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Desiccation and temperature tolerance of green and red *Haematococcus lacustris* (Chlamydomonadales, Chlorophyta) akinetes

Desikační a teplotní tolerance zelených a červených akinet *Haematococcus lacustris* (Chlamydomonadales, Chlorophyta)

RIGOROSUM THESIS

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Declaration

I declare that this thesis is my copyrighted work. All literature and other resources I used while processing are listed in bibliography and properly cited. The thesis was not misused for obtaining the same or different academic degree.

In Prague, 17. 6. 2024

Mgr. Karolína Vávrová

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Abstrakt

Haematococcus lacustris je zelená sladkovodní řasa známá především jako přírodní zdroj silného antioxidantu astaxanthinu. Tento sekundární karotenoid je tvořen především v tlustostěnných odolných akinetách, díky kterým *H. lacustris* přežívá různé nepříznivé podmínky prostředí. V této studii jsme zkoumali odolnost akinet vůči vysychání. Pro sledování fyziologické odpovědi buněk na stres jsme využili metodu měření efektivního kvantového výtěžku fotosystému II. Nejprve jsme porovnávali desikační toleranci zelených a červených akinet při různých rychlostech vysychání. Oba typy akinet přežily i nejrychlejší vysychání při 10% relativní vzdušné vlhkosti. Sledováním ultrastruktury zelených i červených akinet pomocí transmisní elektronové mikroskopie jsme zjistili, že je vysoká odolnost akinet způsobena především silnou buněčnou stěnou. V následující sérii pokusů jsme podrobněji zkoumali desikační toleranci červených akinet v extrémních podmínkách. Akinety přežily i vysušení po dobu 12 týdnů a krátkodobé vystavení teplotám -80 °C a 55 °C. Oproti tomu nevysušené akinety snášely zmrznutí lépe než působení vysokých teplot. Desikace akinet tudíž může sloužit jako způsob pro přečkání horkých dní. Výsledky naší studie prohloubily porozumění desikační tolerance akinet *H. lacustris*, která je pravděpodobně zásadní pro celosvětové rozšíření této řasy v mělkých efemerních tůňkách.

Klíčová slova: akinety, astaxanthin, desikační tolerance, efektivní kvantový výtěžek, *Haematococcus lacustris,* stresová tolerance

Abstract

The green freshwater alga *Haematococcus lacustris* is known as a natural source of a powerful antioxidant called astaxanthin. This secondary carotenoid is mainly formed in the thick-walled, resistant akinetes, thanks to which *H. lacustris* survives various unfavourable environmental conditions. In this study, we investigated the desiccation tolerance of akinetes. To monitor the physiological response of cells to stress, we used the method of measuring the effective quantum yield of photosystem II. First, we compared the desiccation tolerance of green and red akinetes at different desiccation rates. Both types of akinetes survived even the fastest desiccation at 10% relative humidity. By observing the ultrastructure of green and red akinetes using transmission electron microscopy, we found that the high resistance of akinetes is mainly due to the thick cell wall. In the following experiments, we examined the desiccation tolerance of red akinetes under extreme conditions in more detail. The akinetes survived desiccation for 12 weeks and short-term exposure to temperatures of -80 °C and 55 °C. In contrast, non-desiccated akinetes tolerated freezing better than exposure to high temperatures. Desiccation of akinetes may therefore serve as a means of survival on hot days. Our study has deepened our understanding of the desiccation tolerance of *H. lacustris* akinetes, which is probably crucial for the global distribution of this alga in shallow ephemeral pools.

Keywords: akinetes, astaxanthin, desiccation tolerance, effective quantum yield, *Haematococcus lacustris,* stress tolerance

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The aims of the work

- Comparison of the ability of green and red akinetes of *Haematococcus lacustris* to withstand dehydration at different desiccation rates using the effective quantum yield of the photosystem II as an indicator of their physiological response to stress.

- To investigate the impact of different durations of desiccation on the viability of *Haematococcus lacustris*.

- To examine whether and to what extent the exposure of desiccated *Haematococcus lacustris* cells to extreme temperatures affects their ability to recover.

- To explore the possible role of desiccation in the life cycle of *Haematococcus lacustris* as a mechanism that enables cells to survive extreme temperatures.

