Microbial Ecology (2020) 80:559–572
https://doi.org/10.1007/s00248-020-01521-w

MICROBIOLOGY OF AQUATIC SYSTEMS

Annual Cycle of Freshwater Diatoms in the High Arctic Revealed by Multiparameter Fluorescent Staining

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Received: 13 January 2020 / Accepted: 1 May 2020 / Published online: 2 June 2020

Abstract

Diatoms (Bacillariophyceae) are important primary producers in a wide range of hydro-terrestrial habitats in polar regions that are characterized by many extreme environmental conditions. Nevertheless, how they survive periods of drought and/or freeze remains unknown. A general strategy of microorganisms to overcome adverse conditions is dormancy, but morphologically distinct diatom resting stages are rare. This study aimed to evaluate the annual cycle of freshwater diatoms in the High Arctic (Central Spitsbergen) and provide an insight into their physiological cell status variability. The diversity and viability of diatom cells were studied in samples collected five times at four study sites, tracing the key events for survival (summer vegetative season, autumn dry-freezing, winter freezing, spring melting, summer vegetative season [again]). For viability evaluation, a multiparameter fluorescent staining was used in combination with light microscopy and allowed to reveal the physiological status at a single-cell level. The proportions of the cell categories were seasonally and locality dependent. The results suggested that a significant portion of vegetative cells survive winter and provide an inoculum for the following vegetative season. The ice thickness significantly influenced spring survival. The thicker the ice layer was, the more dead cells and fewer other stages were observed. The influence of the average week max–min temperature differences in autumn and winter was not proven.

Keywords Diatoms · Life cycle · Overwintering · Freezing tolerance · Viability · Polar regions

Introduction

Natural conditions in polar regions are characterized by many extremes that are challenging for any organism. Despite this fact, diatoms (Bacillariophyceae) apparently adapted well and became one of the most abundant algal groups in many polar freshwater and terrestrial habitats [1, 2] where they have to face inhospitable and stressful polar conditions represented by low light and nutrient supply, as well as low temperatures causing recurrent freezing events and irregular liquid water availability [3, 4]. These patterns differ in periodicity, amplitude, and synchronicity, and they initiate several different ecological and physiological acclimation and adaptation responses. For example, annual solar irradiance is over the latitude from 30° to 80° reduced by about 50%, which, together with snow and ice layers, limits the total annual production and strongly influences the seasonality of photosynthesis [3, 4]. Polar phytoplankton experiences temperatures around zero for most of the year, which is usually less than the optimal range for physiological processes [5, 6]. Moreover, at temperatures below − 0.6 °C, biological water becomes thermodynamically unstable under isotonic conditions and tends to crystallize [7]. Intracellular freezing is almost always associated with cell injuries, often resulting in irreversible or lethal cell damages [7, 8]. Furthermore, the climatic conditions in the Arctic are changing; long-term increases in winter air temperatures are being observed and are predicted to be faster than those in the rest of the world and more obvious than in summer [9, 10]. Changes in the amount of precipitation and the proportion that falls as rain or snow, as well as its duration,

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00248-020-01521-w) contains supplementary material, which is available to authorized users.

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have important consequences for sub- and supernivian biological activity [10, 11].

Microalgae have had to evolve various universal strategies for successful survival in polar habitats. To maintain an aqueous external environment, microalgae produce numerous extracellular macromolecular substances that act via lowering the freezing point of a solution below the thawing point, changing the viscosity of the brine and increasing the volume of the inhabitable liquid phase and the inner ice–liquid surface area [12, 13]. Equally important effects of these substances are the ability to bind to ice, influence its growth, and inhibit the recrystallization process (growth of large crystals at the expenses of small ones) [12–15]. Most of these mechanisms have been found only in algae from low-temperature environments, which suggest their cryoprotective function [16]. The intracellular responses to temperature downshift include the synthesis of enzyme stabilizing molecules, which are protecting different cellular compartments from freezing injuries [17, 18], production of compatible solutes as sucrose, prolin [19], betaines [20], and dimethylsulfide (DMS) [20, 21], and increase in the proportion of polyunsaturated fatty that helps to keep fluidity of phospholipid bilayer [22, 23].

Another general strategy to survive unfavorable conditions is to create a resistant long-lasting stage. Diatoms are known to form two types of such stages: resting cells and resting spores. Both stages are characterized by reduced metabolic activity and different structure, changes in cellular components leading to a resting state that requires the consumption of small amounts of cellular carbon to survive [24, 25]. Resting cells are physiologically adapted vegetative-looking cells [26–28]. They have dense and dark cytoplasmic matter, and rounder plastids are observed in spores [29], as well as less pigmentation and contracted chloroplasts [24]. Resting cells were observed in many pennate diatoms [30], including freshwater species [31]. It is suggested that resting cells are important for the freezing and desiccation survival of terrestrial diatoms in their variable habitats [31]. Resting spores are morphologically different from vegetative cells. They are characterized by their rounder shape, thicker cell wall, and different patterns [30, 32]. Their formation is usually associated with limited nutrition supplies and winter survival [30, 32, 33]. Resting spores are the most common in centric marine diatoms from temperate neritic habitats [30, 33]. They are generally relatively rare in freshwater and are mostly observed in centric species [25, 30, 34].

Despite the fact that diatoms are a well-studied group of microorganisms, the strategy that enables them to survive polar winters remains unknown. Even though they do not form any morphologically different resting stages, they dominate in many polar habitats. A recent field study of the overwintering strategy of the filamentous cyanobacteria *Phormidium* sp. revealed the high survival of vegetative cells as filaments enclosed in thick polysaccharide sheaths [35]. Another study on filamentous green microalgae *Zygnema* spp. presented their survival as modified vegetative cells called "pre-akinetes" [36]. In this study, we decided to study the annual cycle of freshwater diatom populations at four study sites in the High Arctic using fluorescent staining, which enables us to distinguish the physiological status at the single-cell level [37] and show its seasonal and spatial variability.

Materials and Methods

Study Sites and Experimental Setup

The field study was conducted in two valleys in the central part of Spitsbergen (High Arctic). Four sites (shallow hydroterrestrial tundra habitats) with high abundances of diatoms were selected (Figs. 1 and S1): Bjørndalen 1 (78.217129° N, 15.333050° E, 52 m a.s.l.), Bjørndalen 2 (78.218626° N, 15.339754° E, 93 m a.s.l.), Endalen 1 (78.183551° N, 15.763844° E, 96 m a.s.l.), and Endalen 2 (78.186742° N, 15.791057° E, 60 m a.s.l.). Both Bjørndalen and Endalen are U-shaped valleys cut through the subhorizontal succession of Cretaceous and Tertiary sedimentary rocks. The bedrock is mostly composed of sandstone with siltstone and shale intercalations and, locally, coal seams close to the Cretaceous-Tertiary boundary [39, 40]. Bjørndalen 1 is a stony snow-fed stream, partly moss and grass covered along the banks with an occurrence of cotton grass (*Eriophorum* sp.); Bjørndalen 2 is a seepage in the middle of a moss- and grass-dominated meadow, with frequent occurrence of cotton grass (*Eriophorum* sp.). Endalen 1 is a snow-fed stream situated under steep slopes of the valley and Endalen 2 a seepage/wetland, both covered by mosses and grass with occurrence of polar willow (*Salix polaris*), mountain sorrel (*Oxyria digyna*), alpine bistort (*Bistorta vivipara*), Arctic mouse-ear (*Cerastium arcticum*), whitlow grass (*Draba* sp.), and buttercup (*Ranunculus* sp.). All the study sites have an oligotrophic character.

