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Corresponding authors:
Daniel Remias,
E-mail: daniel.remias@plus.ac.at;
Lenka Procházková,
E-mail: lenka.prochazkova@natur.cuni.cz

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The first cultivation of the glacier ice alga *Ancylonema alaskanum* (Zygnematophyceae, Streptophyta): differences in morphology and photophysiology of field vs laboratory strain cells

Daniel Remias[1,2] and Lenka Procházková[3,4]

[1]School of Engineering, University of Applied Sciences Upper Austria, Stelzhamerstr. 23, 4600 Wels, Austria; [2]Department of Ecology and Biodiversity, Paris Lodron University of Salzburg, 5020 Salzburg, Austria; [3]Department of Ecology, Faculty of Science, Charles University, Viničná 7, 12844 Prague, Czech Republic and [4]Centre for Phycology, Institute of Botany of the Czech Academy of Sciences, Dukelská 135, 37982 Třeboň, Czech Republic

Abstract

Melting glacier surfaces are unique ecosystems for specialized microbes, frequently harbouring blooms of microalgae with pigments contributing to the darkening of ice surfaces, reducing albedo and enhancing melt rates. The main cause of this phenomenon is algae of the genus *Ancylonema*. Prior investigation depended on field-collected material because these algae resisted cultivation. To enhance research on how these algae dominate melting ice, we established a strain of *Ancylonema alaskanum* from an alpine glacier and exposed to temperatures around the freezing point at irradiations of ∼10% of full sunlight. The morphology of the culture changed, with the cells becoming longer and turning green by losing their brownish pigmentation, indicating that these dark phenols are crucial for survival in the cryosphere. Photophysiological comparisons of strain and glacial material showed adaptation of the photosynthetic apparatus to prevailing conditions. This laboratorial strain opens possibilities for a wide range of comparative 'omics' research.

1. Introduction

During the melting season, icy surfaces of alpine and polar regions can be occupied by blooms of glacier ice algae (Williamson and others, 2019; Hoham and Remias, 2020). Recently, these specialized microorganisms have been recognized as significantly contributing to supraglacial darkening due to dark phenolic pigmentation (Stibal and others, 2017). The cells of some glacier ice algae are five-times more effective energy absorbers than snow algae (Halbach and others, 2022), and such algae are dominant albedo reducers and accelerators of ice thawing. This has been particularly recognized at the south-western margin of the Greenland ice sheet, and over the long term will likely eventually influence the rising rate of the global sea level (Cook and others, 2020; Onuma and others, 2022).

The majority of microalgae on melting ice belong to the green algal genus *Ancylonema* (Procházková and others, 2021), part of the Zygnematophyceae that are a sister group to land plants (Busch and Hess, 2022*a*). Unlike conventional red blooms of cryoflora (snow algae), glacier ice algal populations are made of actively dividing (vegetative) cells and not of non-dividing, protective stages like cysts. Despite this, research until now has depended on ephemeral field material because long-lasting cultures of glacier ice algae were not available. This was apparently due to the cessation of cell division once the algae were taken from their natural habitat, maybe due to difficulties in simulating the environmental conditions of wet ice surfaces (Remias and others, 2009, 2012*a*; Williamson and others, 2019). Nevertheless, in order to understand how glacier ice algae can colonize and cope with their extreme habitats, the establishment of laboratorial strains is crucial. Comparisons between cells harvested in situ vs grown ex situ under defined laboratorial parameters are necessary for uncovering the cytological, physiological or molecular aspects of their ability to overcome harsh conditions (i.e. frequent freeze–thaw events, low availability of nutrients or extreme irradiation levels).

For this purpose, we established a culture of *Ancylonema alaskanum* (formerly *Mesotaenium berggrenii* var. *alaskanum*), isolated from a glacier in the Austrian Alps (Procházková and others, 2021). This is a unicellular species commonly causing blooms in cold regions worldwide, such as Greenland (Uetake and others, 2010; Onuma and others, 2022), southern Chile (Takeuchi and Kohshima, 2004) or the European Alps (Remias and others, 2009). A brief comparison between environmental material and this laboratory strain revealed striking differences in cell lengths, the extent of secondary pigmentation and photophysiological light preferences. These traits likely reflect the variety of adaptation mechanisms needed for eukaryotic phototrophs living on ice and need to be elucidated in the future.