Introduction

Freshwater green algae belonging to the *Haematococcus lacustris* (Girod-Chantrans) Rostafinski species complex (Allewaert et al., 2015), formerly known as *H. pluvialis* (Nakada & Ota, 2016), are frequently studied as a natural source of astaxanthin. Astaxanthin is a red pigment that can be used as a source of coloration for aquatic animals (Lorenz & Cysewski, 2000). Additionally, astaxanthin is a very effective antioxidant (Naguib, 2000) and has immunomodulatory (Okai & Higashi-Okai, 1996) and antitumor properties (Jyonouchi et al., 2000). It has been reported to prevent the development of Parkinson's disease and other neurodegenerative disorders (Chan et al., 2009), influence age-related mitochondrial dysfunction (Park et al., 2013), and cardiovascular diseases (Fassett & Coombes, 2012). Furthermore, the biological activities of astaxanthin may be beneficial in the field of dermatology (Davinelli et al., 2018).

Most research on *H. lacustris* focuses on optimizing the production of astaxanthin. This is usually done by comparing different cultivation media (Domínguez-Bocanegra et al., 2004), optimal levels of irradiance, temperature, or pH values (Saha et al., 2013, Sarada et al., 2002) and different types of photobioreactors and cultivation methods (Park et al., 2014; Hata et al., 2001; Del Río, 2005). However, the biology and ecology of *H. lacustris* have not been studied as thoroughly. There are still areas that require further investigation, such as the ability of this alga to survive unfavorable conditions in a desiccated state. This ability seems to be crucial for the worldwide distribution of *H. lacustris*.

Life cycle and natural distribution of *Haematococcus lacustris*

Haematococcus lacustris has a complex life cycle that includes four stages: gametes, zoospores, green, immotile, round palmella stage, and thick-walled, resistant akinetes (Peebles, 1909).
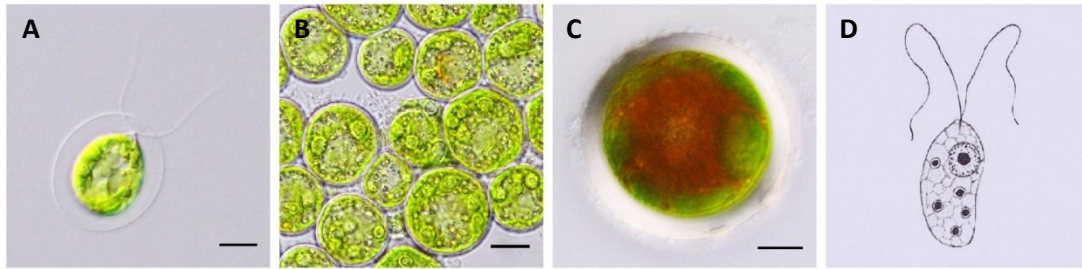
Fig. 1: Different cell types that occur in the life cycle of *Haematococcus lacustris*. A: zoospores; B: palmella cells with distinct pyrenoids; C: thick-walled akinete accumulating the secondary carotenoid astaxanthin; D: a schematic representation of the gamete. Scale bar: 10 µm. (A-C: original photographs, strain CCALA 357; D: Elliott 1934)

The life cycle of *H. lacustris* begins with the akinetes encountering favorable environmental conditions, such as a fresh medium. The akinetes divide multiple times, then their thick cell wall bursts and releases 2-32 daughter zoospores (Hagen et al., 2002; Wayama et al., 2013). These motile cells have two flagella and are enclosed in a glycoprotein matrix. They continue to grow, lose the red color caused by the astaxanthin pigment, and multiply. Eventually, the zoospores transform into immotile palmellae, which can either continue to divide or form akinetes, which are specially adapted to survive various environmental stresses (Hazen, 1899; Elliott, 1934). Alternatively, akinetes can release up to 64 small biflagellate gametes instead of zoospores (Triki et al., 1997). These gametes can then fuse together to form planozygotes, which have four flagella (Peebles, 1909; Pocock, 1960).
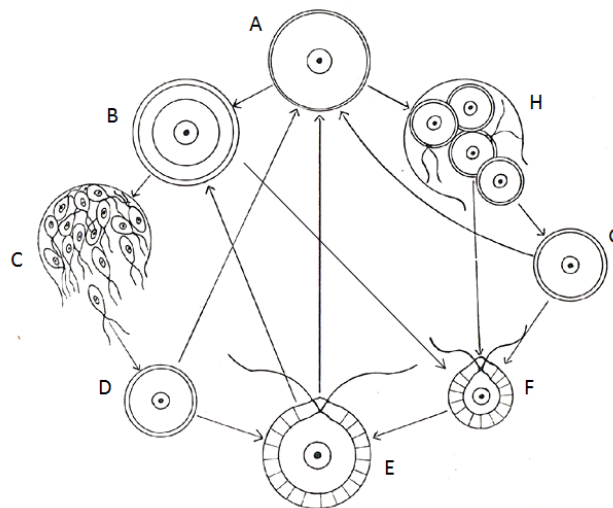

