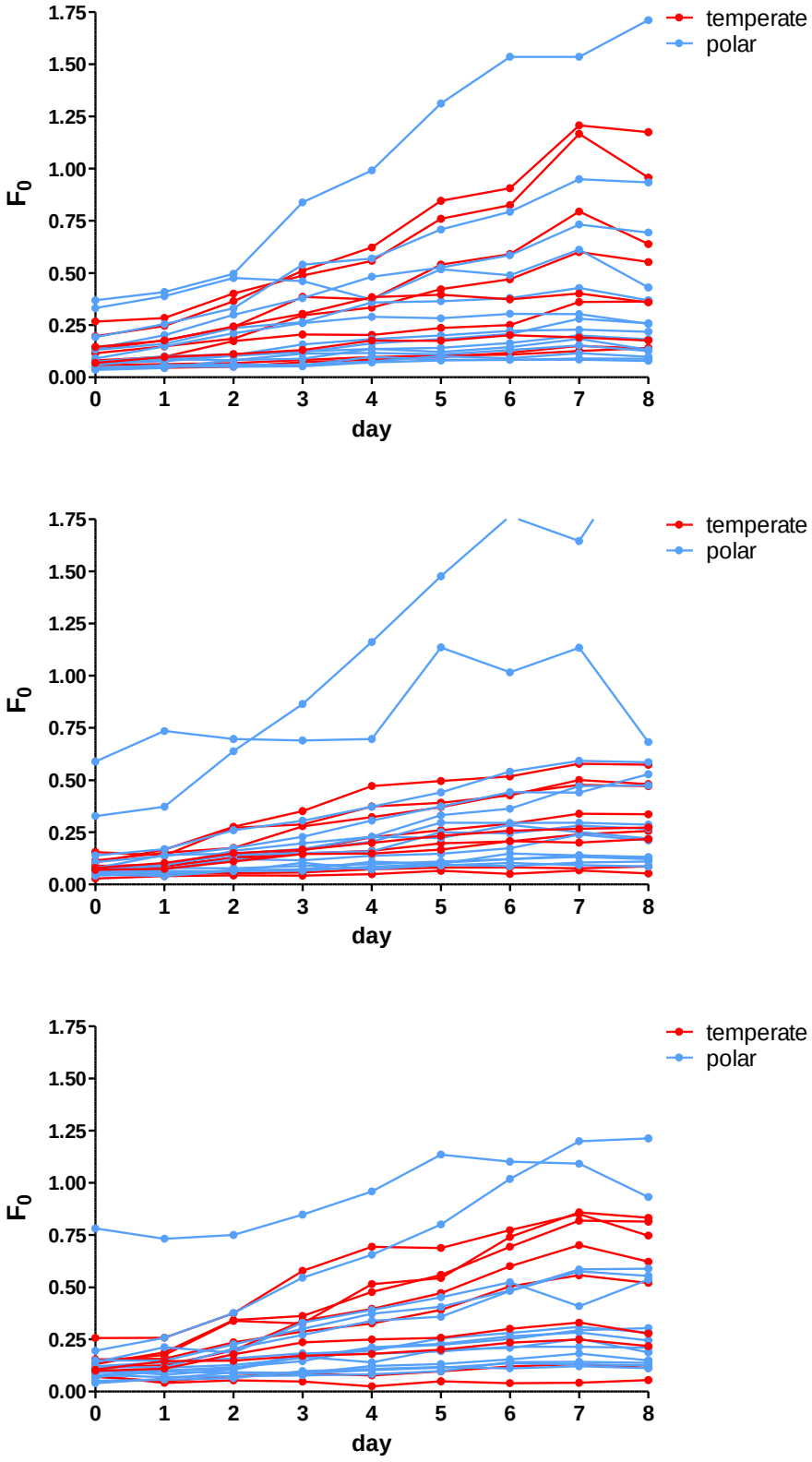
1. a) Continuous freezing to –4 °C followed by fast thawing (resting cells).



