Desert green algae show higher desiccation tolerance than their aquatic sister-species

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Abstract

Diverse algae possess vegetative desiccation tolerance, the ability to recover from extreme desiccation without forming specialized resting structures. Green algal genera such as Tetradesmus (Sphaeropleales, Chlorophyceae) contain both terrestrial and aquatic species, providing an opportunity to compare physiological traits associated with the transition to land in closely related taxa. We subjected six species from aquatic and terrestrial habitats to three desiccation treatments varying in final relative humidity followed by short- and mid-term rehydration. We tested the capacity of the algae to recover from desiccation using the effective quantum yield of photosystem II as a proxy for physiological activity. The degree of recovery was dependent both on the habitat of origin and the desiccation scenario, with terrestrial, but not aquatic species, recovering from desiccation. Distinct strains of each species responded similarly to desiccation and rehydration, with the exception of one aquatic strain that recovered from the mildest desiccation. Cell ultrastructure was uniformly maintained in both aquatic and desert species during dehydration and rehydration, but staining with an amphiphilic styryl dye indicated desiccation-induced damage to the plasma membrane in the aquatic species. These analyses demonstrate that terrestrial Tetradesmus possess the vegetative desiccation tolerance phenotype, making these species ideal for comparative omics studies.

Introduction

Vegetative desiccation tolerance is the ability of organisms to recover from extreme cellular water loss without forming specialized dormant structures (Oliver et al. 2000). Some desiccation-tolerant plants can survive only short periods of desiccation or require desiccation to onset slowly. Others, including some species of algae, bryophytes, and lycophytes, restore their physiological activity even after rapid or lengthy desiccation (e.g., Alpert & Oliver 2002). Vegetative desiccation tolerance is thought to be one of the key traits in the evolution of early land plants as they evolved from aquatic ancestors (Ligrone et al. 2012), and considerable research has focused on advancing our understanding of this capacity in mosses, lycophytes, and their immediate algal relatives. This research showed that multiple cellular processes are affected by desiccation including carbon, protein, and lipid metabolism, cell cycle, stress response pathways, and signaling activity (e.g., Holzinger et al. 2011; Yobi et al. 2012; Gao et al. 2017).

Our current understanding of the mechanisms and evolution of vegetative desiccation tolerance in land plants is mostly based on the comparison of a single terrestrial lineage of land plants (embryophytes) with streptophyte green algae (Farrant et al. 2009; Holzinger et al. 2011), a combined divergence of which spans 900+ MY (Leliaert et al. 2012). Unlike the land plants, which evolved on land once, green algae show multiple origins of terrestriality, including lineages inhabiting extreme environments, such as deserts (Lewis & Lewis 2005; Cardon et al. 2008; Rindi et al. 2009), providing multiple opportunities to understand and characterize the array of adaptations that may facilitate desiccation tolerance. In deserts the surface of the soil can be covered by soil crusts, a complex community of microorganisms, fungi, and bryophytes that together act as ecosystem engineers by powering nutrient cycles, preventing erosion, and enhancing water

holding capacity, as well as influencing the composition of plant communities (Evans & Johansen 1999; Belnap & Lange 2001; Song et al. 2017). Of the bryophyte members of desert soil crusts, many possess cellular mechanisms allowing them to withstand desiccation events and persist in their environment (e.g., Proctor & Smirnoff 2000; Gao et al. 2017). The specific mechanisms of desiccation tolerance in chlorophyte green algae are not as well studied, but species isolated from desert soil crusts can, unlike their aquatic relatives, recover even after long and extreme desiccation (Gray et al. 2007).

Desert green algae arose multiple times during the diversification of Chlorophyta, often in clades containing both aquatic and terrestrial species (Lewis & Lewis 2005; Büdel et al. 2009; Fučíková et al. 2014). For example, *Tetradesmus* G.M. Smith (Sphaeropleales, Chlorophyceae, Chlorophyta), a common planktonic freshwater genus of unicellular or colonial green algae, contains several terrestrial species that are closely related to the aquatic species (Lewis & Flechtner 2005; Mikhailyuk et al. 2019; Terlova & Lewis 2019).

Given the irregularity of precipitation in terrestrial habitats, we hypothesize that desert, but not aquatic, *Tetradesmus* species tolerate desiccation in a vegetative state. We tested this by evaluating the recovery of physiological activity upon rehydration after desiccation. The rate of desiccation and the final value of relative humidity (RH) impact the time required for recovery of desiccation-tolerant plants (e.g., Bartoškova et al. Nauš 1999). Thus, our second hypothesis is that two variables, the rate and maximum intensity of desiccation impacts the extent of recovery of*Tetradesmus* . To test these hypotheses, we subjected six species of *Tetradesmus* (four terrestrial desert and two aquatic) to desiccation under three different scenarios (RH ˜5% over 2-5h, 65% over 8h, 80% over 16h) followed by rehydration. We estimated photochemical yield of photosystem II (Φ_{PSII}) from measurements of chlorophyll fluorescence during the desiccation-rehydration cycle to characterize the level of cell physiological activity. To account for possible within-species variation, which has been demonstrated for terrestrial streptophytic algae (Donner et al. 2017), we used multiple strains per species when possible.

