A new microscopic method to analyse desiccation induced volume changes in aeroterrestrial green algae

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Abstract

Aeroterrestrial green algae are exposed to desiccation in their natural habitat, but their actual volume changes have not been investigated. We here measure the relative volume reduction in *Klebsormidium crenulatum* and *Zygnema* sp. under different pre-set relative air humidities (RH). A new chamber allows monitoring RH during light microscopic observation of the desiccation process. The RHs were set in the range of ~ 4 % to ~ 95 % in 10 steps. Relative volume reduction (RV_{RED}) caused by the desiccation process was determined after full acclimation to the respective RHs. In *K. crenulatum* RV_{RED} (mean ± SE) was 46.4 ± 1.9%, in *Zygnema* sp. RV_{RED} was only 34.3 ± 2.4% at the highest RH (~ 95%) tested. This indicates a more pronounced water loss at higher RHs in *K. crenulatum* vs. *Zygnema* sp. In contrast, at the lowest RH (~ 4%) tested RV_{RED} ranged from 75.9 ± 2.7% in *K. crenulatum* to 83.9 ± 2.2% in *Zygnema* sp. The final volume reduction is therefore more drastic in *Zygnema* sp. These data contribute to our understanding of the desiccation process in streptophytic green algae, which are considered the closest ancestors of land plants.

Introduction

Green algae have the ability to perform photosynthesis and their major distribution is in aquatic habitats making them rather an ecological unit than a taxonomic entity. In fact, according to recent phylogenetic research, green algae can be divided in two main lineages (Leliaert et al. 2012): (1) Chlorophyta, which contains the majority of described green algal species, and (2) Streptophyta, which includes the Charophytes, a paraphyletic group of freshwater and terrestrial green algae, as well as all land plants, the Embryophytes (Leliaert et al. 2012). Among both lineages, several groups with terrestrial representatives occur. In case of the Chlorophyta, the classes Ulvophyceae, Trebouxiophyceae and Chlorophyceae include some taxa living in terrestrial habitats, e.g. the the Trentepohliales of the Ulvophyceae (Leliaert et al. 2012), the members of Trebouxiophyceae ('lichen algae group') and sparse genera in the Chlorophyceae e.g. *Fritschiella* (Holzinger and Karsten 2013). In the streptophyte lineage, terrestrial members can be found in the classes Klebsormidiophyceae, Zygnematophyceae and Coleochaetopyhceae (Graham et al. 2012,

Holzinger & Karsten 2013, Herburger & Holzinger 2015, Mikhailyuk et al. 2015). The closest relatives to land plants can be found among charophyte green algae (Wodniok et al. 2011, Timme et al. 2012, Delwiche and Cooper 2015). Their genome holds primary factors for plant terrestrial adaptation (Hori et al. 2014), and transcriptional changes upon severe desiccation stress demonstrated a land plant like defence reaction in *Klebsormidium* (Holzinger et al. 2014). Raffinose family oligosaccharides (RFOs) have been found to be upregulated upon desiccation stress likely contributing to the osmotic potential (Holzinger et al. 2014). Increasing knowledge on cell wall properties of these algal groups has become available recently, demonstrating the close relationship to land plants (Sørensen et al. 2011, Mikhailyuk et al. 2014, Herburger & Holzinger 2015). However, green algae are poikilhydric organisms that do not have cuticles or similar structures to protect from water evaporation (Delaux et al. 2012).

While the hydraulic parameters (osmotic potential at full turgor and relative water content at the turgor loss point) in pokilohydric plants of the embryophytes, ferns, mosses and lichens has been well investigated (see Table 1) only little information is available in aeroterrestrial green algae (Holzinger & Karsten 2013). This lack of data may be related to the difficulty of determining the volume or weight of terrestrial green algal samples due to their small size, mostly ranging between 5 and 25 μm in diameter (Rindi et al. 2011, Herburger et al. 2015). In contrast, the osmotic potential has been determined in *Klebsormidium* and *Zygnema* (Kaplan et al. 2012, 2013). In these studies, the water potential at the turgor loss point (Ψ_{TLP}) was determined by means of the 'incipient plasmolysis technique'. For plasmolysis fully turgescent plant cells, like the cell filaments of the investigated green algae, were exposed to osmotically active solutions with increasing concentrations and the value where 50% of the cells plasmolysed determined microscopically. At the equilibrium point, the cells have the same osmolarity as the external solution, which allowed determining the osmotic potential. In *K. crenulatum* a Ψ_{TLP} of -2.09 MPa, whereas in *Klebsormidium nitens* a Ψ_{TLP} of -1.67 MPa were found (Kaplan et al. 2012). In the same way, Ψ_{TLP} values of -1.67 and -0.8 MPa were determined in different strains of *Zygnema* sp. (Kaplan et al. 2013).