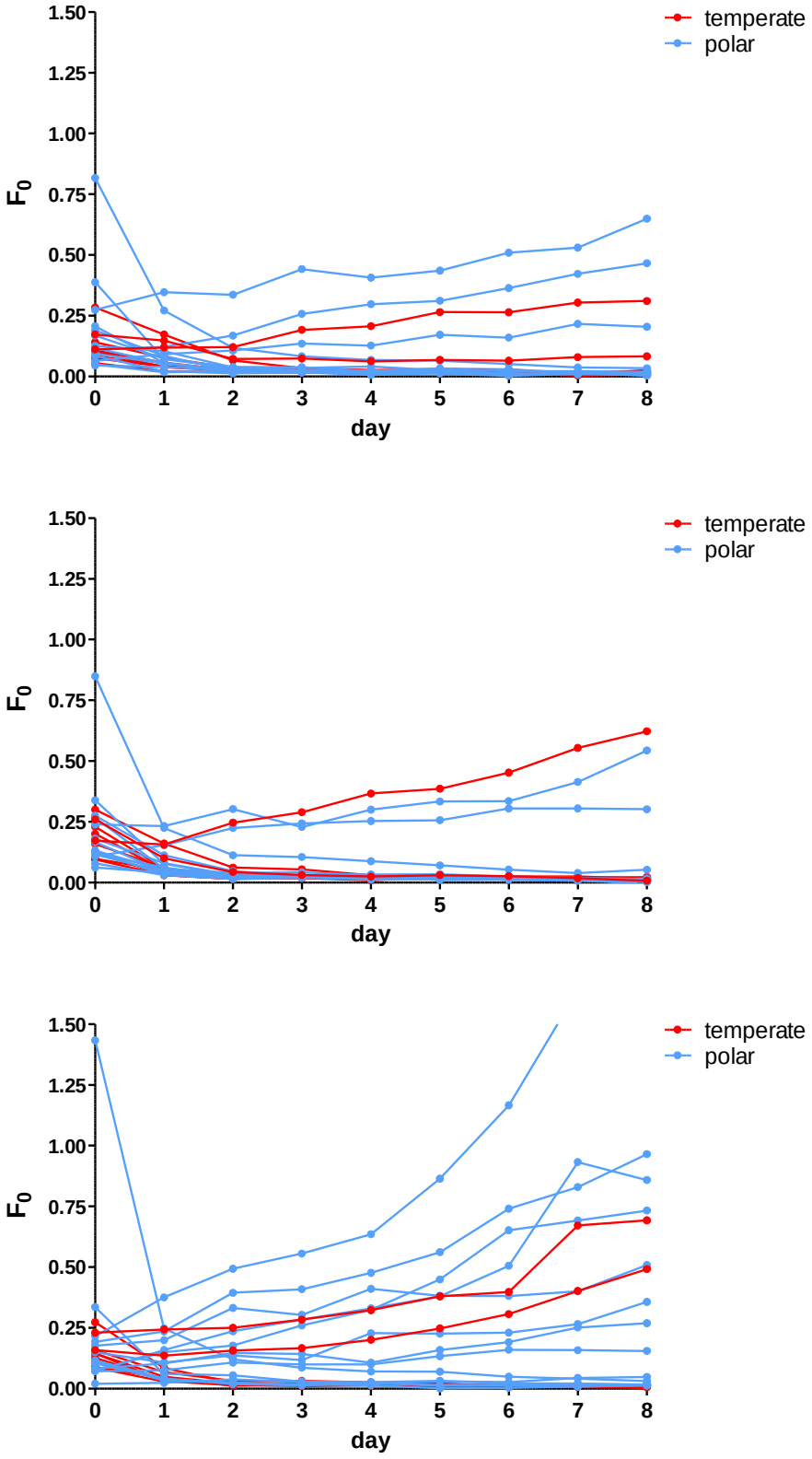




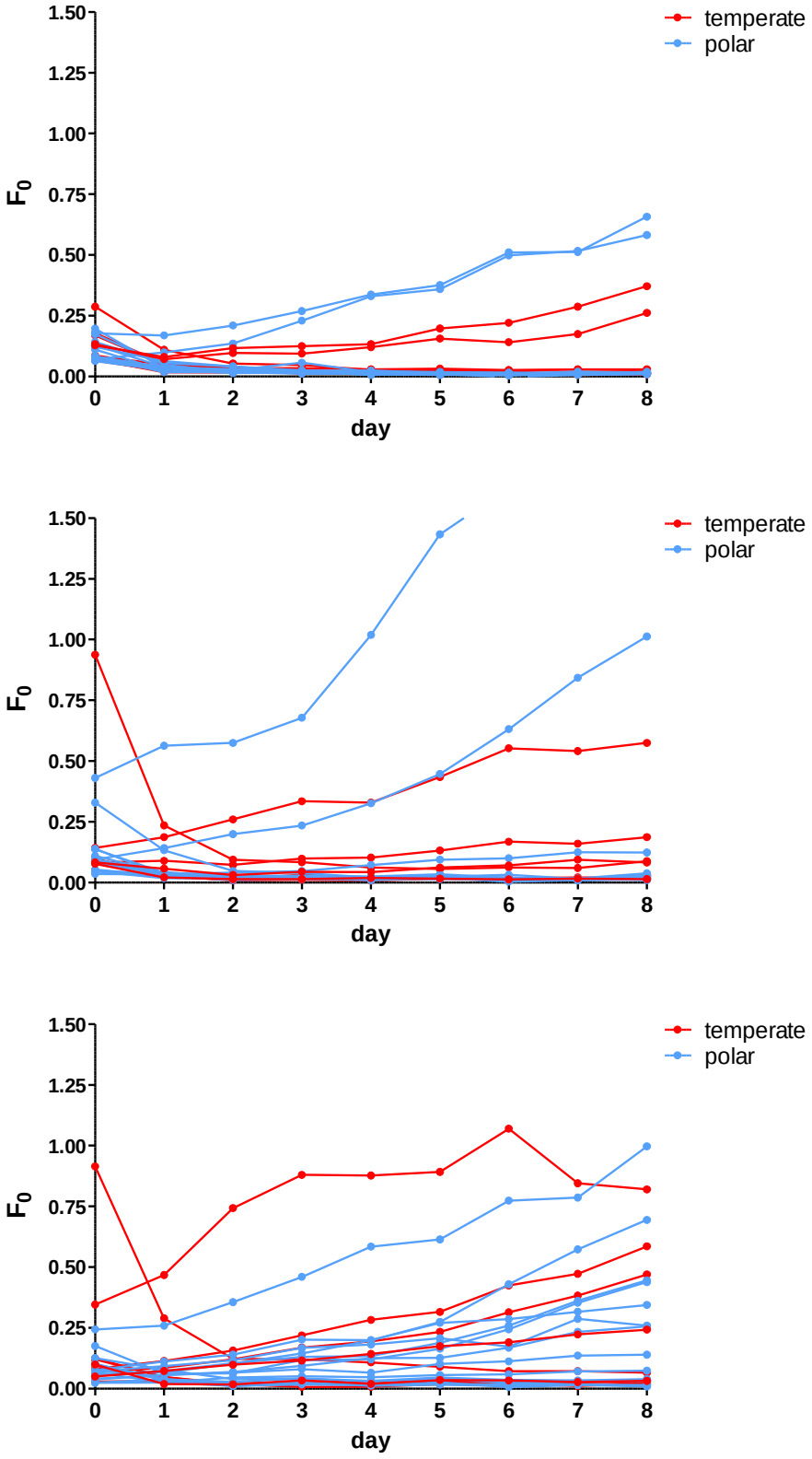
1. b) Continuous freezing to –4 °C followed by fast thawing (vegetative cells).