Desiccation and osmotic stress cause permanent damage to the ultrastructure of desiccation-sensitive cells (e.g., Cheng et al. 2017), however reversible changes in ultrastructure are also known for desiccation-tolerant organisms (Wu et al. 2012; Li et al. 2014; Holzinger et al. 2015). These changes include decrease of the cytoplasm volume, following expansion or undulation of cell wall, and degradation of thylakoid membranes. Therefore, our third hypothesis is that cell ultrastructure will change in both aquatic and desert species after desiccation, and that the change is permanent in the aquatic algae, but reversible upon rehydration in the desert lineages. To test this hypothesis, we compared cell ultrastructure of selected aquatic and desert species in hydrated, desiccated, and rehydrated states using transmission electron microscopy. We also tested the integrity of the plasma membrane under osmotic stress caused by a sorbitol solution, which mimics desiccation, using a vital fluorescent dye and confocal laser scanning microscopy.

Materials and Methods

Study organisms

The response to desiccation and rehydration was studied in 13 strains of*Tetradesmus* belonging to 6 species: aquatic *T. obliquus* , T. sp. (listed at CCAP as "*T. raciborskii* " strain CCAP 276/35), temperate soil species *T. dissociatus* , and desert algae *T. deserticola* , *T. adustus* , *T. bajacalifornicus* . All algae were grown in liquid KSM medium (Clear & Hom, personal communication) under a 12:12 h light:dark cycle (photon flux density ˜200 μmol $m^{-2}s^{-1}$) at 22@C. Mixing of the cultures was achieved by orbital shaking at 0.4 rad/sec.

DNA extraction, amplification, and sequencing

To confirm the phylogenetic placement of *T. obliquus* strain UTEX 72, we obtained the sequences of three DNA loci (*tuf* A,*rbc* L, and ITS2) and analyzed them with data from the other available species. Cells from culture aliquots of the strain UTEX 72 were concentrated, frozen, and then mechanically disrupted. Genomic DNA was extracted using the ZymoBIOMICS DNA Miniprep Kit. The *tuf* A gene was amplified with the primer pair tufAF – tufA.870r (Hall et al. 2010, Famá et al. 2012). For amplification of the *rbc* L

gene we used the primer pair M35 – M650r (McManus & Lewis 2011). The ITS region was amplified using the primer pair ITS1 – ITS 4 (White et al. 1990, Hall et al. 2010). Standard PCR protocol was carried out with GoTaq Green Master Mix (Promega Corporation, Madison, WI, USA) according to manufacturer's recommendations. Prior to sequencing, amplification products were purified with Exosap-IT Express (Life Technologies Corporation, Carlsbad, CA, USA). DNA sequencing was performed by Eurofins Scientific with the same pars of primers used for amplification reactions. Consensus sequences of three genes were obtained from forward and reverse sequences using Geneious 10.2.2 (https://www.geneious.com) and deposited to NCBI with accession numbers MT270139 for *tuf* A, MT270138 for *rbc* L, MT270137 for the ITS 2 region.

Phylogenetic analysis

A three-gene concatenated dataset used in the analysis, included six *Tetradesmus* species, some with multiple strains, and selected taxa from related genera (Supplements Table S1). ITS2 was aligned using sequences together with the secondary structure (inferred by homology prediction) using ITS2 database (Schultz et al. 2006, Koetschan et al. 2012).

Substitution model and parameter values for the phylogenetic analysis were selected with Partitionfinder2 (Lanfear et al. 2017) using algorithms greedy (Lanfear et al. 2012) and PhyML (Guindon et al. 2010). The Akaike Information Criterion (AIC, Akaike 1998) was used to select the best model. Bayesian Interference (BI) was carried out with MrBayes 3.2.6 (Ronquist & Huelsenbeck 2003) available on the CIPRES Science Gateway (Miller et al. 2010). The concatenated dataset was partitioned by gene and by codon position (for protein-coding genes). The HKY+I, F81, and HKY+G models were chosen for the first, second, and third codons of *tuf* A respectively, GTR+G was applied to all three codons of *rbc* L, and SYM+G model was selected for ITS 2. The analysis included two separate MCMC runs, each composed of four chains. Each MCMC chain ran for 2,000,000 generations, sampling trees every 100 generations. Upon completion, the runs were compared using Tracer v. 1.7 (Rambout et al. 2018) and the first 25% of generated trees were discarded as burn-in. A 65% majority-rule consensus topology and posterior probabilities were then calculated from the remaining trees.