Fig. 2: Schematic representation of the asexual life cycle of *Haematococcus lacustris*. A: adult palmella; B: akinete; C: release of gametes; D+G: young palmellae; H: division of palmella into palmellae or zoospores; F: young zoospore; E: adult zoospore. (Elliott 1934)

The natural distribution of *Haematococcus lacustris* is determined by its life cycle and relatively low ability to compete with other species of algae (Proctor, 1957). As a result, H. lacustris typically lives as a pioneer alga in shallow, ephemeral pools (Genitsaris et al., 2016).

Fig. 3: Example of the habitat of *Haematococcus lacustris.* (Chekanov et al., 2014)

The thick-walled, resistant and desiccation-tolerant akinetes play a crucial role in the worldwide distribution of *H. lacustris*. This species can survive in different biogeographical regions in habitats affected by multiple abiotic stress conditions, especially high radiation and periodic drought. Akinetes also allow *H. lacustris* to reach new habitats by air transportation (Genitsaris et al., 2011) and on the legs and feathers of waterfowl (Figuerola & Green, 2002).
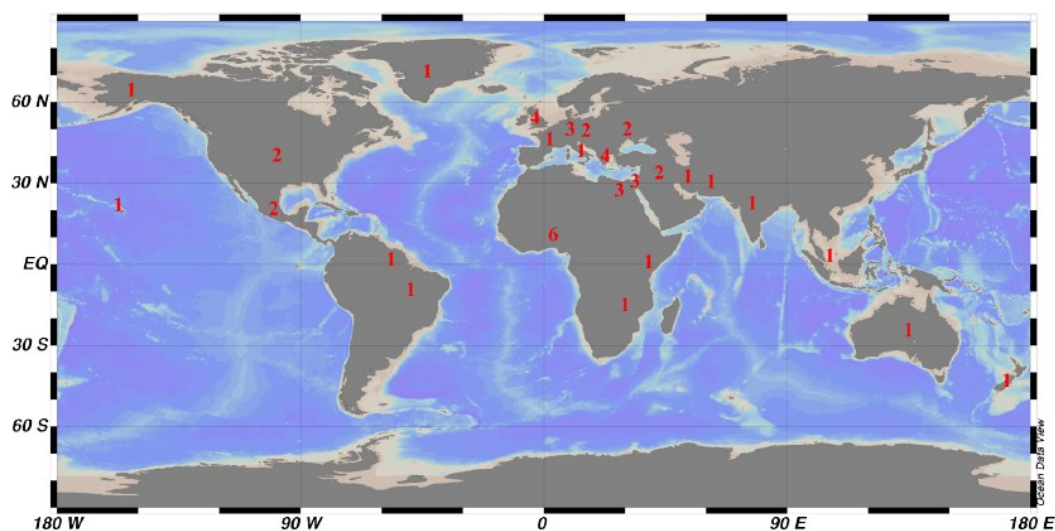

Fig. 4: Map showing examples of the worldwide distribution of *Haematococcus*, with numbers indicating the scientific references for its occurrence in natural systems. (Genitsaris et al., 2016)

Structure and formation of akinetes

Akinetes are a type of resting stage formed from zoospores or palmellae in response to various physiological stresses. Some examples of these stresses are high irradiance (Saha et al., 2013; Chekanov et al., 2014), temperature above 30 °C (Tjahjono et al., 1994), higher salt concentration in the medium (Sarada et al., 2002), and nutrient depletion (Boussiba et al., 1999; Saha et al., 2013).