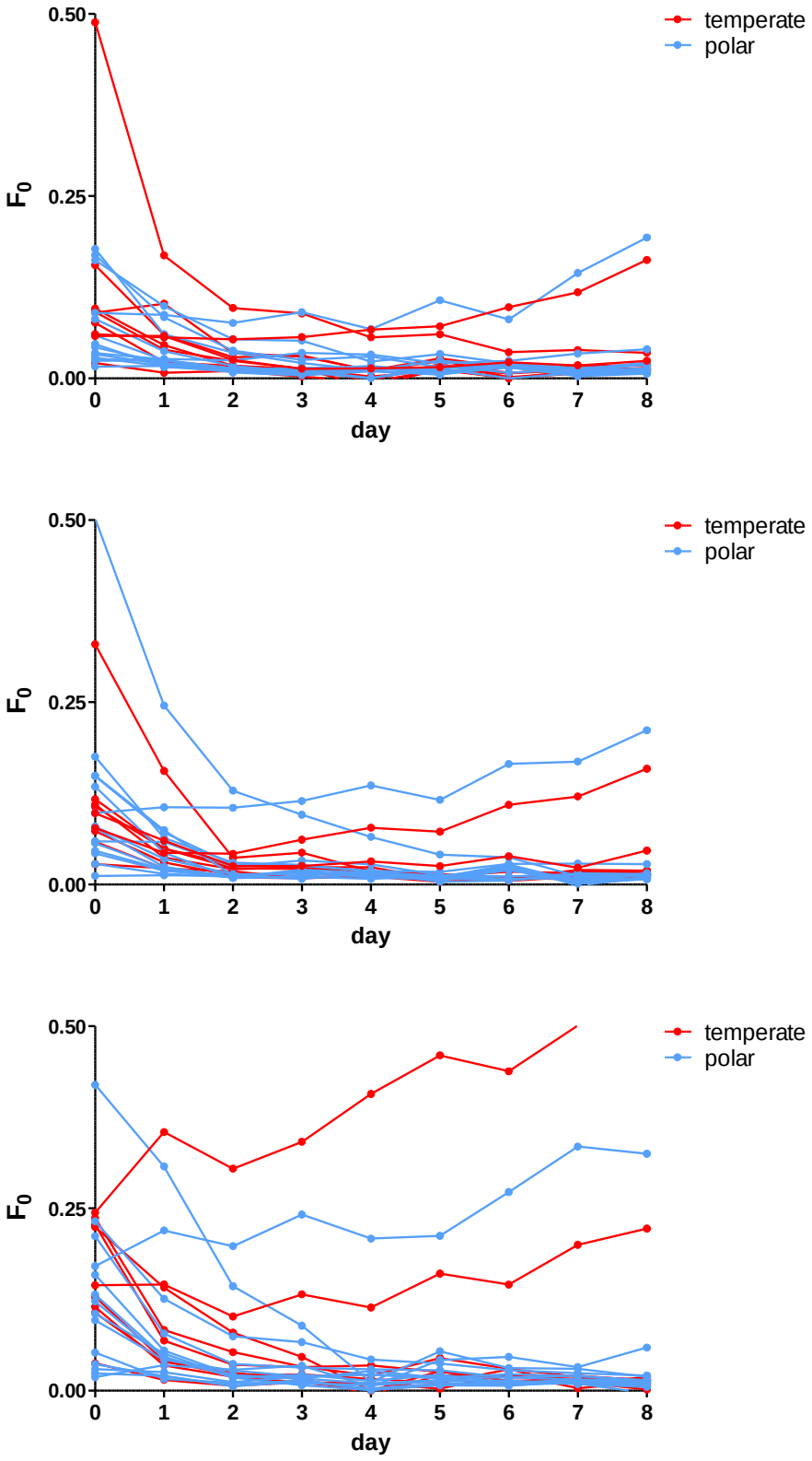
2. a) Continuous freezing to –20 °C followed by fast thawing (resting cells).