Maximum likelihood (ML) analysis of the concatenated dataset was carried out with partitioning by genes. Model GYR+I+G was implemented with following parameters: nucleotide frequencies A=0.30920532, C=0.16801733, G=0.21847705, T=0.3043003; substitution rates: AC=0.40628109, AG=1.567514, AT=2.0983752, CG=0.60725568, CT=5.5017757, GT=1.000000; Pinvar=0.56788907; Gamma shape = 0.86898379. The ML tree was produced from the 1000 bootstrap pseudo-replicates using 65% majority rule. ML analysis and the bootstrap were performed using PAUP* V4.0a (Swofford 2003).

Desiccation and rehydration procedures

Desiccation experiments were carried out using desiccation chambers previously described by Karsten et al. (2014), illustrated in Fig. S1. Different levels of relative humidity (RH) in the chamber were achieved by adding one of three different desiccants to the chambers: 100 g of $CaSO_4$ (W. A. Hammond DRIERITE Co. LTD, Xenia, OH, USA) achieving ˜5 % RH, a solution of 33g LiCl in 100 ml of dH_2O for 65% RH, and a saturated solution of KCl in dH_2O (100 ml) for 80% RH.

Algal cell suspensions (50 μl, approximately 150,000 cells corresponding to chlorophyll concentration 30–40 mg ml^{-1}) were placed onto glass fiber filters (Whatman, Maidstone, United Kingdom) in replicates of four. Three filters at a time were then positioned on a perforated metal grid inside a desiccation chamber containing the appropriate desiccant. RH levels in the chambers were monitored using PCEMSR145S-TH mini data logger (PCE Instruments, Meschede, Germany). The chambers were kept under dim light of ˜10 μmol photons $m^{-2}s^{-1}$ at 22@C. Measurements ofΔ F/Fm' of PSII (Φ_{PSII}) in *T. obliquus, T. deserticola, T. adustus* , and *T. bajacalifornicus* were taken using a PAM 2500 chlorophyll fluorometer (Heinz Walz GmbH, Effeltrich, Germany), the light probe was adjusted outside of desiccation chamber in 12 mm distance from the algal samples. Strains of *T. dissociatus*and *T.* sp. "*raciborskii* " (CCAP 276/35) were acquired later. Experiments involving these species, along with a previously measured aquatic and terrestrial strain, followed the same protocol except that their chlorophyll fluorescence was recorded using a Junior PAM (Heinz Walz

GmbH, Effeltrich, Germany).

Measurements of chlorophyll fluorescence were taken every 10 min during the desiccation period (PAM settings: measuring light 3, saturation pulse 6, actinic light 3). The desiccation was assumed complete when the mean of measured $\Delta F/Fm'$ for the algae on all filters reached zero. The samples were then rehydrated by adding 50 μL of the KSM growth medium to each replicate on the filter and the desiccant in a chamber was replaced with 100 ml of tap water to achieve higher RH ($\sim$96%) after which the measurements were resumed at the same intervals.

Cell structure during desiccation and rehydration

Transmission electron microscopy (TEM). Samples for TEM were prepared following the protocol described in Holzinger et al. (2015). Cells were fixed in 2.5% glutaraldehyde in cacodylate buffer (pH 6.8) for 1.5 h, washed in cacodylate buffer, then postfixed in 1% osmium tetroxide solution in cacodylate buffer overnight at 4°C. The samples were dehydrated in increasing graded ethanol solutions and embedded in Spurr's resin (Sigma-Aldrich, St. Louis, MO, USA). Ultra-thin sections were prepared, then viewed using FEI Tecnai 12 G2 Spirit BioTWIN TEM microscope.

Confocal laser scanning microscopy (CLSM). To test whether the plasma membrane of cells is fragmented during osmotic stress, we subjected cell suspensions of one representative species from each habitat to 4M sorbitol and used the vital fluorescent dye FM 1-43 (green biofilm cell stain, Invitrogen Ltd. Paisley, UK) following the protocol described in Holzinger et al. (2011). Two aliquots of each selected species (aquatic *T. obliquus* strain UTEX 72, temperate soil *T. dissociatus* , desert *T. deserticola* strain SNI-2) were subjected to the osmotic stress in 4M sorbitol solution for 1 h (cells under osmotic stress), after which sorbitol was replaced with deionized H_2O in one of the aliquots (rehydrated cells). Control samples served as hydrated samples. All samples were then exposed to 20μM Film Tracer FM 1-43, prepared from 20mM stock solution in deionized water for 30 min prior to examination with Nikon A1R Spectral confocal microscope (Nikon Inc, Tokyo, Japan). Samples were excited with the argon laser beam at 488 nm, emission was collected at 500-550 nm (false color green) and at 575–625 nm (false color red).

Statistical Data analysis with R

Data analysis and visualization were carried out with R (raw data and full code are available at DRYAD, doi:10.5061/dryad.sqv9s4n1t). We first calculated mean effective photosynthetic yield values from four individual measurements of each strain at each time point, then applied hierarchical cluster analysis to assign the "hydrated", "desiccating", and "desiccated" physiological states to the measurements. The time of rehydration (corresponding to the "rehydrated" physiological state) was recorded during data collection.