The formation of akinetes is accompanied by numerous ultrastructural changes in the cells. A distinctive feature of akinete cells is a thick secondary wall that formes under the primary wall and a layer of extracellular matrix. This secondary wall is covered by a trilaminar sheath containing algaenans (Hagen et al., 2002), which are aliphatic, non-hydrolyzable hydrocarbons that probably contribute to desiccation and UV light resistance of vegetative cells in some algal species (Blokker et al., 1998). The protective function of algaenans has been reported, for example, at certain stages of the life cycle of *Spirogyra*, *Dunaliella* (Blokker, 2000), *Chlamydomonas* (Blokker et al., 1999) and *Mougeotia* (Permann et al., 2021).

Chloroplasts also undergo dramatic changes. The volume of the chloroplast is reduced by up to 9.7 % compared to the original cells, and partial degradation of the thylakoids can occur (Wayama et al., 2013). The chloroplasts then acquire a net-like structure (Nogami et al., 2014). Nevertheless, photosynthetic activity is maintained and energy is probably used for lipid and astaxanthin synthesis (Gu et al., 2013).

The accumulation of large amounts of astaxanthin contained in lipid droplets is typical for akinetes exposed to light (Wayama et al., 2013). It spreads from the center to the periphery of the cell until the entire mature akinete appears red (Santos & Mesquita, 1984; Collins et al., 2011).

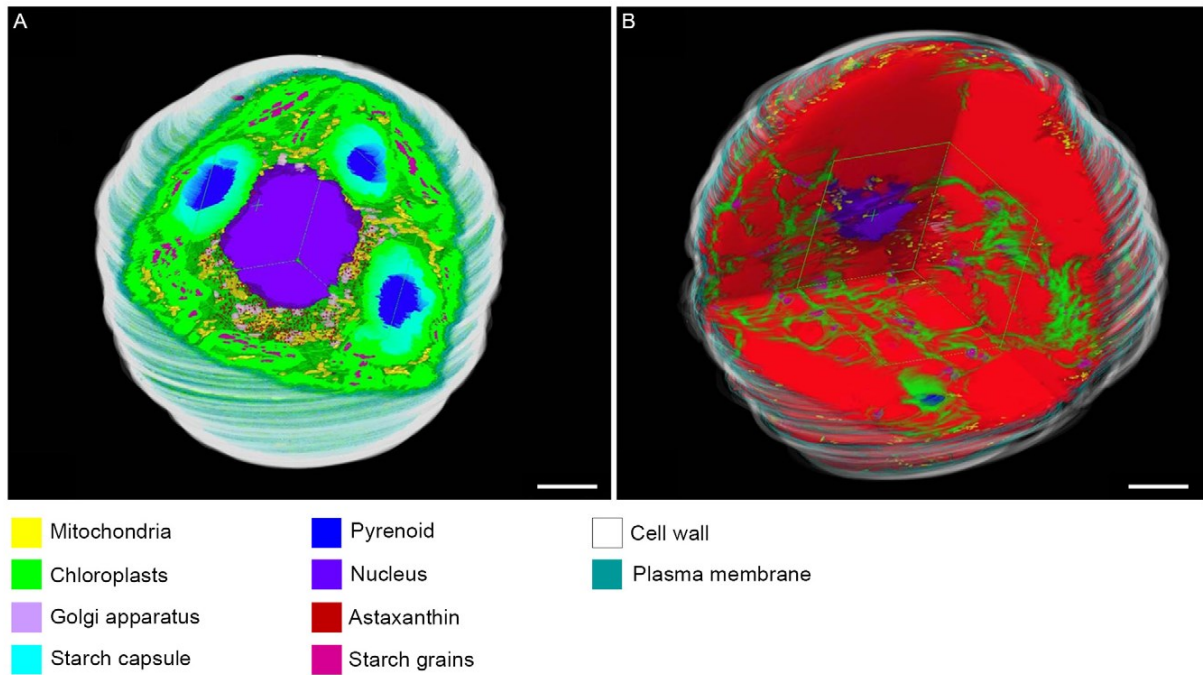| Mitochondria | Pyrenoid | Cell wall |
Chloroplasts	Nucleus	Plasma membrane
Golgi apparatus	Astaxanthin	
Starch capsule	Starch grains	