Climatic conditions in Spitsbergen are according to Köppen-Geiger climate types classified as semiarid polar tundra [41, 42]. Average year precipitation based on data provided by the Norwegian Meteorological Institute reported during 2005–2015 at Svalbard Airport meteorological station was 221.5 mm. The hottest month was July with an average temperature of 7 °C and the coldest was March with a temperature of − 11 °C. The month with the highest mean precipitation was December (35.9 mm) and lowest was June (6.6 mm). Recent measurements of meteorological parameters in this area (Adventdalen) showed that the mean air temperature (2 m above the ground) exceeded 0 °C for about 5 months, from the beginning of May until the end of September (using data provided by the UNIS weather stations [43]). Daylight was unavailable from the end of October to the middle of February (10/26/2017–2/16/2018).

Two metal poles, three Petri dishes, and two or three plastic boxes were installed per location in the summer of 2017 (Figs.

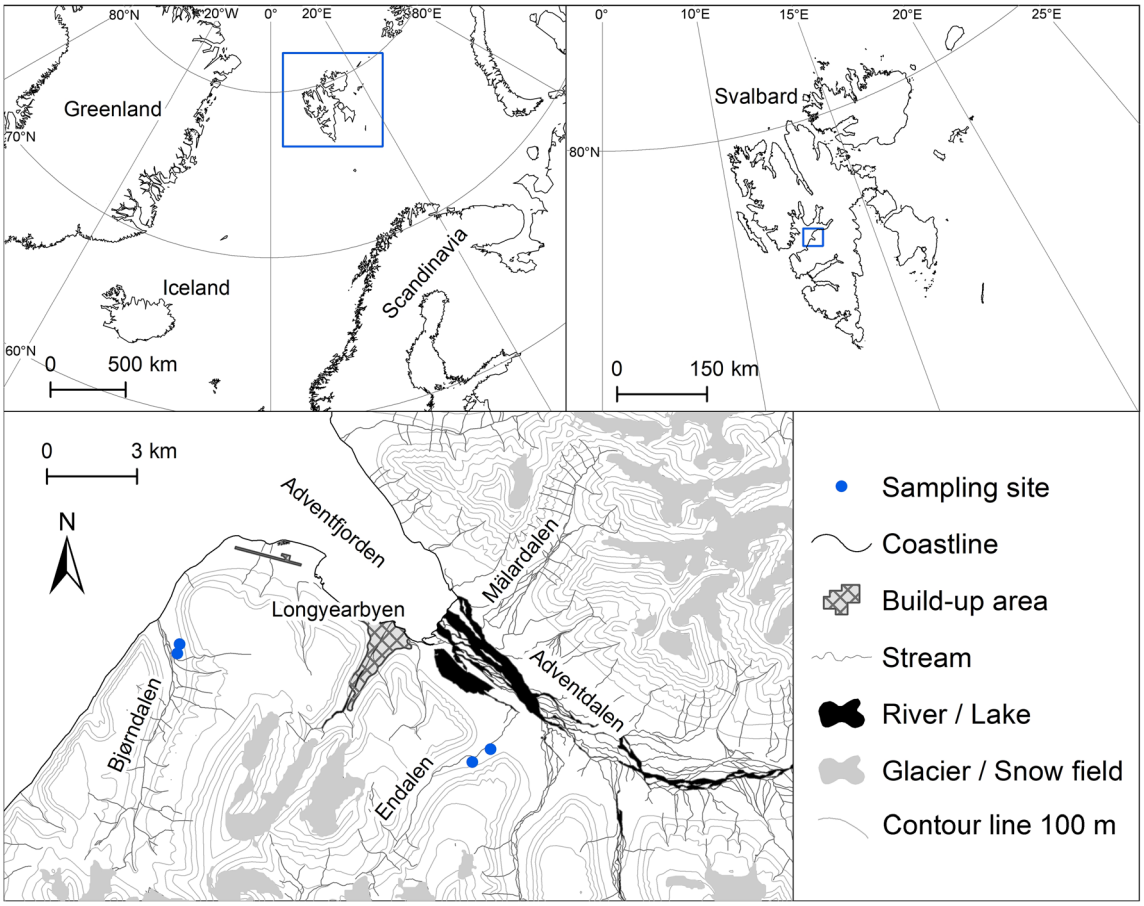

Fig. 1 The Arctic region, with Svalbard highlighted. A detailed map illustrating the study sites situated in two valleys in Central Spitsbergen. Map source: Svalbardkartet, Norwegian Polar Institute [38]

S1 and S2). The dishes and boxes were perforated to ensure water flow inside. Several stones were placed inside the boxes and the installations were fixed by sticks to avoid their loss. Diatoms were caught in the boxes and Petri dishes to ensure multiple samplings of the same population. The poles were installed to find the exact location of installations during the winter season when the surface was snow-covered. To provide temperature characteristics of the studied habitats, three Minikin Tie dataloggers (EMS Brno, Brno, Czech Republic) were installed in each site and measured the temperature at a 1-h interval during the whole period of study (8/8/2017–7/26/2018). In each site, two of the dataloggers were installed in the water body and one in a perforated box for temperature comparison. The average, minimal, and maximal temperatures and max–min temperature difference per week per study site were determined. Data were processed with the program Mini32 (EMS Brno, Brno, Czech Republic).

Sampling

The diatom communities were sampled five times during the period of August 2017–July 2018 to record key events for algal survival: summer vegetative season (8/8/2017), autumn dry-freezing (10/20/2017), frozen state in winter (4/12, 14/2018), spring melting (7/6/2018), and the summer season again (7/26/2018). Three to five samples were collected per study site from the inside of the perforated boxes and Petri dishes (three different spots from each dish/box together made one sample). Material was collected into 15-ml plastic tubes using a Pasteur pipette, knife, or ax, depending on the seasonal conditions. The sampling equipment was cleaned to avoid cross-contamination between samplings. The biomass was immediately used for viability analyses. In total, 79 samples were collected (summer 2017, 17; autumn, 12; winter, 13; spring, 19; and summer 2018, 18). Field samples were transported and processed in the lab at the Czech Arctic Research Station of Josef Svoboda in Longyearbyen (Svalbard). In addition, aliquots of samples were fixed with ethanol for further microscopy investigation.