2. Materials and methods

For cultivation, *A. alaskanum* was harvested at the Gurgler Ferner glacier in Tyrol, Austria on 25 August 2020 (sample WP251; Fig. S1). Four preceding samplings from alpine glaciers were mainly used for cell size studies (samples P39, WP167, AS08, WP213; for details see Table S1). The raw material was taken with a stainless-steel scoop from bare greyish ice and transported frozen to the laboratory. There, it was gently melted at 4°C at an illumination of 30–40 µmol PAR (photosynthetically active radiation) $m^{-2} s^{-1}$ for ~12 hours. Cryoconite particles and other large debris were removed from meltwater by filtration through pre-cooled stainless-steel sieves (800, 400, 200 and 140 µm; Retsch, Germany). This material was used for photophysiological measurements. A second cleaning step was performed by density gradient centrifugation (400*g*, 1°C, 5 min), using layers of 10 ml each of 100, 66 and 33% (v/v) Percoll (Sigma Aldrich P1644) in 50 ml glass tubes. On top, 10 ml of a well-suspended cell pellet (after 20 s of ultrasonication) was applied, and after centrifugation a cell layer of *A. alaskanum* occurred at the border between 66 and 100% Percoll. This layer ('B1') was collected with a sterile pipette and washed two times with pre-filtered (Whatman GF/F) glacial meltwater, performing the same centrifugation steps as before. This Percoll-cleaned field material (sample WP251B1) was used for algal cultivation, molecular sequencing and light microscopy (LM) purposes. For cultivation, a drop of this cell suspension was pipetted into sterile Petri dishes, filled with an inorganic medium used specifically for Zygnematophyceae – SFM (synthetic freshwater medium; pH 6.0, by M. Melkonian, see Durán and others, 2022) – and solidified with 1.5% agar (w/v). Fungal growth was inhibited by the addition of benomyl (10 mg l^{-1}, dissolved in 2 ml ethanol and added to the hot medium directly after autoclavation). The glacier algae were exposed to a diurnal cycle of 12 hour light (PAR ~100–120 µmol $m^{-2} s^{-1}$) at 1°C and 12 hour darkness at −1°C in a Percival Plant Growth chamber (LT-36VL) equipped with 'full spectrum' 36 W fluorescence tubes (Narva 958 bio-vital) including UV-A. Green colonies were observed and evaluated by LM, picked by sterile loops and transferred onto fresh SFM plates without fungicide. For biomass generation, algae were put into liquid SFM with double the nitrogen and phosphorous concentrations (enhanced SFM = eSFM, Table S2), the temperature was set to 5°C, and irradiated at 15–25 µmol PAR $m^{-2} s^{-1}$ (14 hour light/10 hour dark). The medium was refreshed once a month. Cell sizes were measured with a Nikon Eclipse 80i LM equipped with a Nikon MRc5 camera using Nikon Cell Sense entry software. LM was also performed on an Olympus BX43 at 1000× magnification using oil immersion, equipped with an Olympus DP27 camera (Olympus Europe SE, Hamburg, Germany). The strain will be deposited at the Culture Collection of Cryophilic Algae as CCCryo 565-23.

The molecular identity of glacial field material and the newly established strain were compared by Sanger sequencing of three selected markers (18S rDNA, ITS2 rDNA, *rbc*L). DNA isolation was carried out with a DNeasy Plant Mini Kit (Qiagen, Germany) for field material WP167 and the strain WP251B1 as in Procházková and others (2018), or by the Instagene Matrix Kit (Bio-Rad Laboratories, Hercules, CA, USA) for field material WP251. The hypervariable ITS2 rDNA marker for *A. alaskanum* was also generated here for the first time. Detailed descriptions of all conditions for polymerase chain reaction amplification and Sanger sequencing are in the Supplementary material, including the accession numbers for the newly obtained sequences of *A. alaskanum*.

The photophysiology was evaluated by pulse–amplitude modulated fluorometry at different irradiation levels (Walz PAM 2500 with a KS-2500 0.4 ml suspension cuvette, held at 1°C). For more details on the sampling protocol for field material and rapid light curve (RLC) measurement, see Procházková and others (2021). For details on the fitting of statistical data including the detailed explanation of the calculated parameters, see Procházková and others (2018). Prior to RLC measurements, strain cells were acclimated either to low-light (~10–15 µmol PAR $m^{-2} s^{-1}$) or higher-light (~200 µmol PAR $m^{-2} s^{-1}$) conditions, both at 1°C (during day, 12 hour long) to −1°C (during night, 12 hour long) for 1 week. In the case of the field sample, after 30 min darkness adaptation, the sedimented algal cells in the meltwater of surface ice were directly put into the suspension chamber. Three to four independent replicates were measured.