Fig. 5: 3D TEM images of whole *Haematococcus lacustris* cells. A: cut-away image of the green coccoid cell; B: cut-away image of the red akinete. All subcellular components are marked by different colors as indicated in the color legends. Scale bar: 5 μm. (Wayama et al., 2013)

Fluorimetry

Fluorimetry is a method for monitoring physiological processes in phototrophic cells. It is based on the measurement of chlorophyll *a* fluorescence and is fast, non-invasive and highly sensitive. This tool provides both quantitative and qualitative information about the current state of photosystem II (Govindjee, 1995). It can therefore be used to monitor physiological processes in phototrophic cells (Roháček & Barták, 1999). In algological research, the fluorimetry is often used to monitor stress responses of cells, for example response to desiccation (e.g. Karsten et al., 2014; Herburger et al., 2016; Pichrtová et al., 2014, Roach et al. 2022b).

To measure the rate of influence of stress factors through changes in photosynthetic activity, a parameter called maximum quantum yield can be used. However, this parameter requires dark-adapted samples (van Kooten & Snel, 1990), which makes it unsuitable for experiments that require the continuous monitoring of physiological changes in cells. To overcome this limitation, the effective quantum yield, Φ_{PSII}, is used to monitor the photochemical energy conversion efficiency in photosystem II in light-adapted cells (Roháček & Barták, 1999). This relative parameter is considered

a good ecophysiological indicator of how plants respond to environmental stress (Rascher et al., 2000). It is calculated as $(F_M'-F)/F_M'$, where F is the steady-state fluorescence and F_M' is the maximum fluorescence in the light-adapted state measured after application of a saturation pulse. A higher Φ_{PSII} value indicates more effective photochemical processes in the cells under optimal conditions. Conversely, the influence of stress factors leads to lower measured Φ_{PSII} values (Karsten et al., 2014; Pichrtová et al., 2014; Roach et al., 2022b).

Results and discussion

Comparison of green and red akinetes

In experiment I (see original paper Vávrová et al. 2023), we compared the desiccation tolerance of both green and astaxanthin-rich red akinetes. We desiccated samples for 24 hours at three different drying rates, namely 86%, 43% and 10% relative humidity (rh). We observed that the effective quantum yield values (Φ_{PSII}) for both cell types changed similarly over time at all three desiccation rates tested. At lower relative humidity in the desiccation chamber, the measured Φ_{PSII} values decreased faster until they reached 0.03–0.05, indicating that the samples were completely dry. Both green and red akinetes dried at 86% rh after 7 hours, while the samples desiccated at 43% rh were dried after 160 minutes. The highest desiccation rate was observed in the samples placed in 10% rh, which were dried after 90 minutes. However, throughout the experiment, the Φ_{PSII} values of the green akinetes were generally higher than those of the red akinetes.

All rehydrated samples showed a gradual recovery of photochemical processes, as evidenced by an increase in Φ_{PSII} values. The Φ_{PSII} values measured 48 hours after rehydration were even higher in some samples than in the cultures at the beginning of the experiment. This was probably due to the transition to an active phase of the life cycle, demonstrated by the release of zoospores observed 24 hours after rehydration in all samples. The presence of zoospores also confirmed at the least partial viability of *H. lacustris* akinetes even after severe desiccation stress. From this we can conclude that both the green and red akinetes are desiccation tolerant, as the samples survived desiccation for 24 hours even at 10% relative air humidity (Alpert, 2006).

When analysing the of green and red akinetes by transmission electron microscopy, we found no visible differences in the cell wall structure, which was 4-4.5 μm thick in both types of akinetes. However, we found remarkable differences in the protoplast level of the cells. In the green akinetes, the chloroplasts with the starch grains were located at the periphery. This probably led to higher Φ_{PSII} values in our desiccation experiment due to the better-preserved chloroplasts. In contrast, the chloroplasts in the red akinetes were considerably reduced and had a reticular appearance. A similar pattern was observed in experiments with *Zygnema*, where pre-akinetes depleted of nitrogen with reduced chloroplasts showed lower photophysiological activity than young vegetative cells (Herburger et al., 2015; Pichrtová et al., 2016).