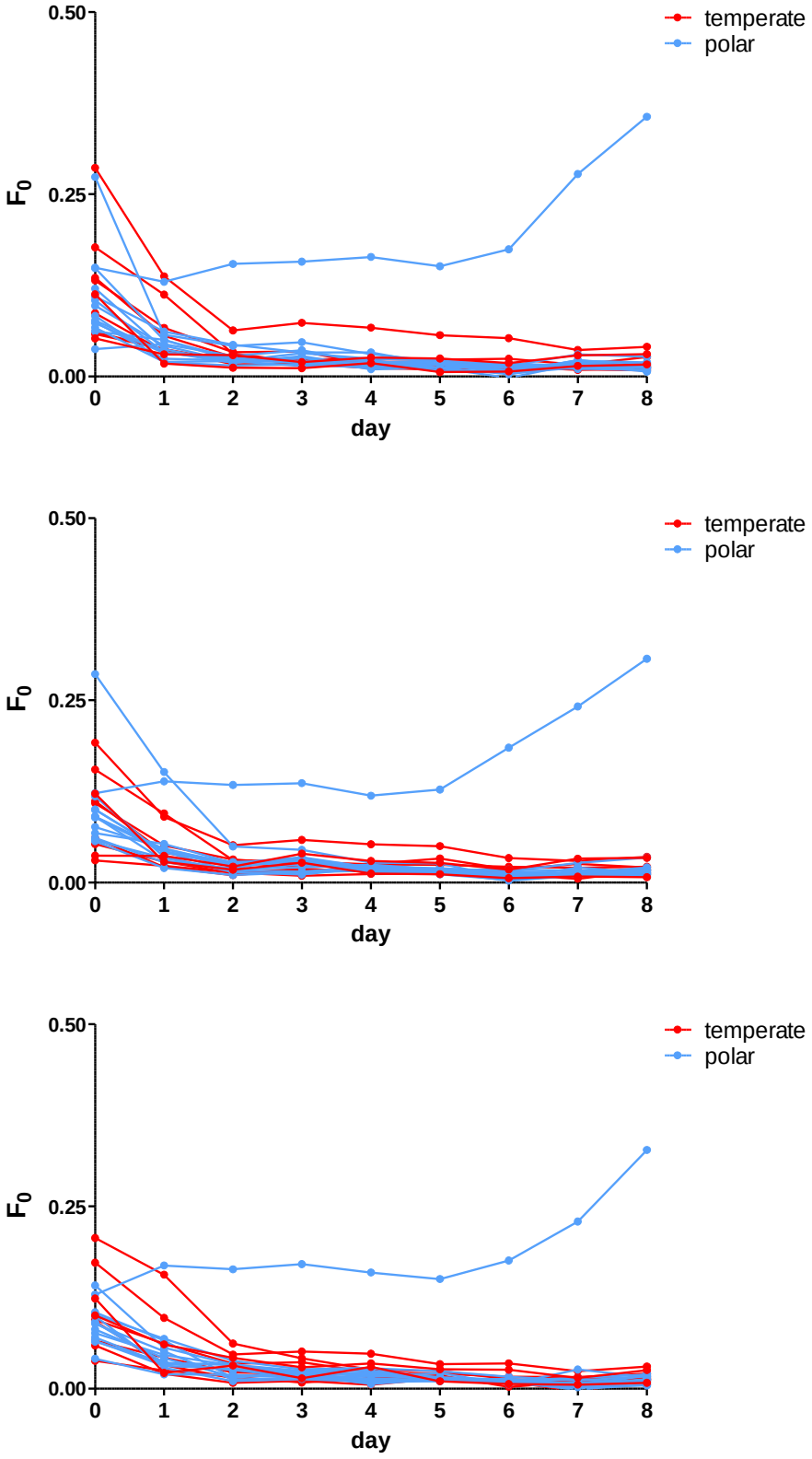
2. b) Continuous freezing to –20 °C followed by fast thawing (vegetative cells).



