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MICROBIOLOGY OF AQUATIC SYSTEMS

Seasonal Dynamics of *Zygnema* (Zygnematophyceae) Mats from the Austrian Alps

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Abstract

Filamentous green algae of the genus *Zygnema* are an essential part of hydro-terrestrial ecosystems. Despite several studies on their resistance to natural stresses, little is known about the composition of their assemblages and the changes they undergo over time. Two sites at altitudes above 2200 m a.s.l. in the Austrian Alps were selected for a 2-year observation period and sampled five times. Molecular phylogenetic analysis of the 152 isolated strains of *Zygnema* sp. was performed based on the *rbcL* and *trnG* sequences. Seven genotypes were found at these sites during the samplings, but their proportion varied throughout the seasons. The site with a more stable water regime also had a more stable representation of genotypes, in contrast to the site with fluctuating water availability. The mats formed resistant pre-akinetes at the end of the season with reduced photosynthetic activity. Contrary to expectations, the mats were not exposed to extremely cold temperatures in winter due to snow cover. Some genotypes have been previously observed at this site, indicating that the population composition is stable. This work highlights the importance of resistant pre-akinetes in surviving winter conditions, the ability of algae to re-establish mats, and the need to address the hidden diversity of the genus *Zygnema*.

Keywords Overwintering · Cryptic diversity · Chlorophyll fluorescence · Freezing · Hidden diversity

Introduction

Periodical changes in environmental conditions during the year are accompanied by weather changes (fluctuations in water availability, high or low temperatures, changing illumination levels, etc.), which often consist of periods of unfavorable conditions. These are particularly challenging for living organisms that must be adapted to surviving in varying environments. Pronounced seasonal differences and long periods of subzero temperatures are characteristic of both polar and high-altitude temperate regions [1]. Long winters act as the main stressor, resulting in a short growing season, which leads to a decline in higher plant vegetation. Thus, tundra ecosystems are dominated by non-vascular plants, including algae, which often form large-scale macroscopic mats [2–5]. Changing environment leads to the development of complex life cycles with different seasonal stages, which can ensure their long-term survival. In addition, patterns of changes in abundances of numerous algal genera have been documented [6].

Wet places in polar and high alpine tundra habitats are dominated by mucilaginous algal mats, often formed by filamentous conjugating green algae (Zygnematophyceae, Streptophyta). They have a simple morphology of unbranched filaments, and the group is characterized by a unique type of sexual reproduction called conjugation [7]. Conjugation results in a dormant zygospore, whose morphology is useful in traditional species determination [8, 9]. Moreover, conjugating algae are considered the closest relatives to land plants (Embryophyta); therefore, elucidating their stress tolerance and life strategies is important from an evolutionary point of view [10, 11].

Many studies have recently investigated the stress resistance of Zygnematophyceae algae in the vegetative state [12, 13]. Zygnematophyceae algae showed resistance to desiccation [14–16], osmotic stress [17, 18], ultraviolet (UV) irradiation [19–21], and freezing [22–24]. However, stress tolerance is often a characteristic of a single cell types. For

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example, there is a difference between the stress tolerance of young cells and hardened pre-akinetes of *Zygnema* sp. [14] or the green and purple morphs of *Zygogonium ericetorum* [21]. In contrast, unspecialized vegetative cells of *Zygnema* sp. showed high resistance to UV irradiation [25]. They are also sensitive to high temperatures [26]. In the genus *Serritaenia*, the UV resistance of vegetative cells was mediated by their pigment-rich mucilage envelopes [27]. *Spirogyra* sp. is known for its resistance in the vegetative stage, and it has been repeatedly found in Antarctic ponds, while no spore formation has been observed [28].

Despite the stress resistance of vegetative cells, field observations in different climatic conditions confirmed the seasonality of the mats formed by Zygnematophyceae algae; characterized by new, fast growing biomass developing every year [5, 28]. Seasonality was observed in several Zygnematophyceae, particularly *Spirogyra*, *Zygnema*, and *Mougeotia* [28–31]. The mass growth of Zygnematophyceae algae begins in spring, and their biomass volume usually peaks between May and September in temperate regions [29, 32]. Algal mats in the Arctic and Antarctic also showed higher abundances in spring and summer; however, this period is noticeably shorter there [5, 28]. The formation of lipid-rich pre-akinetes at the end of the growth season enabled survival through winter freezing [5, 33]. Mats of Zygnematophyceae algae usually grow quickly at the beginning of the growth season. *Spirogyra* can triplicate its biomass within only 3 days [31]. Spring coverage of the total water surface by floating algal mats can reach 70–80% under suitable conditions [29]. Reproduction usually occurs during the spring and summer. In the Netherlands [29], sexual reproduction of *Spirogyra* species was observed from April to August. The abundance of algal communities usually fluctuates throughout the year. The decline in conjugating-algal communities may be caused by summer drought [34] or anoxic conditions under ice [28].

Several studies have investigated the genetic diversity of *Zygnema* in Svalbard [14, 35], New Zealand [36], California [8], and Czechia [9]. As mentioned above, characteristics associated with sexual reproduction are used in species delimitation; however, only sterile populations are often found in field conditions. Thus, the diversity within the genus *Zygnema* is most likely underestimated, and an understanding of the seasonal pattern of individual species (strains) is still lacking. A recent study in Svalbard [4] showed unexpectedly high diversity hidden within the algal mats; the mats were composed up to four different genotypes. Zygospores of *Zygnema* and *Zygnemopsis* were found during sampling, but their occurrence was rare.

In the present study, we focused on the diversity within the mats formed by *Zygnema* sp. in the Alps and its changes within 2 years and different seasons. We hypothesized that, like in the Arctic, zygospores would be very rare, and hidden diversity will be detected. Moreover, we aimed to understand which stress conditions directly affect the annual character of the mats and whether genotypes tended to inhabit one place for a longer time. The results showing trends in long-term persistence are discussed in the context of this alga's environmental conditions and stress resistance.

Materials and Methods

Studied Sites

The field study was performed in the Austrian Stubai Alps, near Kühtai village. This site has an alpine tundra characteristic with a cold temperate climate. Summer stretches from the end of June to September [37]. The average annual temperature is 0.9 °C, and annual rainfall is 1653 mm [37]. Two localities with a high abundance of algal mats, designated "Lake" (47.2273°N, 11.0152°E, 2354 m a.s.l.) and "Streamlet" (47.2223° N, 11.0283° E, 2243 m a.s.l.), were selected for the long-time observation study. "Lake" was the tributary area of a stream flowing into a study site (Fig. 1a). Water was available throughout the year, and the conditions were relatively stable. Algal mats were distributed in the shallow waters of the tributary area and submerged most of the time during the vegetative season (Fig. 1b). "Streamlet" was a wet meadow (Fig. 1c), which was fed by melting water from the mountains above through small, branched streamlet. Algal mats were distributed throughout the wet area in the depressions filled with retained water (Fig. 1d). Water levels fluctuated strongly, from almost complete desiccation to a large amount of available slow-streaming water. The mats were subjected to repeated desiccation and were exposed to direct sunlight for most of the year.