Viability Evaluation and Sample Processing

Upon arrival at the lab, the samples were left in subdued light at 8 °C for 30 min or in darkness at 5 °C for 10 h (winter samples) to thaw slowly if necessary. For the viability evaluation, three to five samples were used per site as replicates.

Table 1 Classification of cells according to fluorescence and light microscopy

Physiological state	Cell content	DAPI	SYTOX Green	CTC	Figure 2 marked as
Active healthy cells	x	x		x	a
Inactive but intact dormant cells	x	x			–
Injured but active cells	x	x	x	x	b
Injured and inactive dead cells	x	x	x		–
Dead cells	x				c

Viability was assessed using a multiparameter fluorescent staining protocol originally developed for filamentous cyanobacteria [37]. The combination of this method and light microscopy enabled us to evaluate the exact physiological state of single cells. The staining protocol combines three fluorescent stains: SYTOX Green Nucleic Acid Stain (S7020, Molecular Probes, Eugene, OR, USA), CTC (5-cyano-2,3-ditolyl tetrazolium chloride, Polysciences Europe GmbH, Eppelheim, Germany), and DAPI (4′,6-diamidin-2-fenylindol, Molecular Probes, Eugene, OR, USA). SYTOX Green is a nucleic acid stain detecting membrane integrity and serving as a useful indicator of dead cells because it does not penetrate living cells. It is expressed as green fluorescence of the cell nucleus. CTC shows respiration activity by accumulation of formazan crystals in mitochondria of active cells, which appears as red fluorescence. DAPI stains dsDNA in both living and dead cells and is detected by white-blue fluorescence of the nuclei.

The homogenized suspension of diatoms and sediment was stained by three fluorescent stains consecutively using 1.5-ml Eppendorf tubes. SYTOX Green Dye was used first, at a concentration of 10 μM. Stained samples were incubated for 15 min in cold (8 °C) and protected from light by two layers of aluminum foil. Samples were washed three times afterward with ambient water. To estimate the respiratory activity, the samples were stained with 5 mM CTC and kept for an additional 120 min under dark and cold conditions. The dye solutions were removed afterward; the samples were rinsed once and stained with DAPI at a concentration of 5 μg/ml, and incubated for 30 min under dark and cool conditions. The samples were then washed three times, after which the

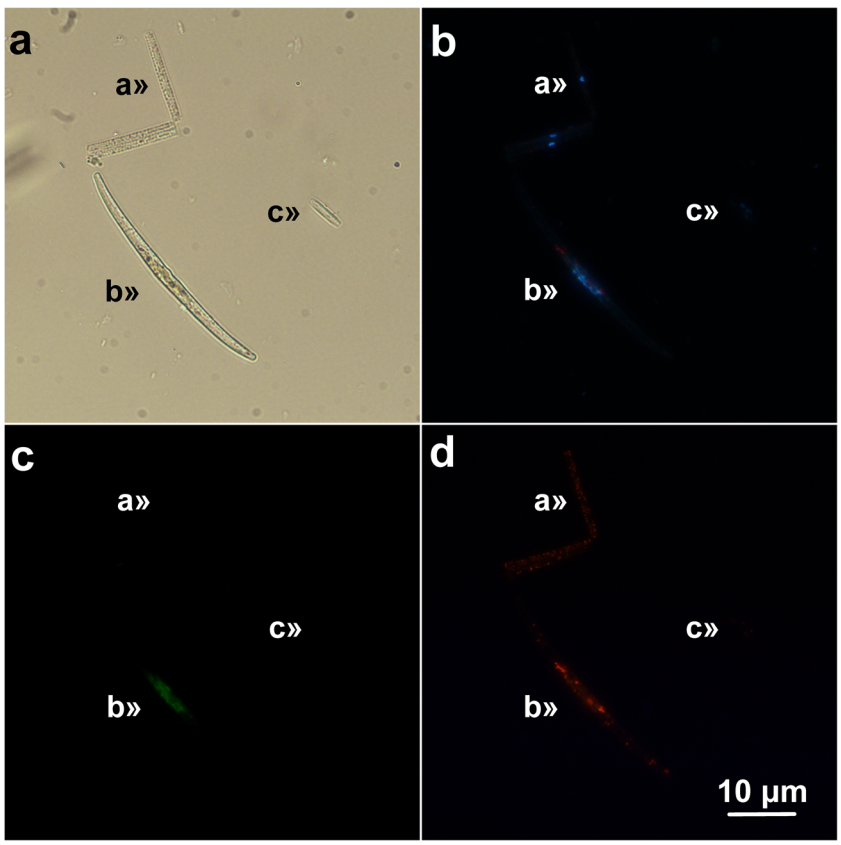

Fig. 2 Slide of the autumn sample **a** under a light microscope, and simultaneously stained with **b** DAPI, **c** SYTOX Green, and **d** CTC fluorescent dyes