To compare recovery across species for each desiccation mode, we calculated recovery indices as the ratio of the mean effective photosynthetic yield value 10 min or 12 h after rehydration to the control hydrated value. We then used hierarchical cluster analysis of Euclidean distances to compare the recovery indices among the strains of desert and aquatic algae. The number of clusters was validated using gap statistics.

Results

Phylogenetic analysis

The *tuf* A and *rbc* L sequences obtained from *T. obliquus* strain UTEX 72 were very similar to these of the strain UTEX 393 and the ITS2 sequence of these strains were identical (see Table S1 for accession number of all sequences used in the analysis). Phylogenetic analyses of the concatenated data set showed that these two strains are grouped into the same clade with high support. Phylogenetic analyses conducted using BI and ML resulted in identical tree topologies (Fig. 1), which agrees with previously published phylogenies of *Tetradesmus* (Fletchner et al. 1998; Lewis & Flechtner 2005; Terlova & Lewis 2019). Species isolated from desert soil crusts do not form a single clade, and instead are dispersed across the tree, with at least one desert species having a strongly supported sister relationship to an aquatic species (Fig. 1).

Desiccation and Rehydration

The effective quantum yield of photosystem II (Φ_{PSII}) of hydrated cells was similar in all species included in this study (approximately 0.6). The desiccation response pattern was also similar in all species and for all desiccation modes used. Specifically, Φ_{PSII} remained constant for a considerable amount of time, but then dropped to zero within 30–80 min following the initiation of desiccation (Fig. 2). The loss of Φ_{PSII} followed similar dynamics in desert and aquatic species of *Tetradesmus* , indicating that the algae are unable to prolong physiological activity under desiccation stress. A striking difference between the aquatic and desert *Tetradesmus*was demonstrated upon rehydration (Fig. 3a), as only strains of terrestrial algae restored their photosynthetic capacity after desiccation (full recovery took 30–180 min). By contrast, the aquatic species showed no recovery of Φ_{PSII} even after 12 h rehydration (with one exception, as discussed below). The response to rehydration varied among terrestrial species depending on the intensity of desiccation (Figs. 3b, 4). Rapid desiccation to ∼5% RH (2-3 h) appears to cause the most severe damage. Initially upon rehydration desert species exhibit a short-term recovery of the photosynthetic activity but did not recover permanently. Less severe desiccation to 65% RH (8 h) and 80% RH (16 h) resulted in long term recovery of all terrestrial species, however the initial level of photosynthetic activity was reached by some strains and not others as described below.

After 10 min of rehydration (Fig. 4a), the most striking difference was between the aquatic species (no photosynthetic activity with an exception of one aquatic strain) and desert species (high photosynthetic activity). Cluster analysis revealed four clusters. All desert species belonged to the same cluster when desiccated at 5% RH (Fig. 4a). Under milder conditions (˜65% RH) *T. bajacalifornicus*exhibited Φ_{PSII} values similar to those in the hydrated state (Fig. 4a), the aquatc taxa did not recover, and *T. deserticola* and *T. adustus* demonstrated 45–75% of their maximum Φ_{PSII}. Under the mildest treatment (i.e., 80% RH, 16 h) one strain of the aquatic *T. obliquus* (UTEX 72) remained inactive, whereas another (UTEX 393) recovered some photosynthetic activity (Fig. 4a). Among desert species, *T. bajacalifornicus* again exhibited higher levels of recovery, with separate strains of T. *deserticola* and *T. adustus* grouped in the same cluster.

After 12 h of rehydration (Fig. 4b) the effect of intense desiccation on*Tetradesmus* cells became more apparent, as neither aquatic nor the desert algae maintained photosynthetic activity under desiccation at 5% RH. Cluster structure of responses from the medium-rate desiccation (Fig. 4b, 65% RH) was similar to that of the 10 min rehydration (*T. deserticola* and *T. adustus* were clustered together, all strains reached ˜75% recovery; *T. bajacalifornicus* was separated with the highest 100% recovery). Under the mildest desiccation at 80% RH, a single aquatic strain (UTEX 393) recovered 75% of its hydrated Φ_{PSII} level, whereas another strain (UTEX 72) failed to recover even after 12 h rehydration (Fig. 4b). The responses of the soil alga *T. dissociatus* and the aquatic species *T.* sp. *"raciborskii"* to desiccation and rehydration followed the general trends described above (these data are presented in Supplementary materials, Fig. S2). Desiccation of these species was carried out separately from the other species, and their chlorophyll fluorescence was measured using a different instrument (Junior PAM, Heinz Walz GmbH, Effeltrich, Germany). The Junior PAM operates with a single fiber optic, and thus yielded lower values for the hydrated cells, however overall patterns displayed by these species agree with original data collected with the PAM 2500. To make these data comparable to the measurements taken for the rest of the species, a strain of aquatic *T. obliquus* (UTEX 393) and dessert *T. bajacalifornicus* (ZA 1-7) were desiccated at the same time.