3. Results and discussion

A laboratory strain of *A. alaskanum* was established, first separating bulk field material from contaminants like pollen, moss parts or other present microalgae by mechanical sieving, followed by density gradient centrifugation. This was done to improve the chances of acquiring significant numbers of dividing *Ancylonema* cells out of the typically rather numerous stagnant individuals, as opposed to picking and inoculating a few individuals at the beginning. Even so, it took almost three months of the incubation of cleaned cells at temperatures around the freezing point until green colonies were macroscopically visible. The majority of cells remained characteristically brownish due to abundant pigmented vacuoles, which contain UV and VIS absorbing purpurogallin-glycoside derivatives (Remias and others, 2012*b*). In the field, many yeast-like cells were observed by means of LM (data not shown). It is well known that glacial surfaces harbour mycobionts, some of them interacting with microalgae (Perini and others, 2022). In producing the strain, the undesirable fungal growth was inhibited with the fungicide benomyl, which did apparently not harm the glacier algae. It is unknown if the selection of SFM pH 6 (dedicated to Zygnematophyceae) as a substrate was crucial for the successful initiation of growth, but once the cells started dividing regularly, they also grew on conventional inorganic Bolt's basal medium (data not shown). It is possible that the constant temperatures around the freezing point were decisive in stimulating activity. Irradiance in the growth chamber was limited to <20% of full sunlight due to technical constraints, but we observed the growth of green cells even at only ~2% of full sunlight (data not shown). To accelerate the acquisition of biomass after successful growth initiation, cultivation was changed from a solid to a liquid substrate, which may 'simulate' a transitional scenario from the dry ice substrate below snow cover to melting wet ice surfaces during the onset of the melting period. Moreover, eSFM and slightly higher temperatures (5°C) were used. As a trial, the new strain was exposed to temperatures of 15°C, but cells bleached and died after 1 week, reflecting the psychrophilic nature of *A. alaskanum*.

While the field material and laboratorial strain had 100% identical marker sequences for 18S rDNA, ITS2 rDNA and *rbc*L (Table S3), the cell morphology was quite different, as seen in Figures 1 and S3. Interestingly, while at the glacier the average length of *A. alaskanum* was 11.4 ± 2.4 µm (WP251, $n = 27$), strain cells were longer, and had a higher length variability (WP251B1, 15.7 ± 3.9 µm, $n = 52$; box plots: see Fig. S2). In contrast, the cell width was similar in both cases (8.4 ± 0.8 µm vs 8.3 ± 0.4 µm). The length differences were likely due to the different abiotic conditions, such as the significantly higher availability of nutrients in the medium. Also, while most field cells had only one parietal chloroplast per cell, in the laboratory strain there were commonly either two chloroplasts, or a large one made of two separate thylakoid-bearing sections connected by a very thin isthmus-like

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stroma bridge. A further morphological difference was that the strain occasionally formed short filaments made of several cells (Fig. 1b), while in the field more than two slightly connected cells (after cleavage) were never seen. Ageing cultures occasionally contained distorted, prolonged cells with more than two chloroplasts (Fig. 1c).

Unlike the field samples, strain cells did not visibly accumulate dark secondary phenols under the applied PAR conditions. However, when the green cells were held at irradiances above 150 µmol photons m^{-2} s^{-1} PAR for more than 4 weeks, a vacuoles re-pigmentation process restarted (Fig. 1e, S3b), indicating that synthesis of the brownish phenols is likely induced by a certain extent of irradiation. It is thus likely that excessive PAR irradiation, maybe in combination with UV, induces enhanced purpurogallin-glycoside-derivative production. This ecophysiological behaviour would be in line with the aero-terrestrial zygnematophyte *Serritaenia* sp., the cells of which respond by the production and secretion of dark secondary pigments towards the direction of UV exposure (Busch and Hess, 2022*b*). The visual absence of broadband UV/VIS screening purpurogallin derivatives in *A. alaskanum* grown below 150 µmol PAR m^{-2} s^{-1} supports the concept that these pigments play a role in preventing harmful photoinhibition in the chloroplasts, or protect against other intracellular damages caused by UV irradiation (Remias and others, 2012*b*). Additionally, the dark phenols have been suggested to repurpose absorbed radiation to melt the surrounding ice, thus keeping the algal microhabitat liquid (Williamson and others, 2020).