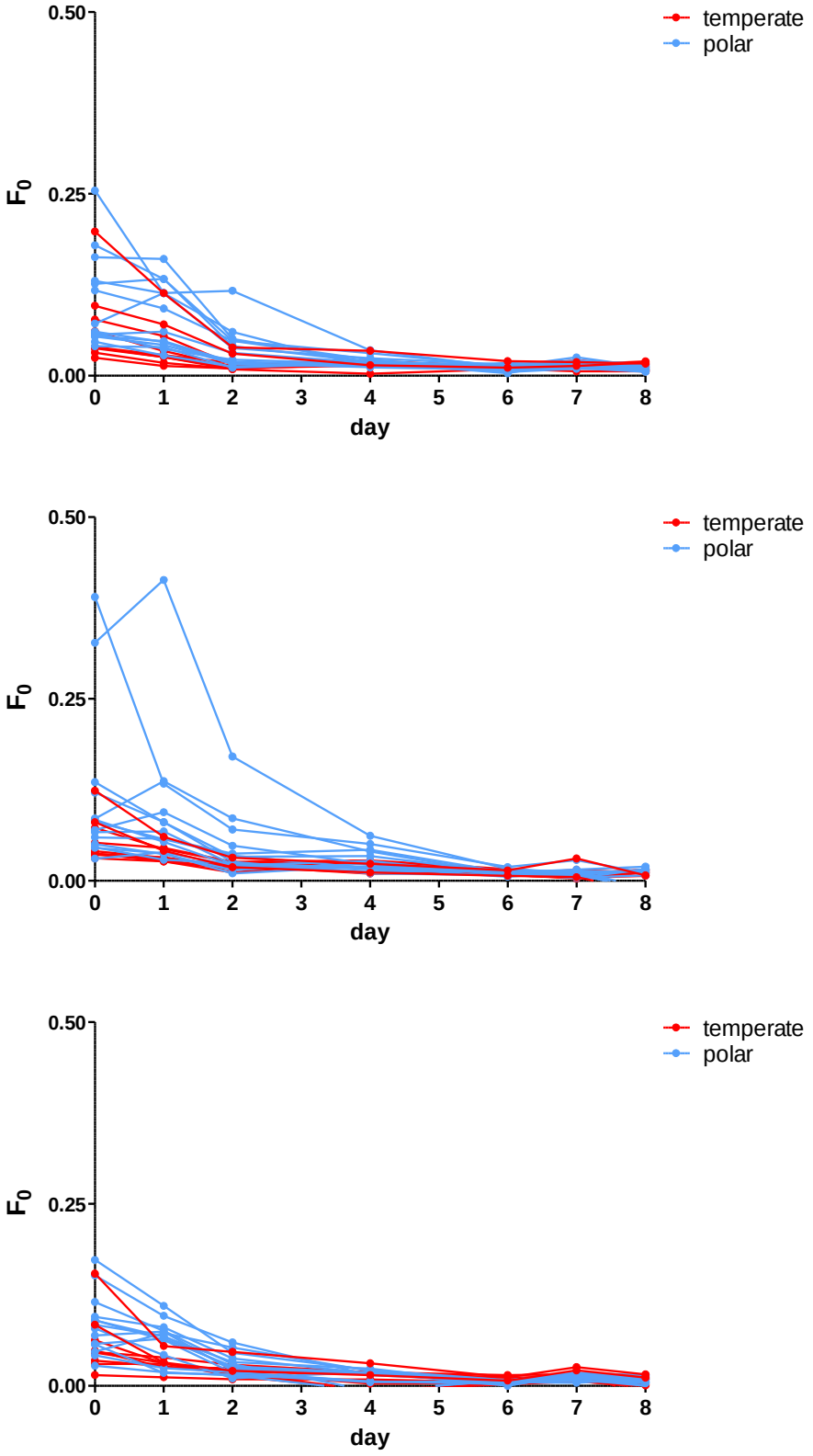
3. a) Continuous freezing to –40 °C followed by fast thawing (resting cells).



3. b) Continuous freezing to –40 °C followed by fast thawing (vegetative cells).



4. a) Abrupt freezing to –180 °C followed by fast thawing (resting cells).



4. b) Abrupt freezing to –180 °C followed by fast thawing (vegetative cells).