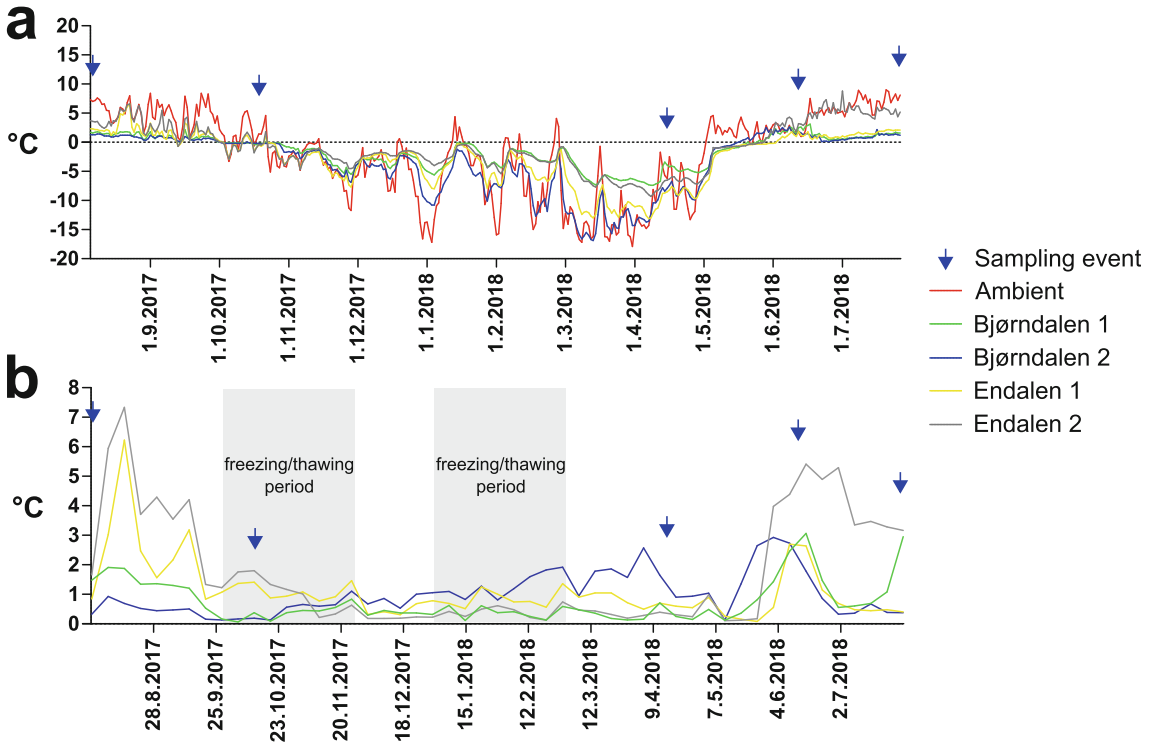

Fig. 3 **a** Average daily temperatures and **b** average weekly difference in the maximum and minimum temperatures per study site measured in 8/8/2017–7/26/2018 with sampling events (arrows) and freezing/thawing periods indicated (shading). The source of ambient air temperature data was the UNIS weather stations (2018)

suspension was transferred onto a slide and studied using the fluorescent microscope Olympus BX53 equipped with a 100-W ultra-high pressure mercury arc lamp (Olympus Corporation, Tokyo, Japan) at × 200 magnification [37]. To assess the proportion of cell physiological states, at least 400 cells per sample were counted on a minimum of 10 random transects. In total, 31,789 cells were studied. According to the staining results, cells were classified in five groups: active healthy cells, inactive but intact dormant cells, injured but active cells, injured and inactive dead cells, and dead cells performing no fluorescence. Cells with no fluorescence but the presence of visible protoplast remains observed under the light microscope were determined to be dead (Table 1; Fig. 2). In addition, a lot of empty silica diatom frustules (50–90%) were observed in samples from all microhabitats. These cells were not included in counting because of the unknown degradation time of the cell content, which means that the real survival could be lower than suggested in this study.

Species Composition

Additional dominant species were determined, and the relative abundances (percentage of total diatom valves per replica and in all five samplings together) were expressed for each study site using stained samples. Only species present at 1% in any single sampling were included. For detailed taxonomical analysis, subsamples were oxidized by adding 37% hydrogen peroxide and incubated overnight. Samples were then rinsed three times with distilled water alternated with centrifugation (10 min at 1200×*g*). The material was dried on microscope

Table 2 Minimum and maximum temperatures measured by dataloggers and natural conditions per season (average snow depth, ice layer, and water conditions)

Study site	Min (°C)	Max (°C)	Summer	Autumn	Winter	Spring
Ambient	− 20.5	11.9	-	-	-	-
Bjørndalen 1	− 16.1	7.2	Wet	Moist, frozen	Snow 50 cm, ice 17 cm	Wet
Bjørndalen 2	− 17.7	7.9	Wet	Desiccated, moist, frozen	Snow 2 cm, ice 16 cm	Wet
Endalen 1	− 18.6	11.3	Wet	Desiccated	Snow 33 cm, ice 22 cm	Wet
Endalen 2	− 9.6	13.8	Wet	Desiccated	Snow 38 cm, ice 16 cm	Wet

cover slips and embedded in Naphrax (Brunel Microscopes Ltd., Chippenham, UK). Microscopic slides were observed using an Olympus BX43 microscope (Olympus Corporation, Tokyo, Japan) equipped with differential interference contrast (Nomarski) optics under oil immersion at × 1000 magnification. Samples and slides were stored at the Department of Ecology, Charles University, Prague, Czech Republic.

Data Analyses

All statistical analyses were performed in R 3.6.1 (R Core Team, Vienna, Austria) using multivariate methods in the "vegan" package [44]. Prior to the analyses, data were standardized across species (mean variance standardization). To analyze the variability within the dataset, principal component analysis (PCA) and redundancy analysis (RDA) were run on the whole dataset. In RDA, the effect of locality and season was tested using the permutation test for RDA under a reduced model. Furthermore, the potential influence of ice thickness, depth of snow layer, and temperature oscillations (average weekly max–min temperature differences in autumn 10/2/2017–11/26/2017 and winter 1/1/2018–3/4/2018) on the cell viabilities in spring was tested using the permutation test for RDA under a reduced model. As the snow depth was highly correlated with winter temperature oscillation, it was not used in further analyses. Therefore, only temperature data were used for statistical analyses. For graphical visualization, R, ArcGIS (Esri, Redlands, CA, USA), Zoner Photo Studio 16 (Zoner Software, Brno, Czech Republic), InkScape 0.92.3 (Software Freedom Conservancy, New York, NY, USA), and GraphPad Prism 5.03 (GraphPad Software, La Jolla, CA, USA) were used.

Results

Environmental Conditions

According to the measurements provided by dataloggers each hour, the temperature reached zero in mid-September in all the studied sites. The temperature courses corresponded with average ambient air temperature fluctuations (Fig. 3a). With the onset of the polar night (October 26, 2017), the study sites became frozen and remained frozen until the end of May, when the snow and ice melted. The minimum temperature of all sampling sites reached − 18.6 °C at Endalen 1 site and the maximum temperature reached was 13.8 °C at Endalen 2 site. The lowest ambient air temperature was − 20.5 °C and the highest was 11.9 °C. During winter, three warm periods were noticeable (in the middle of January and in the beginning and end of February), when the air temperature increased above 0 °C and the temperatures at some of the study sites reached 0 °C. The minimal and maximal temperatures and environmental conditions per study site are given in Tables 2 and S3. The average max–min temperature differences per week measured in "critical" survival periods, when temperatures fluctuated around freezing point, were as follows: in autumn (10/2/2017–11/26/2017)/winter (1/1/2018–3/4/2018) were 1.06/0.86 °C (Endalen 1), 1.11/0.40 °C (Endalen 2), 0.32/0.38 °C (Bjørndalen 1), 0.39/1.29 °C (Bjørndalen 2) (Fig. 3b).

Seasonal Changes of the Habitats

The sampling started in the summer vegetative season, in the beginning of August 2017. Liquid water was available in all of the sampled sites. In autumn 2017, the sediment with diatoms in both the stream and seepage in the Bjørndalen valley was not frozen solid yet; it was slightly moist and easily deformable, but very close to the frozen state. In Endalen, most of the sampled microhabitats were desiccated and frozen (Figs. 4 and 5). A thin layer of ice was visible above the sampled material, where the water level usually occurs, giving evidence of a desiccation event. In the winter season of 2018, all of the localities were frozen and covered by snow. The average thickness of layers observed in the study sites is shown in Table 2. In spring, the sampling sites appeared to be similar to those in both summer vegetative seasons (Figs. 4 and 5). Most of the ice and snow melted, and liquid water was available in all of the localities. The study sites in summer 2018 were very similar to those in spring 2018 and summer 2017.