For photoautotrophs, the light-dependent regulation of photosynthesis is a critical issue to prevent intracellular damage, especially in view of the excessive irradiation on glacial surfaces. Figure 2 compares the rapid light response curves of *A. alaskanum* in adaptation to three different light conditions (low-light, higher-light, in situ conditions). The maximum relative electron transport rates (rETR) of the photosystems differed between among the low- or higher-light bred green strain and the fully sun-exposed, brownish field cells (ambient PAR value of Gurgler glacier during clear summer sky was 2072 µmol photons m^{-2} s^{-1}; Remias and others, 2009). Only the low-light grown strain showed signs of photoinhibition (a decline in rETR), but on the other hand had the best light utilization efficiency (α = 0.23), which is beneficial under shaded conditions. The higher-light grown strain received ten times more PAR in advance and had the highest rETR at higher irradiances, but also the lowest efficiency. Overall, these differences can be explained as typical low-light (i.e. increased α, reduced I_k) or high-light (i.e. lower α, increased I_k) acclimation of the photosystems. The relative light curve of the field cells was intermediate but had only a slightly lower efficiency than the higher-light strain, even though an impairing darkening effect by the phenols could be expected.

Williamson and others (2020) used a similar fluorometric protocol testing the photobiology of the close relative and identically dark pigmented *Ancylonema nordenskioeldii* from the Greenland ice sheet (PAR was ~1700 µmol photons m^{-2} s^{-1} at bright clear sky). They calculated that considering the extent of shading phenols, the chloroplasts remained low-light-adapted