Transmission electron microscopy (TEM) of cells in different hydration states

The cells of two investigated species (*T. obliquus* , UTEX 393 and*T. deserticola* , EM2-VF30) had a single cup-shaped chloroplast with a pyrenoid surrounded by several starch grains of different sizes. Typical for green algae, thylakoids are single or in stacks of 3–4, and are never in larger stacks, contrary to what can be seen in embryophytes. Golgi bodies are composed of 6–8 cisternae with attached vesicles, and numerous mitochondria could be seen in the cytoplasm (Fig. 5). Desiccated cells do not show striking differences in their ultrastructure compared to the controls. The protoplasm did not shrink, thylakoid membranes were intact, and cytoplasm was not denser than in fully hydrated cells. Accumulation of electron dense globules, known as plastoglobuli (50–100 nm) was found inside the chloroplast of both species mainly under desiccated and rehydrated conditions (Fig. 5). Cellular membranes of *T. obliquus* appeared to have more contrast but

did not show noticeable damage (Fig. 5b). No definite changes in cell ultrastructure were noticed upon rehydration in either investigated species. Thylakoid membranes of both species appeared intact (Fig. 5). The cytoplasm of *T. obliquus* was denser compared to the hydrated cells, but the plastoglobuli were smaller compared to the desiccated cells (Fig. 5). Some rehydrated cells of *T. deserticola* contained a larger number of small vacuoles, and chloroplast contained numerous plastoglobuli (Fig. 5b).

CLSM visualized differences in membrane integrity

The fluorescent vital stain FM 1-43 did not penetrate the plasma membrane of the aquatic *T. obliquus* in the hydrated state (Fig. 6a), whereas cells exposed to 4M sorbitol showed clear plasma membrane damage and rehydrated cells contained inner membranes stained with FM 1-43, indicating a severely damaged plasma membrane (Fig. 6b-c). In contrast, FM 1-43 did not penetrate the plasma membranes of the desert *T. dissociatus* and *T. deserticola* in either of the physiological states (Fig 6d-f and g-i, respectively), indicating preservation of membrane integrity.

Discussion

Phylogenetic relationships of aquatic and terrestrial *Tetradesmus*

Of the 25 known species in the green algal genus *Tetradesmus,*five were described from terrestrial habitats (Lewis & Flechtner 2005, Mikhailyuk et al. 2019, Terlova & Lewis 2019). Strain UTEX 72 was confirmed as a strain of *T. obliquus* in the present study.As shown in our study (Fig. 1) and in Terlova & Lewis (2019), not all terrestrial taxa are reconstructed as closest sister taxa, with aquatic *T. "raciborskii "* sharing a common ancestor with terrestrial *T. bajacalifornicus* . The other aquatic taxa *T. obliquus*and *T. distendus* were further separated from the first group by intervening terrestrial taxa. This result supports habitat switches within the genus, although the direction of these are not testable in the present study (Fig. 1). However, given the contrasting habitats of *Tetradesmus* species, we tested the hypothesis of distinct responses to desiccation and rehydration by the aquatic and the terrestrial congeners.

Habitat of origin is predictive of vegetative desiccation tolerance exhibited by the *Tetradesmus* species

Our current understanding of vegetative desiccation tolerance is primarily based on the research of desiccation tolerant land plants, also called resurrection plants, and their streptophyte algal relatives (Farrant et al. 2009; Holzinger et al. 2011), or studies of individual lichenized algae (Banchi et al. 2018). Physiological responses to desiccation of desiccation-tolerant green algae and mosses appears to have much in common (e.g., Proctor & Smirnoff 2000; Proctor et al. 2007; Koster et al. 2010; Karsten et al. 2016; Pierangelini et al. 2017, 2019), including rapid (within minutes after rehydration) recovery even from lengthy and severe desiccation events.

A study by Gray et al. (2007) examined responses to desiccation and rehydration in a wide range of aquatic and desert green algae. They showed dramatic differences in the behavior of species adapted to these habitats; and that only desert algae were capable of recovering their full photosynthetic capacity after being dry for a period of 1–30 days. Conditions under which desiccation and rehydration occur influence the response of the algae. For example, desiccation in the dark resulted in the recovery of more species (Gray et al. 2007). In Scenedesmaceae (a family including *Tetradesmus*), algae isolated from desert soil crusts recover photosynthetic activity three minutes after rehydration and to a much higher degree than the aquatic species (Cardon et al. 2018). They also demonstrated variation in recovery among the desert algae. However, species included in this study belonged to multiple genera and only short-term rehydration (3 min) was recorded, whereas our experiment aimed to investigate closely related species and we measured Φ_{PSII} for ˜12 h after rehydration.