Species Composition and Diversity

A total of 36 taxa (including species, varieties, and forms) belonging to 22 genera were found in all of the analyzed samples. Five taxa were found outside of the counts when scanning the slides for additional species. Several taxa could not be identified up to the species or genus level; additional morphological investigations are necessary to clarify their taxonomic position.

Each of the study sites was dominated by two to four species (Table 3). In Bjørndalen 1, *Hannaea arcus* (50%) and *Meridion circulare* (35%) predominated. *M. circulare* (40%) together with *Diatoma* cf. *problematica* (23%), *Fragilaria tenera* (17%), and *Fragilaria fragilarioides* (11%) occurred most frequently in Bjørndalen 2. Despite the close position of the two Endalen sites, the dominant species were different. In Endalen site 1, *H. arcus* (61%) and *M. circulare* (36%) prevailed, while *Cymbella hantzschiana* (34%), *Pinnularia frequentis* (33%), and *F. tenera* (11%) dominated in Endalen site 2. The determined diatom taxa are shown in Fig. S4. Filaments of green algae were occasionally observed in most samples as well; cyanobacteria were rare.

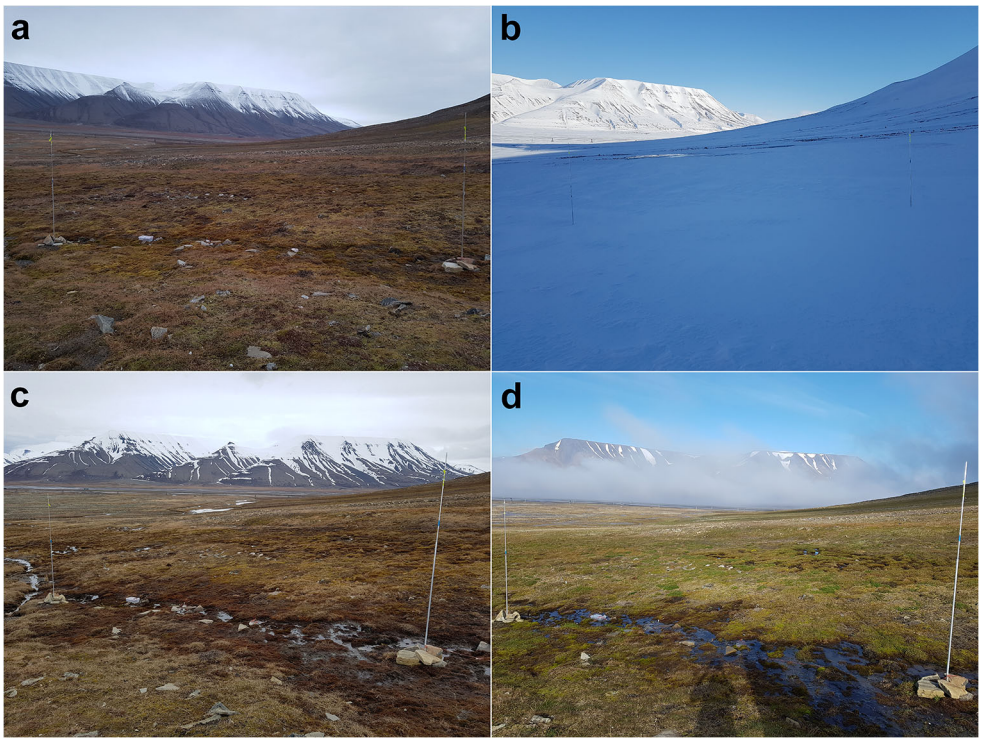

Fig. 4 Natural conditions at the Endalen site 2 during the year. **a** Autumn, **b** winter, **c** spring, and **d** summer sampling

Seasonal Changes of Diatom Viability

During the first sampling in August 2017, diatoms were proven to be highly viable and active. In all the observed sites, the majority of evaluated cells were active with percentages between 50 and 90%, depending on the study site (Figs. 6). A low proportion of cells were shown as dormant because there were no respiration activity and undamaged membranes. Some injured active or inactive cells were observed at all sites. Dead cells were in the minority (less than 3%). Autumn sampling in October 2017 showed a much lower diatom survival in the active stage (1–15%). As expected, most of the cells

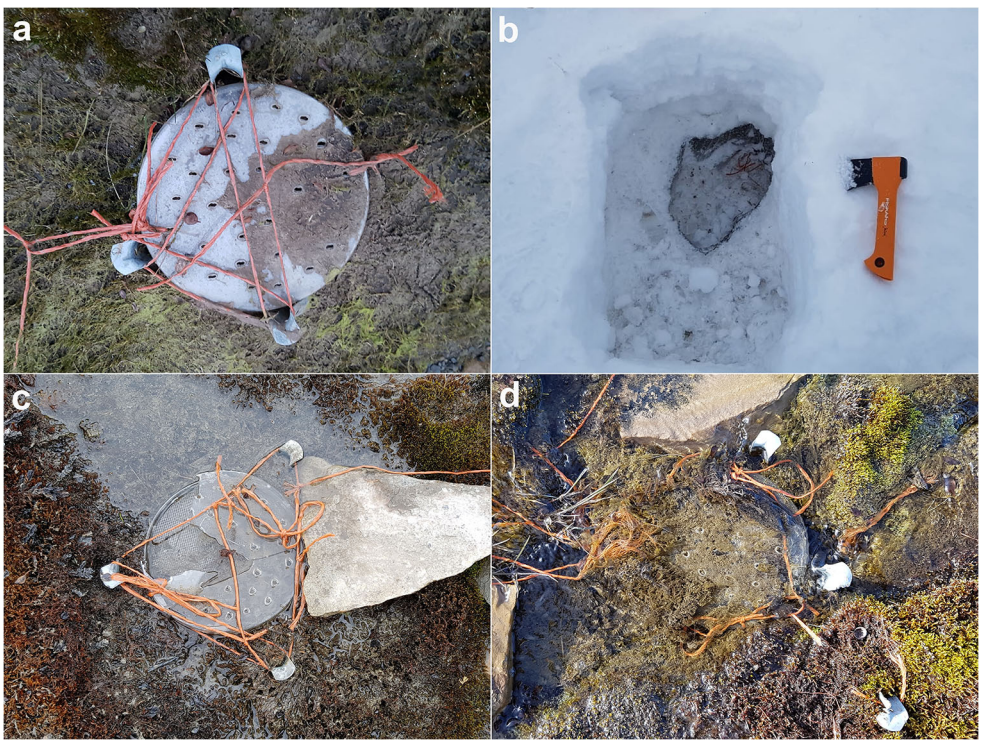

Fig. 5 Detailed pictures of Petri dishes at Endalen site 2 during the year. **a** Autumn, **b** winter, **c** spring, and **d** summer sampling