Sampling Pattern

Both localities were sampled five times: August and November 2018 and July, August, and October 2019. Sampling covered the vegetative seasons in both years, mainly summer and autumn, when the largest biomass was expected. Sampling was performed at multiple locations throughout the mat to ensure that the entire mat was covered. We used 120 mL plastic containers for sampling to collect a sufficient volume of biomass (approx. 20 mL) to estimate the proportion of pre-akinetes. The water temperature in the two observed sites was measured during the study period (from 21 August 2018 to 16 August 2020) using a Minikin Tie datalogger (Minikin Tie, ESM, Brno, Czech Republic) at 1-h intervals during the study. The dataloggers were placed close to the algal mats and secured using a cord. Data from the dataloggers were graphically displayed using Sigma plot 14.0. The pH and conductivity of the water were measured

Fig. 1 Localities in Kühtai where long-term observation was performed. **a** Lake locality, algal mats were located in the tributary area of the Lake in a stream, marked with arrow; **b** detail of the algal mat in the stream water on the Lake locality; **c** Streamlet locality, stream on a wet meadow, algal mats were distributed through the wet area in the depressions in-ground and a branched streamlet, marked with arrow; **d** detail of the algal mat in a depression filled with retained water on the Streamlet locality

during the first sampling (WTW pH/Cond 340i). To correlate the presence of mats and cell types with the water level in the localities, we created a scale of estimated water levels from 1 to 5, where 1 means completely dry, and 5 means high amount of water during the spring snowmelt. Scales applied to localities 1 and 2 are not fully comparable because of the different water regimes in both localities.

Four additional localities around Kühtai were also sampled once to determine the total diversity of *Zygnema* sp. in the region and to enrich the dataset for phylogenetic analysis: Lake Gossenkollesse (47.2302°N, 11.0136°E, 2417 m a.s.l.), Pond 1 Kühtai (47.2206°N, 11.0235°E, 2260 m a.s.l.), Pond 2 Kühtai (47.2214°N, 11.0240°E, 2268 m a.s.l.), and Upper Lake Kühtai (47.2284°N, 11.0089°E, 2436 m a.s.l.).

Cultivation and Origin of Strains, Light Microscopy Observations, and Measurements

The samples were collected and subsequently transferred to the laboratory in a cooled box. They were microscopically examined for *Zygnema* sp. filaments, and the young vegetative cells/pre-akinetes ratio in each locality was estimated. Using a single-filament isolation method, we obtained 152 monoclonal cultures that were cultivated on Bold's basal medium (Merck, Germany). Additionally, four strains from Kühtai were obtained from a previous sampling campaign in 2015 and used in this study. Cultures were incubated at 18–19 °C and with continuous illumination at 40 mmol m^{-2} s^{-1} using 18 W cool fluorescent tubes (Philips TLD 18 W/33, Royal Philips Electronics). The strains were microscopically examined after 3 weeks of cultivation. Microphotographs of the strains representing all clades were obtained using a Leica DM2000 LED equipped with a camera, Leica ICC50W, with the LASX program (Leica Microsystems). Microphotographs were graphically adjusted using Affinity Photo (Serif Ltd.). Thirty cells for each genotype were randomly chosen for width and length measurements in ImageJ software (http://imagej.nih.gov/ij). To reveal possible significant differences between strains, measured cellular width and length were tested by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test in PAST 4.10 [48].

DNA Isolation, PCR Reaction, Sequencing

Genomic DNA was extracted using the Instagene matrix (Bio-Rad), according to a previous protocol [38]. Two molecular markers were used for the phylogenetic analysis: plastid *rbc*L and *trn*G. The *rbc*L region was amplified using the primers RH1 and 1385R [39] or ZygF and ZygR [4]. Primers trnG-uuc-F-5′ and trnG-uuc-R-3′ [40] were used to amplify the *trnG* intron. The PCR reactions were carried out in 20 µL volumes: 13.9 µL of sterile Milli-Q water, 2 µL of $MgCl_2$ (25 µM), 2 µL of PCR Buffer 10 × (Applied Biosystems), 0.4 µL of dNTP, 0.25 µL of each primer, 0.2 µL of AmpliTaq GOLD polymerase (5 U/µL), and 1 µL of template DNA (not quantified). PCR amplification of the *rbcL* region (temperatures and times for the *trnG* intron are given in brackets) was set to an initial denaturation at 95 °C for 10 min (94 °C for 2 min), followed by 35 (40) cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min, elongation at 72 °C for 2 min (2.5 min), and a final extension at 72 °C

for 10 min. The PCR products were purified using AMPure XP beads (Beckman Coulter, Inc.) and sequenced using an Applied Biosystems automated sequencer (ABI 3730xl) by Macrogen (Seoul, Korea). Sequencing reads were assembled and edited using the SeqAssem program [41]. The obtained unique sequences were concatenated using the MEGAX program [42], assigned with capital letters Z1–Z7, and submitted to GenBank under accession numbers ON338067, ON338068, and ON989204 to ON989211 for *the rbcL* region and ON980776 to ON980783 for the *trnG* region.

Sequence Alignment and Phylogenetic Analysis

The concatenated phylogeny of the plastid *rbcL* gene and *trnG* intron was inferred to illustrate the phylogenetic position of the newly obtained *Zygnema* strains. The dataset of 152 newly isolated strains was enriched with 24 sequences collected from Svalbard and the Czech Republic [4] and sequences obtained from public databases. A total of 180 strains were analyzed. Sequences were aligned using MEGA X [42] and trimmed manually. The most appropriate substitution model was estimated for each dataset using the Bayesian information criterion (BIC) with jModeltest 2.1.4 [43]: GTR + I for the first, JC for the second, and HKY + Γ for the third codon position of the *rbcL* dataset and HKY + G model for the *trnG* alignment. The phylogenetic tree was inferred by Bayesian inference (BI) using MrBayes version 3.2.6 [44]. Bootstrap analyses were performed using maximum likelihood (ML) criteria on GARLI version 0.951 [45]. The phylogenetic tree obtained was displayed in FigTree [46] and Mega X [42]. Finally, the displayed phylogenetic tree was graphically adjusted using Affinity Designer (Serif Ltd.).