We first tested the hypothesis that the investigated desert species are capable of recovering from desiccation events compared to the aquatic species. All species studied here lost their photosynthetic capacity upon desiccation. Upon rehydration, desert algae showed an immediate recovery, with 50–80% of initial photosynthetic activity being recovered in 10–30 min, followed by a period of slower change in which the hydrated levels of photosynthesis were reached in 24–48 h. However, fast and severe desiccation at 5% RH

was damaging even for these species (after short-term recovery of the photosynthetic activity immediately upon rehydration, photosynthetic capacity invariably again declined over time). Similar responses characterize desiccation-tolerant mosses (e.g., Proctor et al. 2007) and algae (Gray et al. 2007, Karsten et al. 2016, Cardon et al. 2018).

In our experiments the severity of desiccation is correlated with the time it takes to reach the final humidity level. The duration of water loss from hydrated samples ranged between 4–16 h, which may potentially be experienced by cells in fundamentally different ways. Slow desiccation potentially allows cells to detect the signal and activate desiccation-induced protective pathways, whereas to survive rapid desiccation they must have these protective compounds accumulated prior to desiccation. The difference between these processes is demonstrated by comparisons of desiccation-tolerant angiosperms and bryophytes. Angiosperms can only survive slow desiccation and demonstrate activation of protective pathways during desiccation (Farrant 2000, Alpert 2006, Farrant et al. 2009). Bryophytes, on the other hand, can recover after rapid desiccation events due to constitutive production of osmolytes and other protective compounds during the hydrated state (Oliver et al. 2011). Interestingly, accumulation of protective compounds may depend on the growth conditions and on the age of plants under the stress. For example, a differential expression study showed that older cells of streptophyte algae were less stressed by desiccation compared to younger ones (Rippin et al. 2017).

Our measurements of Φ_{PSII} suggest that the rate of decrease in photosynthesis is similar in all three desiccation modes, but that the different desiccation modes varied in the timing of the onset of the decrease in photosynthesis. In other words, the treatments differed in how long Φ_{PSII} remains stable at the beginning of the experiment following the initiation of the desiccation treatment. We suggest that the cells were initially surrounded by a liquid layer of culture medium, which is attached to cells by surface tension forces. Cells start losing water only after this water film is absorbed by the desiccant.

The modes of desiccation (final humidity level and duration to reach it) have an impact on the desiccation tolerance phenotype of desert *Tetradesmus* (Fig 4). The strongest desiccation (5% RH, 4-5 h) inflicted damage even to terrestrial algae. Despite a short partial recovery of Φ_{PSII} after 10 min of rehydration in terrestrial algae was observed, in all of the studied desert strains the Φ_{PSII} subsequently dropped to zero. A short-term initial recovery of Φ_{PSII} with a subsequent decline of the signal was also reported for other green algae exposed to desiccation (e.g., Pierangelini et al. 2019). A lack of capacity of desert algae to fully recover from this harsh treatment is likely related to the difference between our experiment design and what the cells experience in nature. Desert soil crusts were shown to remain humid for longer than the surrounding bare sand (Belnap & Lange 2001), which prolongs the hydration time and slows down desiccation. Despite of how extreme our treatment was, clear differences in response to it between the aquatic and desert species is apparent and indicates underlying physiological differences. Gentler modes of desiccation (RH 65% and 80% reached in 8 or 12 h, respectively) resulted in complete or almost complete recovery in all desert species. However, only one species (*T. bajacalifornicus*) recovered its maximum photosynthetic yield within 10 min of rehydration under both of these conditions. Among desert species, variation in recovery was higher for the strains of *T. deserticola* and *T. adustus* . Some fully recovered within 10 min when desiccated at RH 80%. Others required more time. Algae desiccation at RH 65% resulted in recovery of 45–75% of the maximum in the first 10 min of rehydration, and did not reach the maximum even 12 h later. The desiccation/rehydration profiles demonstrate variation in desiccation phenotype in this genus. The re-decline of photosynthetic activity after short-term recovery upon rehydration in the rapid and severe desiccation experiment shows the importance of extended data collection to completely understand the response of a species to desiccation and rehydration. Variation in the desiccation phenotypes among the desert *Tetradesmus,* belonging to separate clades within the genus, may indicate differences in the mechanisms of vegetative desiccation tolerance in these species and opens the possibility for future development of the genus as a model group for investigation of evolution of this complex trait.

To investigate variation among the strains of *Tetradesmus* species we included multiple strains (2–4) for the species, where possible. In most of the cases the within-species variation was minimal and different strains

of a species clustered together (Fig. 4). The only exception was UTEX 393, a strain of the aquatic species *T. obliquus* , which recovered 75% photosynthetic activity after 12 h of rehydration following the mildest desiccation of our treatments (80% RH, 12 h). This result is similar to the findings of Cardon et al. (2018) during desiccation of the same strain. Consistent recovery of UTEX 393 from mild desiccation suggests the presence of protective pathways even in the generally desiccation-sensitive strains. The genes responsible for these pathways are likely shared by all species of *Tetradesmus*and may prove key to discovery of the mechanisms of desiccation-tolerance in the desert species.