In the present study, we developed and test a special chamber that allows microscopic observations at several pre-set relative air humidity (RH) levels. To illustrate the capacities of this chamber, we analyzed the desiccation induced volume changes of the cytoplasm in streptophyte green algae. We hypothesised, that green algae from distinct classes (Klebsormidiophyceae, Zygnematophycae) show different desiccation patterns. We measured the relative protoplast volume reduction (RV_{RED}) at different pre-set RH levels under controlled conditions. This allowed us to get information on the water loss at different RHs and to enhance our understanding of desiccation processes in the studied organisms.

Materials and methods

Plant material

Two strains from different charophyte classes were selected for the experiments, as contrasting desiccation behaviour was expected from previous studies (Kaplan et al. 2012, 2013). (1) *Klebsormidium crenulatum* (Kützing) Lokhorst (Lokhorst and Star 1985) was previously isolated from an alpine soil sample (Obergurgl, Tyrol, Austria, 46° 50' 99.8'' N,

11° 00' 90.3'' E; 2,350 m a.s.l.; Karsten et al. 2010; Culture Collection of Algae Göttingen, SAG 2415). *K. crenulatum* was cultivated in liquid modified Bold's Basal Medium (Starr and Zeikus 1993) at a light dark regime of 16-8 h at 20°C during light- and 15°C during dark phase (Kaplan et al. 2012).

(2) *Zygnema* sp. (C.Agardh) 'Saalach' (SAG 2419), previously collected from the sandy littoral zone of the river Saalach near the City of Salzburg (Austria, 47°47' 8.70" N, 12°56' 42.66" O; 440 m a.s.l.; Herburger et al. 2015). *Zygnema* sp. was cultivated in conventional Bold's Basal Medium (BBM; Bischoff & Bold 1963) at the same light- dark regime and temperature as described above.

Test chamber

A test chamber that allowed (1) maintaining a constant RH for desiccation experiments and (2) microscopic observation of the samples was constructed (Fig. 1). The test chamber was two-storied, where the 'upper storey' consisted of a 120 x 120 mm square transparent polycarbonate Petri dish (Nr. 18252, Gammarad, Bologna, Italy). There, the salt solution or silica gel and in a separated space (created by a 10 mm plastic delimitation) the air humidity sensor (mini data logger PCE-MSR145W-THPA, PCE Instruments, Germany) were placed. This 'upper storey' had a circular hole, where a smaller, circular Petri dish (60 mm Ø, Nr. 628160, Greiner Labortechnik, Kremsmünster, Austria) was attached as the 'lower storey' which fitted into the mechanical stage of the microscope. In this 'lower storey' the algal samples were directly placed for observation. To separate the sample from the salt solution or silica gel, a plastic ring (9 mm high, made from the lid of round Petri dish), was attached around the hole of the upper Petri dish. The test chamber was covered by a square lid with a rubber seal (140x140 mm, 4 mm thick, transparent acrylic glass). Two motorised mini fans (MagLev Motor Blower, Spec. No. D01000940G-00, 17x17x3 mm, Sunonwealth Electric Machine Industry, Kaohsiung City, Taiwan) were attached to the lid, and ensured a constant ventilation of the chamber.

Experimental setting of the relative air humidity

The precise setting of the relative air humidity inside the hermetically sealed test chamber was achieved by different non-saturated lithium chloride (LiCl)-solutions (98%, Sigma Aldrich, Vienna, Austria) according to Hay et al. (2008), see Tab 2. Alternatively, a saturated K_2CO_3-solution (99.0%, Sigma-Aldrich) was used. The lowest RH generated by saturated LiCl solution is 11.2% at 20°C (Hay et al. 2008). Therefore, silica gel (with moisture indicator, Sigma-Aldrich) was used to generate RH values of 3.7-4.2% (Tab. 2).

Experimental procedure

The test organisms (*K. crenulatum*, *Zygnema* sp.) were placed in a volume of 20 µl culture medium in the 'lower storey' of the test chamber. In the 'upper storey', the respective salt solution or silica gel was filled. The cell filaments were spread and microscopic images were taken immediately. The cells were then allowed to completely equilibrate for up to 12 h at 20°C to the respective RH before 10-15 random images were taken. The measurements of the diameters at individual protoplasts were performed only on filaments that were found solitary after desiccation, where the protoplasts as well as the cell lumen diameter (CLD)

were clearly visible. In *K. crenulatum* an average of 22 protoplasts per RH step, and in *Zygnema* sp. an average of 16 protoplasts per RH step were measured.

Determination of the relative volume reduction RV_{RED}

The desiccation process was monitored by an inverted Zeiss Axiovert 200M microscope (20x, NA = 0.50; 40x, NA = 0.75; Carl Zeiss AG, Oberkochen, Germany). To determine the protoplast volume, the cell lumen diameter (CLD) and the diameter of the desiccated protoplast (PD_d) were measured in individual cells and the reduction of the protoplast volume was quantified (Zeiss AxioVision 4.7.1 software).