Chlorophyll a Fluorescence

The photosynthetic activity was assessed using chlorophyll fluorescence. Biomass samples for fluorescence measurements were harvested from Lake and Streamlet in August and October 2018 and transported to the laboratory. Prior to the measurement, the samples were dark-adapted for 30 min. The maximum quantum yield (F_V/F_M) and relative electron transport rate (rETR) curves were measured using a pulse-amplitude-modulated fluorometer (FluorCam, PSI, Czech Republic). The light response curves showed photoinhibition and were fitted according to Webb et al. [47]. The parameters rETRmax (µmol electrons m^{-2} s^{-1}), α (electrons $photon^{-1}$), and I_k (µmol) were calculated. The difference in the values of the calculated fluorescence parameters between seasons was tested using the t-test in PAST [48].

Results

Phylogenetic Diversity of Mats Formed by Zygnema sp.

Phylogenetic analyses revealed seven *Zygnema* sp. genotypes in the Kühtai region (Fig. 2). These genotypes were distributed among other *Zygnema* strains in both major clades of the genus. Most strains isolated during sampling in Kühtai belonged to genotype Z1 (Fig. 2). This lineage mainly comprised isolates from the Lake (66 isolates), Streamlet (10), Lake Gossenkollesse (7), and two in ponds 1 and 2. Genotype Z2 was represented by fewer isolates from the Lake (8), upper lake (2), and pond 2 (2). Lineages Z3 and Z5 comprised exclusively the isolates from Lake and Streamlet. Genotype Z4 comprised isolates from Streamlet (2) and upper lakes (2). Genotypes Z6 and Z7 belonged to Clade 2. Lineage Z6 comprised isolates from Lake (1) and Streamlet (16), together with the two isolates from 2015. Genotype Z7 comprised exclusively isolates from Lake (2) and Streamlet (4). The number of isolates found in all localities and their lineages are summarized in Table 1. Four isolates of *Zygnema* sp. found in 2015 by Martina Pichrtová clustered together with those identified during the observation period. Isolate Kühtai 8 was identical to other isolates of genotype Z1, and isolates Kühtai 5 and 7 belonged to genotype Z6. Isolate Kühtai 4 was not detected during the observation period.

Vegetative Morphology

Isolated strains from seven Kühtai lineages shared a similar vegetative morphology typical of this genus (Fig. 3a-g). The young vegetative cells had two stellate chloroplasts, and vacuoles occupied most of the remaining space in the protoplast. Pre-akinetes showed only imperceptible vacuolization, while the cells were full of storage lipids, and chloroplasts were reduced. We did not observe any conjugation or zygospore formation despite our sampling efforts.

Width of cells varied from 24.5 µm in Z5 (mean value, ± SD 0.72) to 26.6 µm in Z3 (mean value, ± SD 1.63). Cell lengths varied from 37.4 µm in Z6 (mean value, ± SD 9.81) to 52.5 µm in Z1 (mean value, ± SD 8.16). Widths and lengths of cells measured for each genotype are given in Supplementary Information 1. Cell length varied significantly among some strains, but cell width, on the contrary, was quite uniform. Only the difference between strains Z3 and Z5 was proven to be weakly significant ($p = 0.01061$). Complete results of statistical analyses are given in Supplementary Information 2.

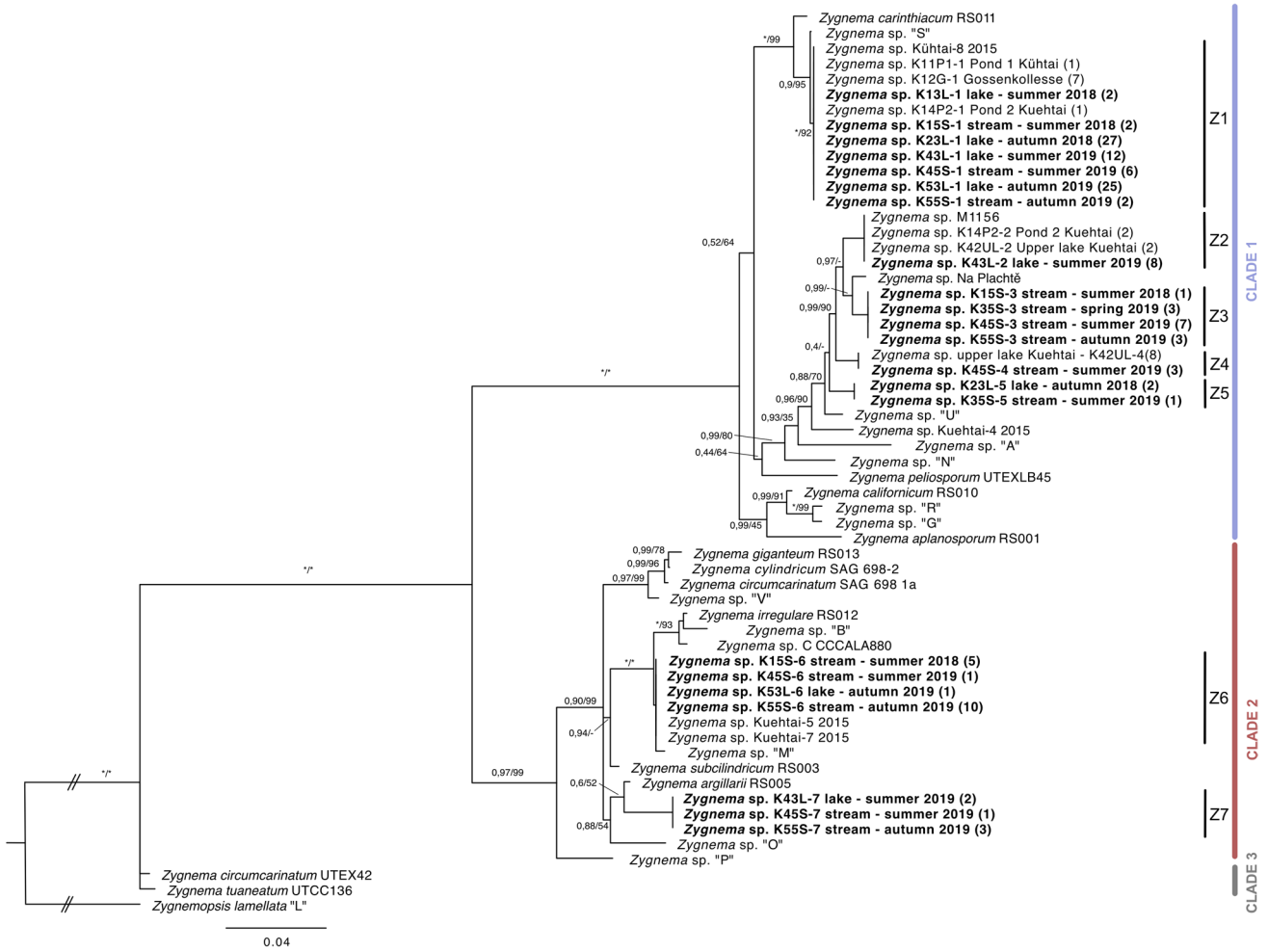

Fig. 2 Phylogenetic tree based on the Bayesian analysis of the concatenated *rbcL* and *trnG*^{UCC} intron sequences. Strains of *Zygnema* sp. collected during the long-term observation in Kühtai are shown in bold. Values at the nodes indicate statistical support estimated by MrBayes posterior node probability and maximum likelihood bootstrap. Number of isolates per locality and sampling is given in brackets. Full statistical support is marked with an asterisk. Branch lengths within the outgroup were shortened to improve visualization