Table 3 Relative abundances of diatom species per site (average percentages from all the samplings)

Species	Bjørndalen 1	Bjørndalen 2	Endalen 1	Endalen 2
Cymbella hantzschiana Krammer	1.3	-	-	*33.7*
Diatoma cf. *problematica* Lange-Bertalot	0.7	*23.4*	-	0.9
Diploneis ovalis ssp. *arctica* Lange-Bertalot	-	-	-	0.8
Eunotia cf. *praerupta* Ehrenberg	-	-	-	0.4
Fragilaria fragilarioides (Grunow) Cholnoky	-	11.1	0.3	0.5
Fragilaria tenera (W.Smith) Lange-Bertalot	4.2	*16.8*	-	*11.2*
Gomphonema sp.	-	-	-	0.1
Hannaea arcus (Ehrenberg) R.M.Patrick	*50.1*	0.2	*60.7*	3.0
Hantzschia sp.	-	-	-	0.3
Meridion circulare (Greville) C.Agardh	*35.1*	*39.5*	*36.2*	3.5
Navicula sp.	*6.7*	2.8	0.6	0.6
Nitzschia sp. 2	0.6	4.9	0.1	7.2
Pinnularia frequentis Krammer	1.0	-	*1.9*	*33.5*
Pinnularia sp. 1	-	1.0	-	0.1
Pinnularia sp. 2	-	-	-	0.1
Pinnularia sp. 3	-	-	0.1	0.3
Rossithidium petersenii (Hustedt) Round & Bukhtiyarova	-	0.1	-	0.3
Tabellaria flocculosa (Roth) Kützing	-	-	0.1	-
Undetermined	0.3	0.2	-	3.4

Values of most abundant species are italicized

were dormant (19%–61%) or dead (19%–70%). Injured and inactive cells were detected in all sites with percentages between 8 and 14%. Dead cells prevailed in winter samples from April 2018, with percentages of between 54 and 88%. Dormant cells were present at 9–33%. Remarkably, a relatively high proportion of cells were active after winter sampling in both Bjørndalen sites. Hardly any injured active cells were observed, with the exception of those in Bjørndalen site 1. The June 2018 spring sampling showed a high proportion of dead cells (27–83%), which was, in the case of the Endalen site 1, even higher than in winter. A significant ratio of cells in dormant stage was detected (14–49%), and the proportion of

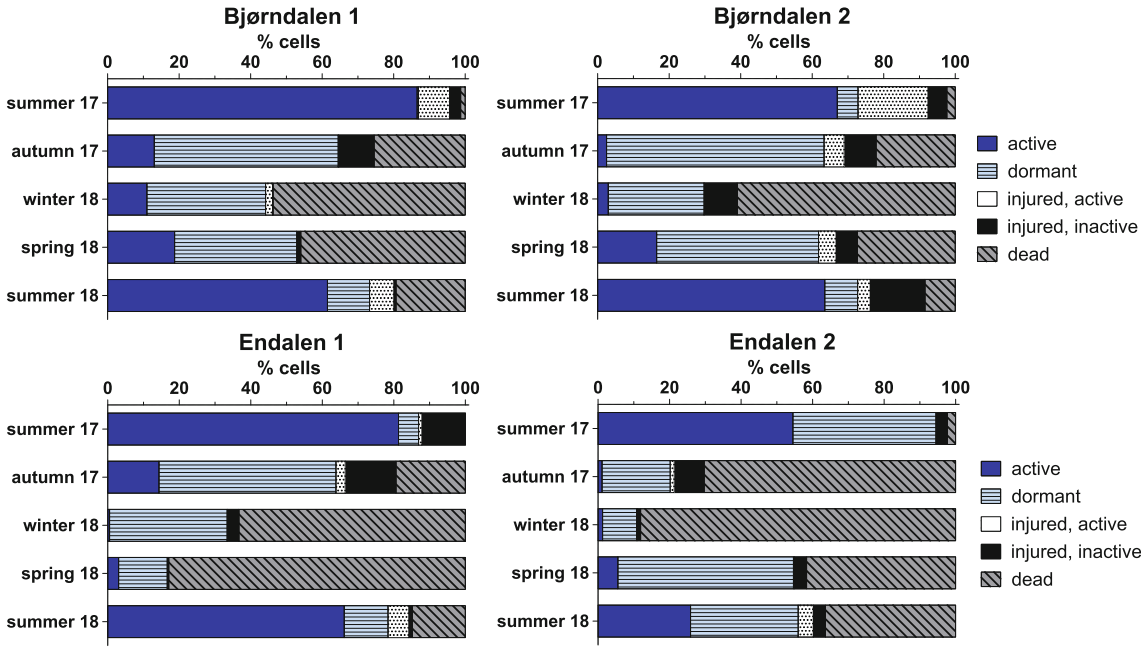

Fig. 6 Seasonal development of diatom viabilities for each site (average percentage per site)

active cells increased (3–19%) when compared with the winter sampling results. The last sampling in the end of July 2018 indicated that most of the cells were active (61–66%) and a minority was dead (8–19%), with the exception of Endalen site 2, where most of the cells were dead (36%) and only 26% of cells were active. Dormancy was detected in 9–30% of cells, 4–7% were shown as injured but still active, and 1–15% were injured but inactive.

The effect of season and locality on the proportion of cell types was highly significant (permutation test for RDA under reduced model, $P < 0.001$). Correlations between the physiological cell status, sampling site, and season using standardized data visualized by principal component analysis (PCA) and redundancy analysis (RDA) are shown in Fig. 7. From the tested factors potentially influencing cell viability in spring, only ice thickness had a significant effect (permutation test for RDA under reduced model, $P < 0.007$). The thicker ice layer, the more dead cells and fewer other stages were observed (Fig. 8). The influence of average weekly max–min temperature differences was not significant in autumn and winter.

Discussion

Physical stresses common in polar environments, such as freezing and desiccation related to reduced water availability, play an important role in the microbial species composition and their biological activity [45, 46]. During the winter season especially, snow cover regulates terrestrial habitats by insulating them, which decouples the fluctuations of the surface layer soil temperature from air temperature and affects many thermally sensitive processes [10, 47, 48]. In our study, the lowest minimal temperature (− 17.2 °C) was documented at Bjørndalen site 2, where the thinnest snow cover (2 cm) was observed during winter sampling. In contrast, the highest minimal temperature (− 7.5 °C) occurred at Bjørndalen site 1, where the snow depth was 50 cm, which was the highest of all the sites (Table S3). According to temperature measurements, the freezing began in the mid-September after which the temperature fluctuated around zero. The study sites were frozen until the end of May which corresponds to previously published data from similar freshwater bodies (slow-flowing shallow stream and a shallow pool) in Svalbard [35].