The main difference between green and red akinetes that we observed is due to the very high content of electron-dense lipid droplets containing astaxanthin, which fills most of the cell volume in red akinetes. In green akinetes, however, the lipid droplets were only present in the central part of the cell around the nucleus. The accumulation of astaxanthin is caused by nitrogen deficiency and high irradiation (Gwak et al., 2014; Boussiba, 2000; Roach et al., 2022a). Its main function is to protect cells from high irradiation and oxidative stress rather than desiccation tolerance (Wang et al., 2003; Hagen et al., 1993). It is also pointed out that secondary carotenoids have a similar function in red, thick-walled spores of snow algae in the order Chlamydomonadales (Hoham & Remias, 2020).

We conclude that *H. lacustris* is desiccation tolerant mainly due to its thick cell wall containing algaenan (Montsant et al., 2001), based on the similar desiccation abilities of both types of akinetes and their observed ultrastructure.

However, recent study by Roach and colleagues (2022a) has shown that green, non-motile *H. lacustris* cells from an actively growing culture supplied with excessive nitrogen are not constitutively desiccation tolerant. In their study, desiccation tolerance was only acquired later by slow desiccation in combination with nitrogen limitation (Roach et al., 2022a). In contrast, the green akinetes used for the experiments in our study were taken from a two-month-old culture in the stationary growth phase and had fully developed secondary cell walls containing algaenans. Therefore, our results suggest that nitrogen limitation may serve as an inducing factor for the development of desiccation tolerance in green akinetes. This has also been observed in *Zygnema*, where nitrogen starvation induced the formation of desiccation-tolerant pre-akinetes, even in liquid

cultures (Pichrtová et al., 2014). Additionally, the observed difference in desiccation tolerance of green akinetes in the two studies could be caused by the use of different strains of *H. lacustris.*

The effect of various combined stress conditions

For the next series of experiments, we used only red akinetes. In experiment II the combined effects of desiccation pretreatment at 30-50% relative humidity for 1, 2, and 4 weeks, and exposures to different temperatures (-1 °C, 25 °C, and 40 °C for one hour were investigated. We found no evidence of a mutual effects of the two selected factors. Red akinetes from all samples survived and transformed into zoospores after rehydration. The recovery of Φ_{PSII} followed a similar pattern for all samples, except for those kept in the desiccated state for 4 weeks, which showed a comparatively slower recovery.

We continued our investigations with experiment III, which was designed to test the effects of very long desiccation periods (8 and 12 weeks) and extreme temperatures (-1 °C, -18 °C, -80 °C, 45 °C, 50 °C, and 55 °C). All samples studied survived even under these severe test conditions. The measured increase in Φ_{PSII} values after rehydration was very similar compared to the control sample. However, the results show that the longer the desiccation period lasts, the slower the increase in Φ_{PSII} values. The presence of released zoospores was observed in all samples, confirming the ability of *H. lacustris* to survive even 12 weeks in a desiccated state. However, the number of zoospores was lower in the samples desiccated for 12 weeks than after 8 weeks. Other samples with comparatively lower numbers of zoospores observed were those exposed to 55 °C, suggesting that the effect of high temperature is more severe for *H. lacustris* than freezing. A similar pattern was previously shown for diatoms (Souffreau et al., 2010).

In experiment IV, we compared the recovery of Φ_{PSII} values after rehydration of desiccated control samples with non-desiccated red akinetes exposed to temperatures of -18°C and 50°C for one hour. Our results confirmed that akinetes survived short-term freezing well. In fact, the Φ_{PSII} values measured after the addition of fresh BG-11 medium were even higher than those of the control sample. However, the akinetes were more severely damaged when the non-desiccated samples were exposed to high temperatures. Even 72 hours after the addition of fresh medium, we did not observe any released zoospores, and the Φ_{PSII} values increased only slightly.

We believe that our results can be explained by the fact that desiccation and freezing have similar effects on the cells, as both lead to water loss and osmotic stress (Smirnoff, 1993). On the other hand, high temperature leads to protein misfolding, for which the cell requires specific molecular chaperones, called heat shock proteins, for protection (Kotak et al., 2007). We conclude that desiccation of akinetes can increase their resistance to extremely high temperatures, which is similar to the adaptation observed in *Botryococcus braunii* cells (Demura et al., 2014).