Seasonal Changes of Zygnema sp. Mats and Their Persistency

The two sampling sites differed in diversity throughout the year (Fig. 4). Genotype Z1 prevailed in the Lake during all sampling periods (Fig. 4a). Additionally, the less abundant genotypes Z2, Z5, Z6, and Z7 inhabited the Lake. However, they occurred only in small volumes and were not present year-round. The Streamlet showed a higher diversity of *Zygnema* sp. In total, six different genotypes were identified during sampling on this site. The prevailing genotype differed among samplings on the Streamlet locality (Fig. 4b). Genotypes Z3 and Z6 prevailed during the four sampling periods when the algal mats were present. Sampling sites differed in the average number of genotypes found during the sampling; we found 1–3 genotypes in the Lake contrary to the 2–5 genotypes in the Streamlet. No algal mats were found at the sites during the two sampling events: in the Streamlet in November 2018 and the Lake in July 2019.

Pre-akinetes were found in both areas. Their proportion in mats fluctuated from 5 to 95%, depending on the season and locality. The lowest number of pre-akinetes (5–10%) was observed during the vegetative period in July and August. We observed massive production of pre-akinetes during the autumn, up to 95% of cells in the pre-akinete stage in October and November (Fig. 5).

Seasonal Changes of the Habitats

Changes in water availability at the two observed localities were recorded during each sampling. The sampling sites differed in their water regime. Water availability in the

Table 1 Complete list of new strains isolated from mats on localities during five samplings from 2018 to 2019

Observation	Month of collection	Locality	Genotype	Number of isolates	Code in the phylogenetic tree	Coordinates	*rbcL* Genebank accession number	*trnG* Genebank accession number
Isolates found during the observation period								
1	August 2018	Lake (1)	Lineage Z1	2	K13L-1	47.2273°N, 11.0152°E	ON338067	ON980776
		Streamlet (2)	Lineage Z1	2	K15S-1	47.2223°N, 11.0283°E	ON338067	ON980776
		Streamlet (2)	Lineage Z3	1	K15S-3	47.2223°N, 11.0283°E	ON989204	ON980778
		Streamlet (2)	Lineage Z6	5	K15S-5	47.2223°N, 11.0283°E	ON989207	ON980781
		Lake Gossen-kollesse (3)	Lineage Z1	7	K12G-1	47.2302°N, 11.0136°E	ON338067	ON980776
		Pond 1 Kühtai (4)	Lineage Z1	1	K11P1-1	47.2206°N, 11.0235°E	ON338067	ON980776
		Pond 2 Kühtai (5)	Lineage Z1	1	K14P2-1	47.2214°N, 11.0240°E	ON338067	ON980776
		Pond 2 Kühtai (5)	Lineage Z2	2	K14P2-2	47.2214°N, 11.0240°E	ON338068	ON980777
2	November 2018	Lake (1)	Lineage Z1	27	K23L-1	47.2273°N, 11.0152°E	ON338067	ON980776
		Lake (1)	Lineage Z5	2	K23L-5	47.2273°N, 11.0152°E	ON989206	ON980780
3	July 2019	Streamlet (2)	Lineage Z3	3	K35S-3	47.2223°N, 11.0283°E	ON989204	ON980778
		Streamlet (2)	Lineage Z5	1	K35S-5	47.2223°N, 11.0283°E	ON989206	ON980780
4	August 2019	Lake (1)	Lineage Z1	12	K43L-1	47.2273°N, 11.0152°E	ON338067	ON980776
		Lake (1)	Lineage Z2	8	K43L-2	47.2273°N, 11.0152°E	ON338068	ON980777
		Lake (1)	Lineage Z7	7	K43L-7	47.2273°N, 11.0152°E	ON989208	ON980782
		Upper lake Kühtai (6)	Lineage Z2	2	K42UL-2	47.2284°N, 11.0089°E	ON338068	ON980777
		Upper lake Kühtai (6)	Lineage Z4	8	K42UL-4	47.2284°N, 11.0089°E	ON989205	ON980779
		Streamlet (2)	Lineage Z1	6	K45S-1	47.2223°N, 11.0283°E	ON338067	ON980776
		Streamlet (2)	Lineage Z3	7	K45S-3	47.2223°N, 11.0283°E	ON989204	ON980778
		Streamlet (2)	Lineage Z4	3	K45S-4	47.2223°N, 11.0283°E	ON989205	ON980779
		Streamlet (2)	Lineage Z6	1	K45S-6	47.2223°N, 11.0283°E	ON989207	ON980781
		Streamlet (2)	Lineage Z7	1	K45S-7	47.2223°N, 11.0283°E	ON989208	ON980782

Table 1 (continued)

Observation	Month of collection	Locality	Genotype	Number of isolates	Code in the phylogenetic tree	Coordinates	*rbcL* Genebank accession number	*trnG* Genebank accession number
5	October 2019	Lake (1)	Lineage Z1	25	K53L-1	47.2273°N, 11.0152°E	ON338067	ON980776
		Lake (1)	Lineage Z6	1	K53L-6	47.2273°N, 11.0152°E	ON989207	ON980781
		Streamlet (2)	Lineage Z1	2	K55S-1	47.2223°N, 11.0283°E	ON338067	ON980776
		Streamlet (2)	Lineage Z3	4	K55S-3	47.2223°N, 11.0283°E	ON989204	ON980778
		Streamlet (2)	Lineage Z6	8	K55S-6	47.2223°N, 11.0283°E	ON989207	ON980781
		Streamlet (2)	Lineage Z7	3	K55S-7	47.2223°N, 11.0283°E	ON989208	ON980782
Other isolates used in the study								
	2015		Kühtai 4	1	Kühtai-4 2015	N/A	ON989211	N/A
	2015		Lineage Z6	1	Kühtai-5 2015	N/A	ON989210	ON980783
	2015		Lineage Z6	1	Kühtai-7 2015	N/A	ON989210	ON980783
	2015		Lineage Z1	1	Kühtai-8 2015	N/A	ON989209	ON980778

Lake fluctuated between levels 3 and 5 (Fig. 5), where 3 meant a stable water availability, while 5 referred to a high amount of water during spring snow melting. We observed stable conditions in the Lake, with no periods of drought, except in winter, characterized by frozen water.