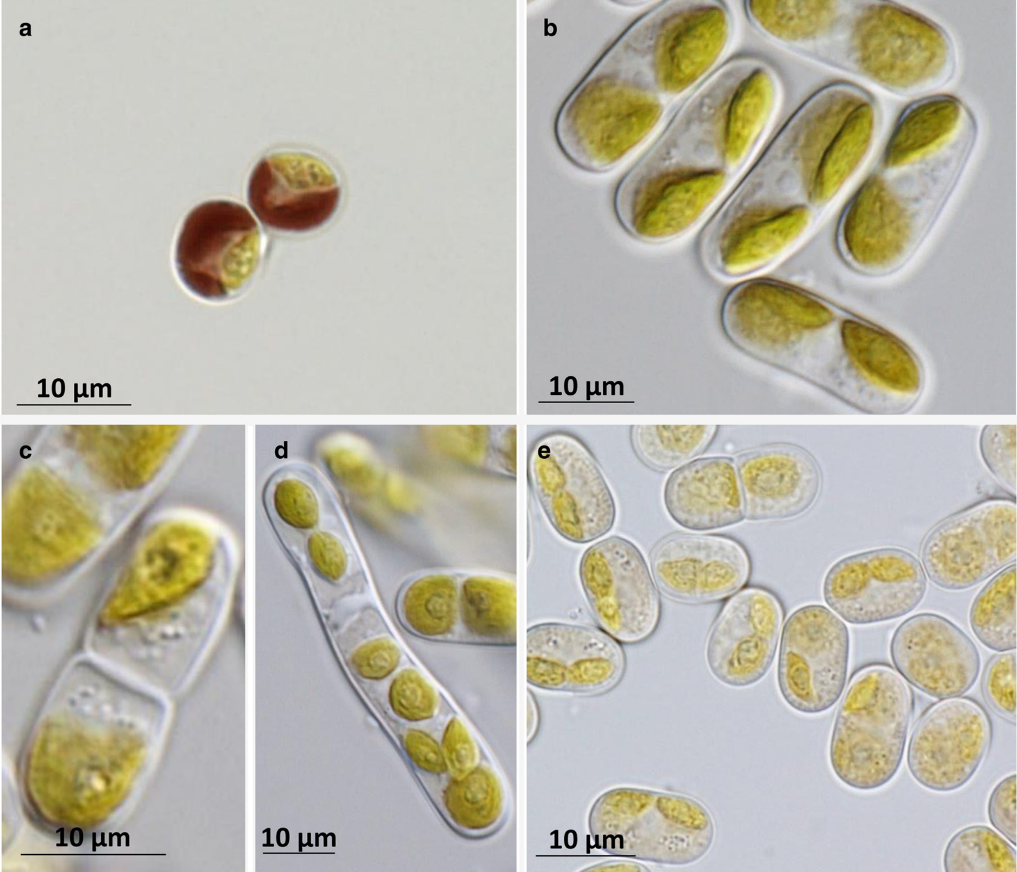

Figure 1. Light photomicrographs of *A. alaskanum* from the glacial surface of Gurgler Ferner, Austrian Alps (a) vs the new laboratorial strain WP251B1 (b–e). (a) A field sample showing two young cells, each with one chloroplast and prominent brownish (phenolic) vacuoles. (b) Representative strain cells with two shovel-like, parietal plastids, a central nucleus and non-pigmented vacuoles. (c) Occasionally, strain cells with only one chloroplast per cell were observed. (d) Distorted, elongated cells with an abnormal higher number of chloroplasts occurring in an ageing culture. (e) Cells exposed to 200 µmol PAR m^{-2} s^{-1} for four weeks show signs of vacuolar re-pigmentation.

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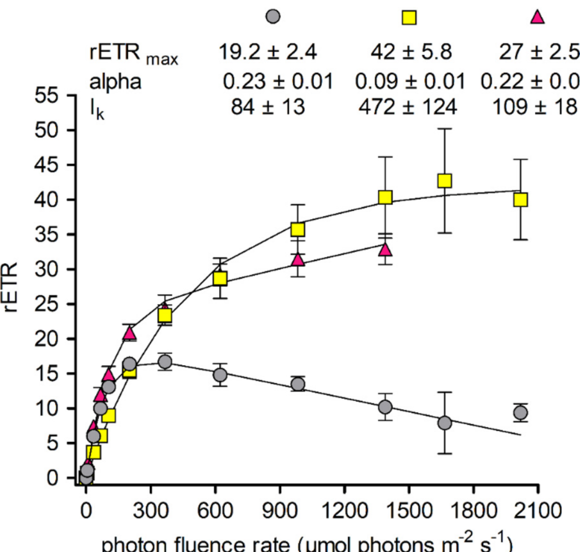

Figure 2. Photosynthetic RLCs of the glacier ice alga *A. alaskanum*. The strain with green cells (WP251B1) cultivated either at low light (grey circles, this study) or higher light (yellow boxes, this study) is compared with the brownish cells from the field (WP167) (pink triangles, Procházková and others, 2021). Values of maximum relative electron transport rates ($rETR_{max}$; it reflects the process of light-induced electron transport for generating chemical energy and reducing equivalents), low-light utilization efficiency (α; efficiency with which alga converts captured/absorbed radiation into organic dry matter through photosynthesis) and light saturation points (I_k; the onset of light saturation) are indicated at the top. Only the low-light grown strain showed photoinhibition (a decline in rETR) at higher irradiances. The higher-light grown strain performed worst at low-light (low α) but best at high irradiance, while the field cells showed a combination of both good low-light performance in combination with no signs of rETR impairment at higher irradiances. However, the light curve of the field cells was maybe not fully saturated, thus probably underestimating rETR and I_k.

($I_k = 46\,\mu mol\ m^{-2}\,s^{-1}$). Native measurements of fully exposed cells had an I_k of 938, which was ninefold higher than *A. alaskanum* from the Alps measured here, but only twice higher than *A. nordenskioeldii* from the Alps (Procházková and others, 2021). However, while they performed an in situ re-incubation study on ice with algae in meltwater, our algae were exposed to low light for 12 hours during melting prior RLC measurement, which could short-term affect the photobiology. In agreement with their study, cells grown under lower light than ambient conditions (yellow boxes in Fig. 2) had higher rETR values than fully exposed ones (pink triangles in Fig. 2).

4. Conclusion

Using a combination of several cultivation protocols including the initial purification of field material, a strict temperature regime close to the freezing point, light conditions <10% of maximum ambient VIS and liquid inorganic medium with the fungicide benomyl, a laboratory strain of *A. alaskanum* was established, with continuous cell growth after a lag-phase of 14 weeks. Three molecular markers confirmed the identity of the new strain to environmental cells. The striking brownish vacuolar phenols seem to be essential for coping with their exposed ice surface habitat, and they were absent under low-light cultivation conditions. Nonetheless, the capacity of photosynthetic machinery to tolerate extreme incident irradiance was demonstrated for non-brownish cells. This first laboratory strain of *Ancylonema* glacier ice alga will open vast possibilities for a wide range of molecular, physiological and metabolomic research.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/jog.2023.22.

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Conflict of interest. The authors declare that they have no conflict of interest.

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