In recent winters, short warm periods (in order of days) occur more frequently when a higher proportion of precipitation falls as rain rather than snow [10]. Rain-on-snow events are mostly common in the maritime Arctic and are associated with water penetration through the snowpack and subsequent ice layer creation on the soil surface [49]. Three warmer periods were documented during this study by temperature measurements. The occurrence of some warm spells was noticeable from the thick ice layer/s present under the snow cover (Table 2). The consequences of such ice crusts could last for years and heavily affect the ecosystem by changing the soil nutrient composition, respiration, and winter gas efflux [10, 49–51]. Repeated spring freeze–thaw events were found to act as a bottleneck for the survival of microalgae, suggesting the importance of a few stress-resistant cells under adverse

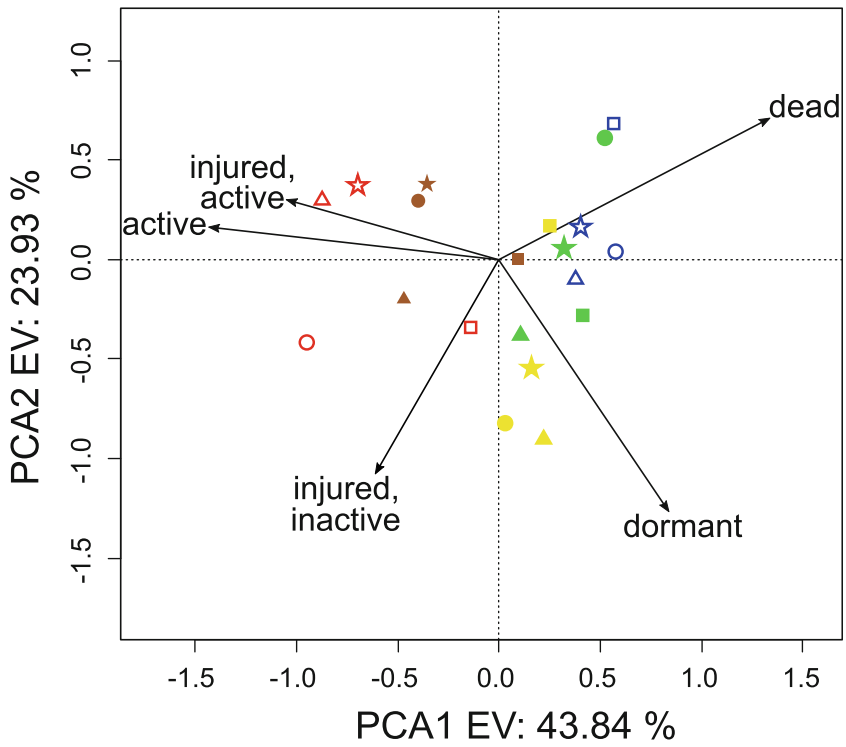

Fig. 7 Principal component analysis (PCA) showing the correlation between sampled sites, sampling season, and the physiological status of cells. Sampled sites: star, Bjørndalen 1; triangle, Bjørndalen 2; dot, Endalen 1; and square, Endalen 2. Sampling season: summer 2017 (red contour), autumn (yellow), winter (blue contour), spring (green), summer 2018 (brown)

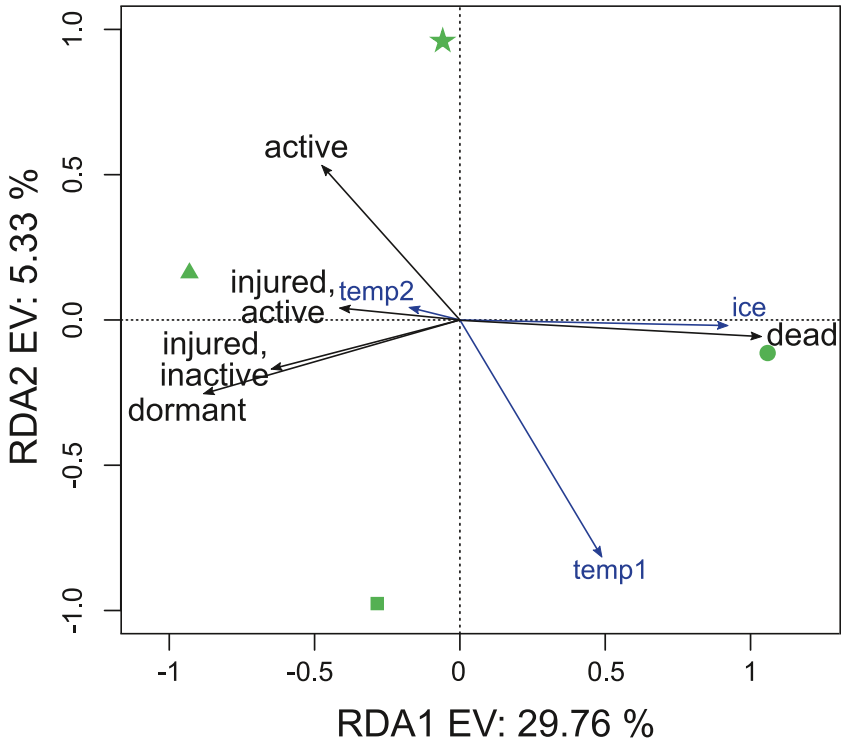

Fig. 8 Redundancy analysis (RDA) showing the influence of ice thickness (ice), and average weekly max–min temperature differences in autumn 10/2/2017–11/26/2017 (temp1) and winter 1/1/2018–3/4/2018 (temp2) on the physiological cell status in spring and sampled sites (star, Bjørndalen 1; triangle, Bjørndalen 2; dot, Endalen 1; and square, Endalen 2)

conditions [52–54]. The increased occurrence of freeze–thaw cycles by winter warm spells could have fatal consequences for their persistence.

Microalgae inhabiting terrestrial and hydro-terrestrial polar environments form annual or perennial communities [46, 55]. A common strategy for eukaryotic microorganisms, e.g., green algae, is a reestablishment of communities every year from a few resistant cells capable of surviving winter, while the majority are not [55, 56]. Beyond this, snow algae from the order Chlamydomonadales (Chlorophyta) represent an example of a group successfully applying the strategy that involves the formation of specialized metabolically active cyst-like stages with a resistant cell wall, which are believed to be resistant to freezing and other unfavorable environmental conditions [57, 58].

Some filamentous centric diatoms from freshwater lakes evidenced resting stages formation (resting cells or spores) to overcome unfavorable conditions. After the spring high cell abundance on the ice bottom, ice breaks up and the diatom filaments are destroyed by water currents to free-floating flakes, which sink to the bottom [59, 60]. The cells could be able to continue division [61] and create resting stages that survive in cool, intermediate depths (50–150 m) during summer stratification [62] or they remain in the bottom sediment until an autumn overturn [59–61].