TEM does not reveal changes in cell ultrastructure during desiccation and rehydration .

Our observations of cell ultrastructure in two selected *Tetradesmus* species in different hydration states are drastically different from what can be found in the literature (e.g., Domozych et al. 2003; Proctor et al. 2007). We did not detect cell plasmolysis (i.e., decrease in cell volume and retraction of the protoplast from the cell wall caused by a loss of water). The cell wall retained the same thickness and none of its layers expanded. An undulation of the cell wall reported for *Klebsormidiuim crenulatum* (Holzinger et al. 2011) was not observed; slightly wrinkled cell walls were seen across all treatments and in both species, which may indicate an artifact of sample preparation. No specific products were seen accumulating in studied cells, and the plastoglobuli detected in the chloroplast do not show distinct patterns when cells of each species are compared across different treatments and with the control. Despite the apparent intact nature of cells of *T. obliquus,* they do not recover Φ_{PSII} after desiccation and therefore must be damaged in a way that is not manifested in the ultrastructural morphology of cells.

Changes in membrane integrity uncovered with CLSM

We tested the functional integrity of *Tetradesmus* cells using the amphiphilic styryl dye FM 1-43 as a vital stain. This stain binds to lipids and therefore will only be seen along the plasma membrane of intact cells, whereas in damaged cells with a fragmented plasma membrane, the membranes of the organelles will be stained as well. Cells of the aquatic *T. obliquus* had intact membranes only when hydrated (Fig. 6a). In cells under osmotic stress only the plasma membrane was stained, but a heterogeneous pattern of staining was found (Fig. 6b). The most damage was done by rehydration: intense staining of intracellular material (Fig. 6c) suggests that this species is vulnerable to osmotic stress. By contrast, cells of the temperate soil alga *T. dissociatus* and the desert alga *T. deserticola* had only the plasma membrane stained (Fig. 6d-f and g-i respectively) in all three physiological states (hydrated, under osmotic stress, rehydrated), indicating preservation of membrane integrity through the osmotic stress-relief cycle.

An absence of changes in cellular volume after desiccation or plasmolysis in *Tetradesmus* species may be related to the fact that these algae lack a large central vacuole that is typical for many land plants and streptophyte green algae. In streptophyte plants, aquaporins located on the vacuolar and plasma membranes were shown to play a prominent role in desiccation tolerance (reviewed in Becker et al. 2020). The absence of a large vacuole may mean that even when fully hydrated cells of *Tetradesmus* do not possess a storage of free water, which can be lost. In this case, the definition of desiccation tolerance as the ability to survive 80–90% water loss (Oliver et al. 2000) may not be applicable to these algae. Moreover, it is impossible to determine cell water content of hydrated *Tetradesmus* cells due to their microscopic size; water creates a film outside the cells which cannot be removed without risking dehydration of the cells. Thus, the method of subtracting dry plant mass from the hydrated mass that is commonly used for land plants (e.g., Bartoškova et al. 1999, Proctor & Smirnoff 2000) cannot be used in unicellular algae. However, these algae exhibit the hallmarks of vegetative desiccation tolerance, as actively metabolizing cells can equilibrate with very dry air and recover from it upon rehydration.

Future directions

The strong association of habitat of origin with the physiological ability to recover from rapid desiccation in the vegetative stage provides a context to formulate hypotheses about the evolution of vegetative desiccation tolerance in *Tetradesmus* , ranging from a single origin in the terrestrial species (in which case the aquatic *T.* sp. *"raciborskii* " returned to the aquatic habitats secondarily), to the evolution of vegetative desiccation

tolerance independently in each of these desert taxa.

Recovery of one aquatic strain (*T. obliquus* , UTEX 393) at 80% RH reveals that aquatic algae tolerate desiccation stress to some extent. This ability may prove to be important for discovering evolutionary and molecular mechanisms of the emergence of desiccation tolerance in *Tetradesmus.* Aquatic *Tetradesmus* are common in fresh waters from around the world (e.g., Ismagulova et al. 2018), including regions where they experience seasonal or intermittent water restrictions, which should require an ability of cells to survive periodic environmental stresses, including desiccation. Many aquatic algae form tolerant dormant stages either as vegetative cells or as reproductive stages, such as zygotes (e.g., Starr 1955; Evans 1958; Rengefors et al. 1998). The genes imparting tolerance to these dormant stages may have been co-opted for vegetative desiccation tolerance by terrestrial *Tetradesmus* in a similar way that genes providing desiccation tolerance to seeds were co-opted in the vegetative tissues in desiccation-tolerant angiosperms (e.g., Illing et al. 2005; Costa et al. 2017; VanBuren et al. 2017). Future comparative omics studies of the closely related species of *Tetradesmus* from different habitats and having distinct desiccation tolerant phenotypes will enable investigations of the origins of the desiccation machinery in this group, and may reveal the evolutionary transitions that enable these algae to occupy such diverse environments.