In contrast, the water regime in the Streamlet differed fundamentally. The sampling site experienced significant changes in water availability (Fig. 5). Water levels fluctuated from levels 2 to 4, where 2 meant almost dry, while 5 meant a completely wet locality with a strong current of the streamlet and depressions filled with a large amount of retained water. Algal mats were exposed to desiccation events when water levels dropped, and the biomass was exposed to air. Water conductivity and pH measured during the first sampling were 0.04 mS cm^{-1} and 6.8 in the Streamlet and 0.06 mS cm^{-1} and 7.6 in the Lake, respectively.

The temperature of the water measured by the Minikin sensors and the average air temperature in Kühtai are shown in Fig. 6. The lowest and highest recorded water temperature at the Lake and Streamlet were – 0.2 °C (21 November 2018) and 20.7 °C (25 July 2019) and – 0.04 °C (26 November 2018) and 21.3 °C (31 August 2019), respectively. The average winter temperature of the water in the Lake and Streamlet was 0.31 °C and 0.9 °C in 2018 and 2019 (from November to April), respectively. The average winter temperature of the air in Kühtai measured from November to April was – 5.3 °C (data collected from 1999 to 2019) [37]. The average daily minimum and maximum air temperature was – 13.7 °C in January and 15.4 °C in August, respectively [37].

Photosynthetic Activity During the Year

Both localities showed similar declines in photosynthetic activity throughout the season. The maximum quantum yield reached significantly lower values at the end of the season than that in summer at both localities, although the actual values differed only slightly in the Lake (Table 2). A clear difference in photosynthetic performance was revealed by the rETR curves (Fig. 7, Table. 2). At both sampling sites, autumn samples showed clearly different kinetics from that of summer samples: mean rETR max values decreased significantly in both the Lake and Streamlet. The decrease in α and I_k values was significant only for the Streamlet. All parameters and t-test results are summarized in Table 2.

Discussion

In this study, mats of *Zygnema* sp. growing in the alpine tundra habitat were thoroughly investigated for the first time. We found eight different genotypes within the study area, seven during this investigation, and one additional unpublished genotype collected in 2015. In both studied localities, pre-akinetes formed at the end of the vegetative season. This adaptation helps the population overcome an unpleasant winter period.

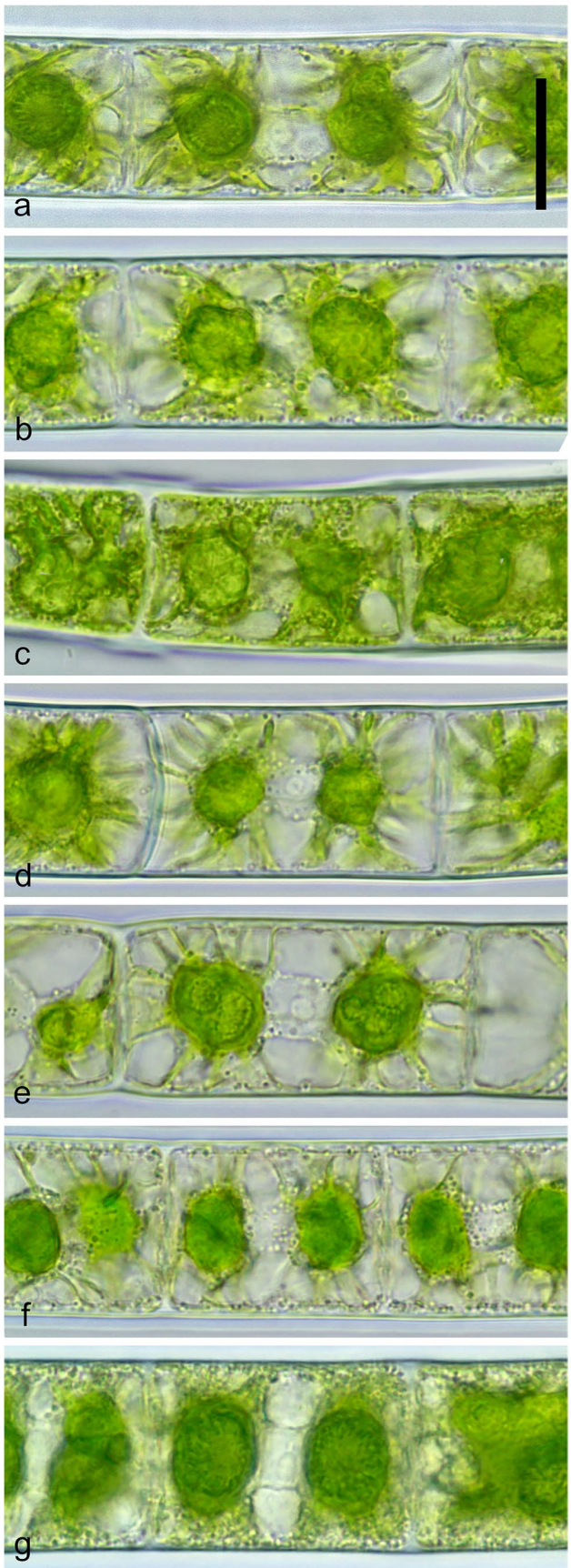

Fig. 3 Young vegetative cells of the genotypes found during the long-term observation. Each image represents a unique genotype from Kühtai lineages Z1–Z7; genotype name is written in the brackets. **a** Lineage Z1 (K23L-1); **b** lineage Z2 (K43L-2); **c** lineage Z3 (K45S-3); **d** lineage Z4 (K45S-4); **e** lineage Z5 (K23L-5); **f** lineage Z6 (K53L-6); **g** lineage Z7 (K45S-7). Scale bar = 20 µm in all images

Diversity of Zygnema sp. in the Studied Region

The diversity within the mats of *Zygnema* sp. growing in the alpine tundra habitat was unexpectedly high. Seven genotypes, assessed by *rbcL* and *trnG* sequences, grew in the Kühtai streams. Another unique *rbcL* genotype was reported in the same study area in 2015 and added to our dataset. None of the 152 isolated strains exhibited any morphological characteristics related to sexual reproduction, restricting species determination. Therefore, we used the term "genotype" instead of "species." Genotypes growing in Kühtai were distributed in clades 1 and 2 (Fig. 2). Although the DNA sequences of most of the genotypes were not identical to any *Zygnema* strains from public databases, they should not be considered endemic to the region because the diversity of this genus has not been satisfactorily investigated yet, and recent molecular diversity studies revealed previously unknown genotypes [4, 8]. Moreover, there is no working species concept based on which species can be reliably distinguished. Traditional taxonomy based on morphological characters associated with sexual reproduction is not applicable to most natural samples because of the rarity of sexual reproduction. Furthermore, the conjugation of *Zygnema* has not yet been successfully induced. In contrast to our previous study from the Arctic, the Alpine strains did not show considerable variation in cell width [4]. In the current study, only a small area was investigated, while the sampling sites in the Arctic covered a larger region with a high variation of abiotic conditions [4] which might be reflected by morphological differences. Variations in cell size can also be explained by differences in genome size. However, only little is known about genome size variation in *Zygnema* [49], and its correlation with *Zygnema* phylogeny and morphology is currently under investigation.