In contrast, a filamentous green alga from alpine and polar environments, *Klebsormidium* sp., was proven to be able to survive freezing stress in a vegetative state without forming any specialized cells [63, 64], and it was suggested that some other microalgae and cyanobacteria from the Arctic can survive as vegetative cells with thick cell walls and accumulated storage products [65]. This annual study of diatom survival in the High Arctic showed a remarkable viability of diatom cells during winter season. More than 20% of cells were found in the inactive resting cell stage, and nearly 5% were active after thawing, suggesting that a significant portion of cells survive winter. It is assumed that the inactive cells represent resting cells which could re-activate in the beginning of vegetative season and provide an inoculum for the following vegetative season. No morphologically distinct stages were found. Even a higher winter survival of morphologically and/or ultrastructurally non-modified (vegetative) cells was observed in a similar recent field study that focused on Arctic filamentous cyanobacteria of the genus *Phormidium* [35]. Although, cyanobacterial dormant cells, akinetes, are thought to be more resistant to desiccation stress, freezing, and prolonged dark periods than vegetative cells [66]. The proportion of viable cells from the frozen samples was more than 80% and resumed respiration within minutes after thawing, suggesting that most of the vegetative cells acquired resistance to winter stress [35]. However, polar cyanobacteria were generally shown to have a higher resistance to freezing and desiccation stress than green algae from similar habitats [45, 67]. Nevertheless, the production of thick polysaccharide sheaths was observed as a protecting mechanism from freeze damage to avoid the contact of the cell surface with ice crystals. Only a small percentage of cells among trichomes without polysaccharide sheaths survived freezing [35]. The formation of

dense mucilaginous mats is known to enable stream and soil communities to withstand temperature fluctuations, desiccation, and short summer freeze/thaw cycles, and to maintain metabolic activity over harsh environmental conditions [46, 68].

Another field study of Arctic filamentous algae *Zygnema* spp. also showed the ability of vegetative cells to survive an entire annual cycle without forming any stress-resistant cells (zygospores or stationary-phase cells filled with storage products [akinetes]). However, harsh periods were survived as modified stationary-phase-like vegetative cells, called pre-akinetes (cells filled with storage material, characterized by reduced chloroplast lobes and thickened cell walls but still forming a filament). These cells are able to quickly recover under favorable conditions and are physiologically active immediately after thawing [36]. Though, a high mortality of newly produced vegetative cells because of frequent freeze–thaw cycles was observed in the early spring [36]. Recent experimental study revealed that the formation of pre-akinetes in polar *Zygnema* spp. and *Zygnemopsis* sp. is induced by nitrogen limitation at the end of the summer season and can be hardened by naturally slow desiccation stress to survive rapid drying. Naturally hardened pre-akinetes play a key role in stress tolerance and dispersal under the extreme conditions of polar regions [53, 54].The results of our field study are supported by several laboratory experiments. Some terrestrial diatoms also showed their ability to survive adverse conditions as vegetative cells [31]. A terrestrial diatom, *Pinnularia* sp., was even able to maintain near-maximum growth rates over a wider range of stress conditions (freezing and desiccation). After repeated freeze–thaw cycles down to −3 and −10 °C, survival rates between 80 and 100% were observed [56]. In contrast, in freshwater benthic diatoms, both vegetative and laboratory-induced resting cells were shown to be sensitive to desiccation, abrupt heating, or freezing, suggesting their habitat dependency [31, 69, 70]. In −20 °C treatments, the tolerance of freshwater polar strains was higher than that of temperate ones [70], and diatoms from aquatic habitats were less tolerant than terrestrial strains [69]. In addition, cyanobacteria isolated from Antarctic seepages were demonstrated to be less tolerant to freezing and desiccation than cyanobacteria from wetland habitats. One possible explanation is that water levels in freshwater seepages are more stable and provide protection against freezing and desiccation than in other habitats, so the communities of microorganisms may not be acclimated to these stresses [67]. Acclimation is known to increase the tolerance to unfavorable conditions in various organisms, including diatoms [71]. Resting cells of terrestrial diatoms induced under laboratory conditions by hardening under lower temperatures, darkness, and nitrogen limitation showed higher survival of stress tolerance, especially desiccation [31]. In addition, the importance of resting stages for freezing tolerance was emphasized for the survival of mild freezing (−4 °C treatment), which could indicate its relevancy for diatoms from temperate regions [70]. Furthermore, even without nutrient deficiency, changes in the physical and chemical environmental factors changing during the year (e.g., temperature, light intensity, conductivity) can result in reserve accumulation and resistant cell formation [72].

Generally, a similar species composition to those in other studies from non-marine habitats in the High Arctic was found in this study [2, 73, 74]. *Hannaea arcus*, as a typical representative of streams [73, 75], dominated in two of the study sites, each from a different valley. *Meridion circulare* was highly common in the entire study area and was also found in many other Arctic lotic ecosystems [73, 74]. *Diatoma* cf. *problematica*, a principal species in one of the study sites, was found as a common main component in many Arctic habitats [2, 74]. In addition, *Cymbella hantzschiana*, which dominated in one of the study sites, is also mentioned as a common species occurring in polar areas [2, 73, 74]. Differences in the species composition could have been caused by the character of each study site (e.g., flowing vs low flow). In any case, the results obtained within this study give an evidence of the survival only for the most abundant species. Other species were represented by a low number of cells, which prevented from making any clear conclusion concerning their physiological performance during the annual cycle.

Conclusion

In conclusion, this study provided a first detailed insight into the physiological status of diatom cells throughout the annual cycle in the extreme conditions of the High Arctic. The adaptation of vegetative cells is crucial for the winter survival of polar freshwater diatoms; a significant proportion of cells were able to survive winter without forming any morphologically distinct stages and served as the inoculum for the following vegetative season. Remarkably, a relatively high number of cells were active immediately after thawing in the winter season. It was shown that the survival is seasonally and locality dependent. The influence of ice thickness to spring survival was also important. The average max–min weekly temperature differences in autumn and winter were not significant.

Acknowledgments The authors wish to thank the Czech Arctic Scientific Infrastructure of the University of South Bohemia, Josef Svoboda Station, Svalbard (CzechPolar2 Project LM2015078 supported by the Ministry of Education, Youth and Sports of the Czech Republic), and the institutional long-term research plan RVO67985939 of the Institute of Botany of the Czech Academy of Sciences. Eveline Pinseel and Bart Van de Vijver are

acknowledged for identification of diatoms and Jana Duchoslavová for a statistical help. The authors wish to thank Paul Hamilton and three anonymous reviewers for their constructive comments on an earlier version of the manuscript.

Funding Information The research was supported by the Grant Agency of Charles University (project no. 20217).

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