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Declarations

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Availability of data and material . New DNA sequence data are accessed in NCBI GenBank. Raw physiological data and scripts for data analysis were deposited to DRYAD repository and GitHub/eterlova/TetradesmusPhysiology.

Figure legends

Fig. 1 Phylogenetic tree of desert and aquatic *Tetradesmus* species based on BI of *tuf* A, *rbc* L, and ITS 2 rDNA. Numbers associated with the nodes indicate support values for BI and ML analysis, respectively. Strains used in the desiccation experiments indicated with a dot (a black dot for the strains from the original experiment, gray dots indicate strains desiccated in a second experiment, see the supplementary materials). Habitats of origin (aquatic, temperate soils, or desert soil crusts) are indicated by the color of a bar (blue, brown, and orange, respectively).

Fig. 2 Example trace of the desiccation/rehydration cycle. In black (left y-axis): representative example of raw measurements of PSII effective yield of a desert alga (*T. adustus* , strain LG2-VF29) taken every 10 min during desiccation to 65% RH, and rehydration. Data points correspond to the mean of four Φ_{PSII}measurements, error bars indicate one standard deviation. In gray (right y-axis) is RH recorded during desiccation and rehydration. A rapid decrease in humidity coinciding with the shift from desiccation to rehydration was by the opening of the desiccation chamber to replace the desiccant with water for algae rehydration.

Fig. 3 Examples of typical responses to desiccation and rehydration (a) by aquatic and desert *Tetradesmus* and (b) to different desiccation modes by a desert species. Each data point represents mean Φ_{PSII} (n=4),

measurements were taken every 10 min. (a) Behavior of *T. obliquus* (aquatic species), *T. deserticola* , and *T. bajacalifornicus* (desert taxa) when desiccated at 65% RH. (b) Response of *T. deserticola* to desiccation under three conditions (RH 5%, 65% and 80%). We used cluster analysis to differentiate among cell physiological states (hydrated, desiccating, or rehydrated).

Fig. 4 Hierarchical cluster analyses of recovery indices (ratio of a rehydrated value to the initial hydrated photosynthetic yield) of *Tetradesmus* across desiccation treatments demonstrate that the habitat of the species (aquatic or terrestrial) as well as the desiccation mode influence their ability to recover from desiccation. (a) After 10 min of rehydration all desert algae are able to re-initiate their photosynthetic activity rapidly upon rehydration, and aquatic algae do not, although there is variation among strains of most species). (b) After 12 h of rehydration the difference in recovery from different desiccation modes became apparent. Within-species variation in the aquatic taxon is clear under the gentlest desiccation at 80% RH. Cluster number on the x-axis represents distinct groups (identified in cluster analysis of Euclidean distances, verified with gap statistic). Symbol shape indicated the native habitat of each species (circles for aquatic and triangles for terrestrial), color indicates the individual species. Multiple symbols of the same color correspond to different strains of each species.

Fig. 5 TEM photographs of an aquatic and terrestrial *Tetradesmus* in hydrated, desiccated, and rehydrated states. (a) Ultrastructure of the aquatic species *Tetradesmus obliquus* (UTEX 393). (b) Ultrastructure of the desert species *T. deserticola* (EM2-VF30). Arrows point at plastoglobuli. GA - Golgi body, Chl - chloroplast, M - mitochondrion, N - nucleus, P – pyrenoid.

Fig. 6 Fluorescence photomicrographs of an aquatic and terrestrial *Tetradesmus* in hydrated state, under osmotic stress, and rehydrated. Effect of osmotic stress by 4M sorbitol on the cells of *T. obliquus* UTEX 72 (a-c), *T. dissociatus* SAG 5/95 (d-f), and *T. deserticola* SNI-2 (h-i) visualized by CLSM and lipid-soluble fluorescent dye FM 1-43. (a) *T. obliquus* fully hydrated control cells, only plasma membrane was exposed to the dye. (b) *T. obliquus* cells under osmotic stress, arrow points to the fracture in the cell membrane. (c) *T. obliquus* rehydrated cells with the dye binding to the intracellular material, indicating the damage to the plasma membrane by desiccation. (d) *T. dissociatus* hydrated cells. (e) *T. dissociatus* under osmotic stress, no indication of membrane damage. (f) *T. dissociatus* rehydrated cells the membrane preserved its integrity. (g) *T. deserticola* hydrated control cells. (h) *T. deserticola* under osmotic stress, no indication of membrane fracturing. (i) *T. deserticola* rehydrated, the membrane integrity was preserved. Chl — chloroplast, P — pyrenoid.

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