Spatial and Temporal Dynamics of Zygnema sp. Diversity

The mats were usually formed by several *Zygnema* sp. genotypes growing together (Fig. 4). A similar phenomenon of up to four genotypes in one mat was previously observed in the tundra habitats of Spitzbergen, which are very similar to high alpine habitats [4]. In the genus *Spirogyra*, up to three genotypes have been observed to grow together in one location [50, 51]. Sympatric occurrence has also been described for other microalgae such as desmids [52] or diatoms [53].

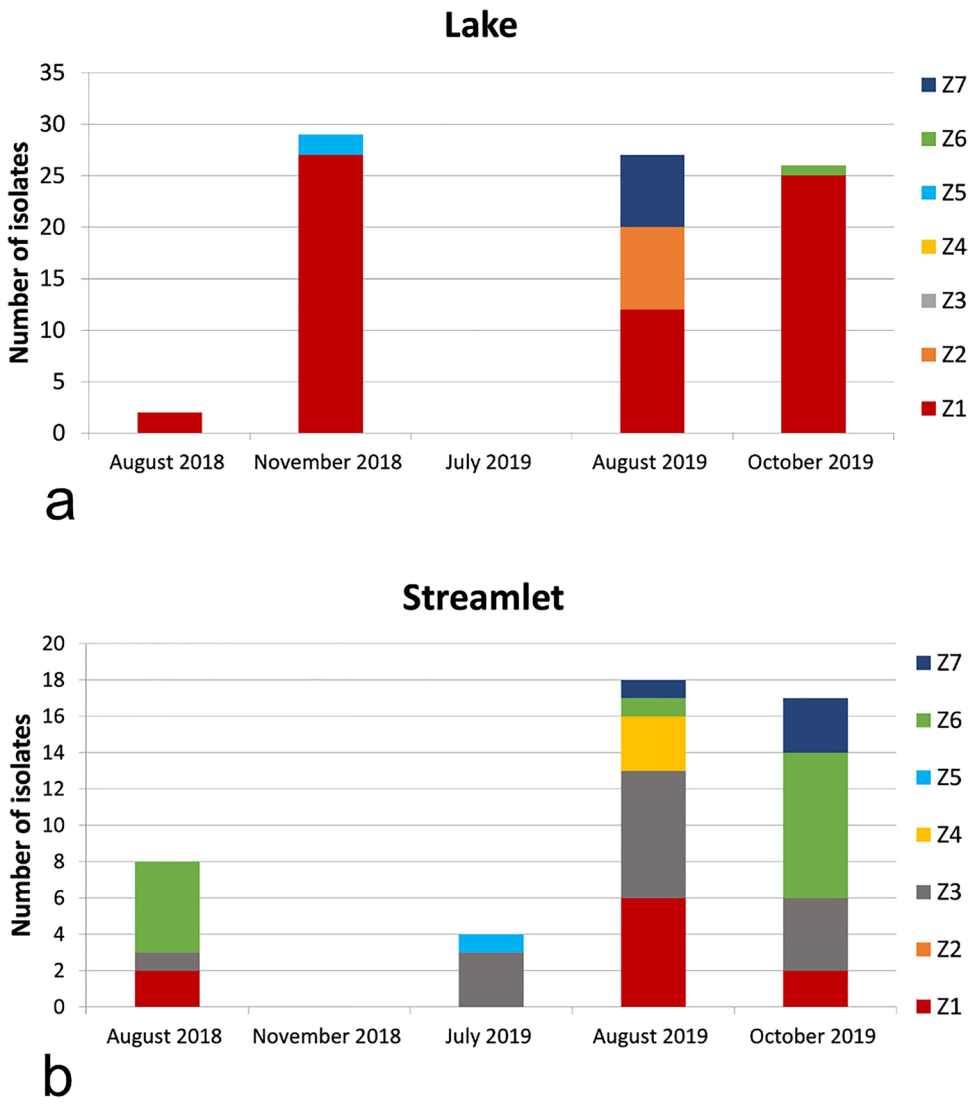

Fig. 4 Number of *Zygnema* sp. isolates of seven genotypes found during the five samplings in the two observed localities

Seasonal variation in the proportion of genotypes differed among the two localities. The total diversity was higher at the Streamlet than that at the Lake, and the Lake showed higher stability in genotype occurrence than the Streamlet (Fig. 4). This difference cannot be explained based on climatic conditions, which are identical over such small areas. However, both localities differed in the fluctuation of water availability. The algae do not have enough time to grow to large volume of biomass in the Streamlet because of the fluctuation in water level, resulting in the mats being constantly renewed from a small number of cells. This is in congruence with the intermediate disturbance hypothesis, a general ecological rule describing the positive effects of a certain level of disturbance on species diversity [54, 55]. Nevertheless, the rediscovery of two identical genotypes that occurred at the site Streamlet 4 years prior to this research indicates that the species pool is stable at this site for a longer period.

Moreover, the Streamlet sampling site showed greater diversity compared to the Lake and had several distinct microhabitats, which may have contributed to the persistence of this diversity, as different genotypes might have different microhabitat preferences. For example, this phenomenon has been observed in the diversity of Arctic *Prasiola* sp., whose occurrence was correlated with natural conditions [56]. Ryšánek et al. [57] suggested that different lineages of *Klebsormidium* are adapted to the substrate on which they originally occur, independent of their evolutionary distance, and closely related lineages differ ecophysiologically to the same extent as unrelated clades. Hainz et al. [58] observed larger vegetative *Spirogyra* sp. filaments in nutrient-rich waters, whereas morphotypes with narrower cells occurred in nutrient-poor water.