Our experiments have shown that the desiccated *Haematococcus lacustris* akinetes exhibit very high resistance to different stress factors. In fact, the stress levels we used were probably lower than the actual limits of akinetes survival. However, we found that a three-month desiccation was lethal for a large proportion of the cells. Nevertheless, even a small number of surviving cells can ensure the continuity of the algal population in the next growing season (Pichrtová et al., 2016). According to Roach et al. (2022b), storage of desiccated *Haematococcus* akinetes at 50% relative humidity in subzero temperatures can ensure their viability for centuries.

The role of desiccation tolerance in *Haematococcus lacustris* ecology

Drought is a significant stress factor affecting the survival of many green algal species (Lüttge & Büdel, 2010; Lewis & Trainor, 2012; Gray et al., 2007). The ability to survive desiccation is even more crucial for *H. lacustris*, as this species tends to lose out in competition with other algae in larger water bodies (Proctor, 1957; Genitsaris et al., 2016). The desiccated akinetes of *H. lacustris* can survive the effects of extreme low and high temperatures and remain desiccated for long periods of time. This allows them to survive in ephemeral pools in both cold and warm, relatively dry areas. *Haematococcus lacustris* can be found in flooded shallow depressions in rocks, known as lithotelms (Hazen, 1899; Proctor, 1957; Pocock, 1960; Chekanov et al., 2014), cemeteries (Hazen, 1899; Proctor, 1957), birdbaths (Proctor, 1957; Pocock, 1960), or gutters (Pocock, 1960) all over the world (Genitsaris et al., 2016). Desiccation tolerance is not only crucial for survival in pools that frequently dry out, but also for colonization of these relatively isolated environments. Desiccated akinetes of *H. lacustris* can be transported by the wind (Genitsaris et al., 2014) or on the legs or feathers of birds (Figuerola & Green, 2002; Cellamare et al., 2010).

Our research results can also provide an explanation for a fascinating phenomenon that is frequently observed both in nature and in the laboratory. A dense red biofilm of *H. lacustris* akinetes accumulates on the walls of pools that gradually dry out, rather than sinking to the bottom to remain in the water for as long as possible (Hazen, 1899; Peebles, 1909; Droop, 1956; Wan et al., 2014) The water in shallow pools can become too warm for non-desiccated akinetes on hot sunny days. However, our experiments have shown that desiccated akinetes can withstand elevated temperatures effectively. Furthermore, the formation of a biofilm can lead to a slower desiccation rate and decreased exposure of the cells in the inner layers of the biofilm to high radiation.

Conclusion

Our study shows that both green and red *Haematococcus lacustris* akinetes can effectively survive even fast desiccation at 10% relative humidity. Their desiccation tolerance is enabled by a thick cell wall containing algaenans. The photochemical processes are more effective in the green akinetes, possibly due to the better-preserved chloroplasts.

We have shown that some akinetes can survive for a very long time, up to 12 weeks, in a desiccated state. Longer desiccation periods are rather lethal, but even a small number of surviving akinetes can ensure the survival of *H. lacustris* in its habitat.

The desiccated akinetes can cope well with extreme temperatures. However, freezing appears to be less severe for *H. lacustris* than exposure to high temperatures.

Our results suggest that desiccation may serve as a mechanism for survival at high temperatures, whereas the akinetes of *H. lacustris* can survive short-term freezing even without prior desiccation.

All these observed features probably play an important role in the worldwide distribution of *H. lacustris* in small ephemeral pools and basins. Furthermore, the highly desiccation-resistant akinetes of *H. lacustris* can have several laboratory and commercial applications. Rapid drying of contaminated cultures can eliminate less resistant algal species. Cultivation of *H. lacustris* on a moistened membrane can save a considerable amount of water. Additionally, subjecting the cells to mild desiccation stress and higher irradiation can lead to faster accumulation of astaxanthin (Wan et al., 2014; Zhang et al., 2014). It has also been observed that desiccation-tolerant algae cope better with salinity

stress. Therefore, *H. lacustris* could a suitable candidate for nutrient removal from slightly saline wastewater (Bácsi et al., 2024).

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Annex – article

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