Seasonal Changes in Morphology and Physiology

No sexually formed zygospores were observed at these sites. However, their presence cannot be completely ruled out given that we did not study soil samples. Nevertheless,

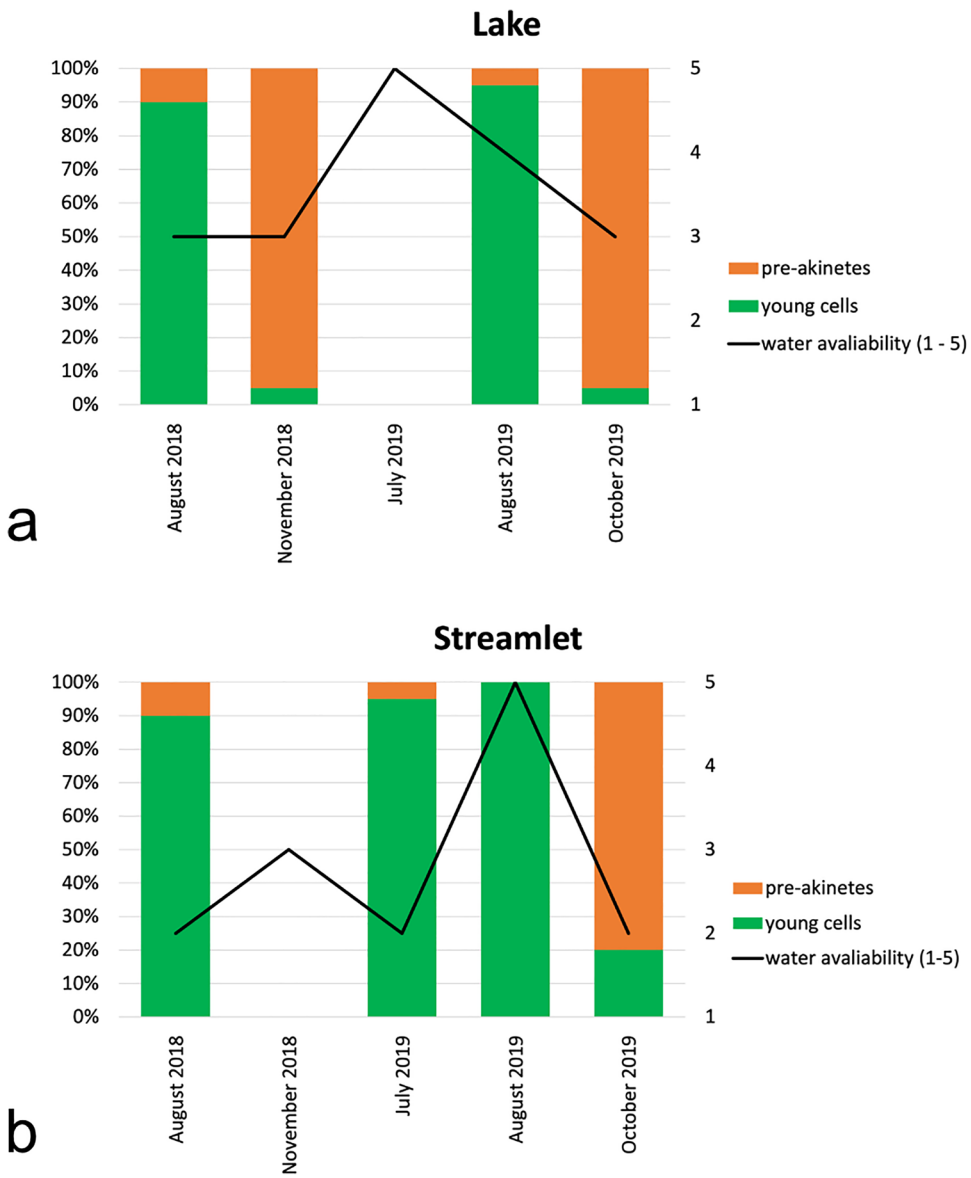

Fig. 5 Proportion of pre-akinetes vs. young vegetative cells and water availability during five samplings in two localities in Kühtai. Proportion of the cell types was estimated for 200 living cells in each sample

at both sites, pre-akinete formation occurred at the end of the growing season (Fig. 5). Pre-akinetes are mature lipid-rich vegetative cells with thick cell walls and distinct mucilage layers [16, 18]. Pre-akinete formation is generally associated with nutrient deficiency [14], as metabolism shifts from the formation of nitrogen-rich proteins toward nitrogen-free sugars and lipids [59]. In addition, acclimation via slow desiccation promotes stress tolerance in pre-akinetes [14]. Pre-akinetes are resistant to many stresses, including drought [14] and frost [22, 24], and their growth ensures the population's survival under adverse conditions.

Seasonal production of pre-akinetes has been observed several times in algae of the genus *Zygnema* and seems to be independent of climatic conditions, as has been observed at both the Arctic [5] and Texas [60]. The decrease in temperature in autumn (Fig. 6) and the associated slowing of metabolic processes may lead to cell starvation, promoting this process. A decline in physiological activity was also observed in their photophysiological response. Pre-akinetes always reach a lower maximal electron transport rate ($rETR_{max}$) than young vegetative cells [61]. Nevertheless, the decline in F_V/F_M, alpha, and I_K was not significant in the Lake despite pre-akinete formation. This was due to water availability. Similarly, pre-akinetes were formed in all types of localities in the Arctic, but populations that had experienced natural drought were photosynthetically less active and stress-tolerant [18].

At both sites, periods of macroscopic disappearance of *the Zygnema* sp. populations were observed. In the Streamlet, no biomass was present in the autumn of 2018, despite there being sufficient water at the site (Fig. 5). The algal population was visibly present in noticeably

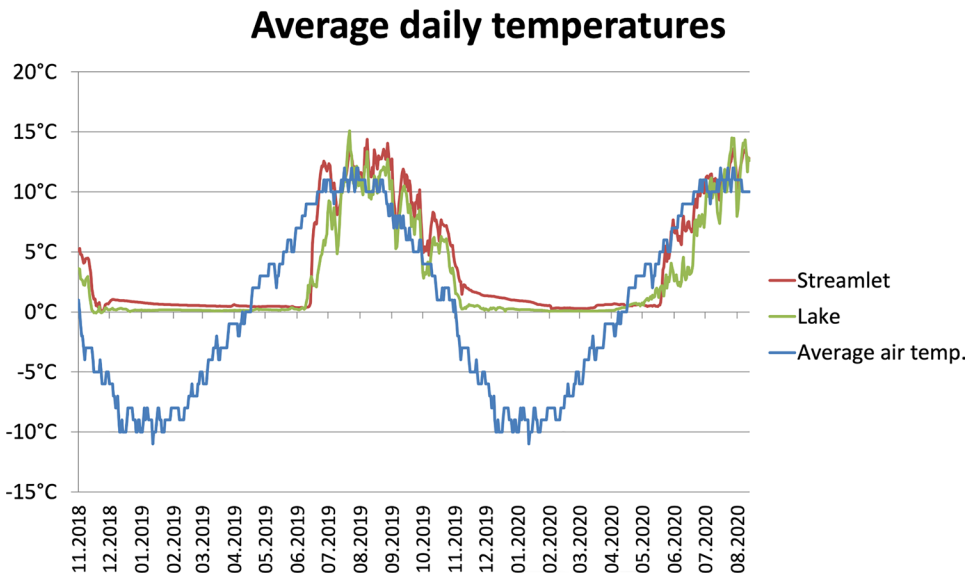

Fig. 6 Average daily temperature of water measured in situ on the observed Lake and Streamlet localities during November 2018–August 2020. The blue line shows average daily temperature of air in Kühtai

Table 2 Summary of the calculated chlorophyll a fluorescence parameters. All values are averages derived from 6 independent measurements ($n=6$). The difference between August and October values was tested by t-test, and *p*-values were given. Parameters of the light curves are described from the curves fitted according to Webb et al. [47]

Locality	Season	F_V/F_M	*p-value*	$rETR_{max}$	*p-value*	alpha	*p-value*	I_k	*p-value*
Lake	August	0.69 ± 0.03	0.0076	108.8 ± 8.1	< 0.0001	0.5 ± 0.03	0.052	274.2 ± 58	0.16
	October	0.64 ± 0.02		61.1 ± 8.1		0.4 ± 0.06		210.4 ± 66.8	
Streamlet	August	0.74 ± 0.01	0.0008	134.4 ± 12.9	< 0.0001	0.6 ± 0.01	0.0001	359.1 ± 40.8	< 0.0001
	October	0.61 ± 0.01		41.5 ± 6.9		0.4 ± 0.06		141.3 ± 28.5	

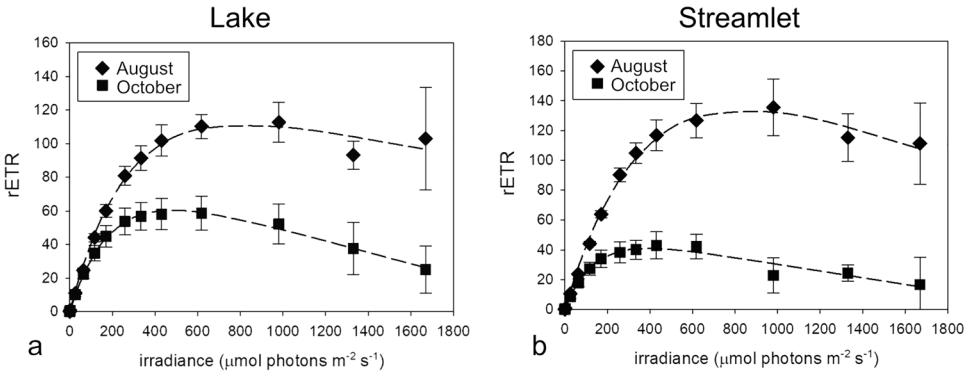

Fig. 7 Relative electron transport rate (rETR) curves of natural population of *Zygnema* sp. from the Lake and Streamlet localities. Samples for the measurements were collected during summer sampling (August 2019) and at the end of vegetative season (October 2019)

smaller numbers at the following observation in July 2019 and gradually increased over the year (Fig. 4). This could be due to the previous drying of the site in the summer or a strong current in the stream that carried biomass away [34]. In the Lake, the biomass disappeared in July 2019 but rejuvenated quickly (Fig. 5). This rapid recovery is due to resilient pre-akinetes. Their presence was observed at 5–10%, even during the period when young vegetative cells were predominant (Fig. 5). This small proportion of mats is constantly prepared for unfavorable conditions, even during the vegetative season, which substantially increases the chances of long-term survival [5, 24].

How Zygnema sp. Survives Winters

Because the observed sites were used as ski slopes during winter, it was impossible to carry out sampling under the snow. However, considering the small amount of biomass present at the sites in spring (Fig. 4), it can be assumed that most of the cells in the mat do not survive winter likely due to freezing

stress. Therefore, we hypothesized that winter temperatures in Kühtai are too low for the survival of most *Zygnema* sp. cells. However, this hypothesis could not be proven, as snow cover effectively insulated the soil surface and protected it from subzero temperatures (Fig. 6). Similarly, Hawes [3] reported that the air temperature of the Antarctic study sites fell to − 25 °C, while the temperature under thick ice (30–50 cm) was only − 4 °C. Steiner et al. [62] reported winter survival of *Micrasterias denticulata* (Zygnematophyceae) in unfrozen peat bog pools despite air temperature minima went down to − 17.3 °C. Pichrtová et al. [5] also showed survival of *Zygnema* sp. under ice.

Moreover, recent laboratory experiments showed that *Zygnema* sp. vegetative cells can survive at –8 °C and pre-akinetes at even lower temperatures [22]. Therefore, we can assume that even direct exposure to air temperature at our sampling sites would not be lethal to the entire population of *Zygnema.* Adaptation of vegetative cells to winter is crucial and has been observed not only in the genus *Zygnema* but also in other algae such as *Spirogyra* sp. [28], *Klebsormidium* sp. [63, 64], the genus *Tribonema* [65], and diatoms [66]. Adaptation to frost is related to the ability of cells to acclimatize to it, which has been observed in the genus *Klebsormidium* [63]. *Klebsormidium crenulatum* showed reinforcement of cells by additional wall layers during freezing stress [64]. Resistance to frost is universally present among organisms [67], so it can be assumed that algae of the genus *Zygnema* have this ability, although this has not been studied yet.

In addition to low-freezing conditions, other stress factors are also associated with winter. Hawes [28] observed a winter decline in *Spirogyra* population in a lake in Antarctica, resulting in the death of almost the entire population because of the lack of light and anoxic conditions. Although the winter conditions in the Alps are not extreme, there is undoubtedly a lack of light owing to shorter days and snow cover. However, low or undetectable respiration at low temperatures in Alpine *Zygnema* [61] and other Alpine or polar terrestrial green algae [68, 69] indicated good adaptation against potential cellular carbon loss during long winter periods in the dark under snow cover.

Conclusion

This study provides the first insight into the seasonal dynamics of mats formed by *Zygnema* sp. in the Alps, based on phylogenetic classification. Algae of this genus exhibit distinctive seasonal dynamics. The largest stands were observed during the summer growing season, followed by gradual dieback and the formation of pre-akinetes. During autumn, virtually the entire population forms resistant pre-akinetes, which have reduced photosynthetic activity and can survive under adverse conditions. Annual mats are renewed each year because of the small number of cells that survive winter.

Contrary to our expectations, the algae in this habitat were not exposed to extreme winter temperatures. However, snow cover that insulates the algae also prevents access to light throughout the winter, which is a probable reason for the survival of a small number of cells after winter. The individual mats consisted of up to five genotypes, which varied in their proportions during the observation period. Seasonal changes in genotype composition probably reflect only local site conditions, while diversity remains stable over a more extended period. Even after 4 years, the same genotypes were found at the observed sites. However, further work is needed to study the ecology and diversity of this alga, specifically for future taxonomic use.

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Author Contribution Kateřina Trumhová and Martina Pichrtová together planned the study conception and design. Material preparation and data collection were performed by Kateřina Trumhová. Analyses were performed by Kateřina Trumhová, Martina Pichrtová, and Vanda Klimešová. The first draft of the manuscript was written by Kateřina Trumhová, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing Interests The authors declare no competing interests.

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