In this way, assuming a cylindrical shape, the protoplast volume at saturation (V_s in Eq. 1) and after desiccation (V_d in Eq. 2) was calculated by the following equations (for abbreviations see Fig. 1):

$$V_{s[\mu m^3]} = \left(\frac{CLD}{2}\right)^2 * \pi * CLL \qquad Eq.1$$

$$V_{d[\mu m^3]} = \left(\frac{PD_d}{2}\right)^2 * \pi * PL_d \qquad Eq.2$$

The relative volume reduction (RV_{RED}, %) of the desiccated protoplast related to the protoplast volume at the initial, saturated state was determined according to equation 3.

$$RV_{RED\,[\%]} = \frac{V_s - V_d}{V_s} \times 100 \qquad Eq.3$$

RV_{RED} were then plotted against RH values of both studied species.

Statistical evaluation

Significant differences between mean values of RV_{RED} [%] for each investigated genus (*K. crenulatum*, *Zygnema* sp.) were calculated with one-way ANOVA followed by Games-Howell's post-hoc test; P<0.01) using SPSS software. Significant differences of RV_{RED} [%] between the two genera at a certain relative air humidity level were calculated by Student's t-test (P<0.05) by SPSS software.

Results

Test chamber

We first tested our newly constructed test chamber for functionality at different RH. The chamber was filled with the respective LiCl-solutions of different concentrations or the saturated K_2CO_3-solution or with silica gel to generate different levels of RH (Tab. 2). At high levels of relative air humidity (> 80%), the RH inside the test chamber reached the

saturation point within 3-4 hours (Fig. 3), over silica gel, the saturation point was achieved in less than 3 hours (see Fig. 3). The recorded relative air humidity differed only slightly between experiments (Tab. 2).

Measurements of volume reduction

After transfer of algal suspensions into the test chamber, an initial phase was observed, where only culture medium evaporated and the cells did not show any change in volume or shape. Then, the desiccation process started, in which the algae lost water for about ~15-20 min until they reached an equilibrium state. The image series in figure 4 shows cell filaments of *K. crenulatum* (78.3% RH, 90-140 min after start of the experiment, Fig. 4 A) and *Zygnema* sp. (56.4% RH, 155-190 min after start of experiment, Fig. 4 B) during the desiccation phase.

The diameter of the protoplasts experienced a drastic reduction during the desiccation process, while the length of the protoplasts usually did not change and the protoplasts remained attached to the cross walls (Fig. 2, Fig. 4 A). Only in *Zygnema* sp., protoplasts occasionally were detached from the cross walls, which lead to shrinkage in length during the desiccation process (Fig. 4 B). As in these cases, severe damage was expected; these cells were not included for volume calculations. In *Zygnema* sp., the strongest shrinkage of the protoplasts was observed in the central area and therefore a mean value was used to calculate the volume reduction (see Fig. 5 A & B). In some cases, the desiccated protoplasts even disintegrated into portions, resembling the chloroplast centres and pyrenoids (see Fig. 5 C-E); such protoplasts were not used for measurements.

Calculations of the relative volume reduction (RV_{RED}) were performed by measuring the protoplast diameter after a constant level of RH inside the test chamber was achieved and cells did not show further changes in protoplast volume or shape. In *K. crenulatum* the RV_{RED} values (mean±SE) ranged from $46.4 \pm 1.9\%$ at 95.4% RH to $75.9 \pm 2.7\%$ at 3.7% RH (Fig. 6). In *Zygnema* sp. the RV_{RED} values of $34.3 \pm 2.4\%$ at the highest RH was significantly higher (P<0.01) when compared to *K. crenulatum*. At the lowest RH the value of $83.9 \pm 2.2\%$ was significantly (P<0.05) lower in comparison to *K. crenulatum*. The development of volume reductions differed between the two genera. While in *K. crenluatum* a drastic water loss was observed already at highest RH tested, in *Zygnema* sp. the initial water loss was less pronounced. However, in *Zygnema* sp. the water loss at RH values < ~80% was more drastic indicated by higher RV_{RED} values.

Discussion

In the present study, a new test chamber allowed the monitoring of desiccation effects at certain pre-set RH during microscopic observation. This device was used with two green algae, *K. crenulatum* and *Zygnema* sp., for which the relative volume reduction (RV_{RED}) was experimentally determined. A distinct desiccation behaviour was found by quantitative analysis of the RV_{RED} at different RHs. The RV_{RED} values at the highest RH tested (~95% RH) indicated a stronger water loss in *K. crenulatum*. In contrast, at lower RHs *Zygnema* sp. showed a stronger water loss. There are also qualitative differences in the shape of the retracted chloroplasts between the two studied green algal genera.

In general, these observations correlate well with physiological parameters, e.g. reduction of the effective quantum yield as a consequence of desiccation in *K. crenluatum* (Karsten et al. 2010) and *Zygnema* sp. (Herburger et al. 2015). Herburger and Holzinger (2015) investigated these two genera in a parallel setup and monitored the desiccation and recovery effects of individual filaments by imagining PAM. In both genera desiccation at ambient air (RH ~ 65%) leads to a rapid decrease of the effective quantum yield of photosystem II (YII). Upon rehydration for 180 min, the Y II is re-established, in *Klebsormidium cenulatum* to nearly the initial value and in *Zygnema* sp. to only half of the initial value (Herburger and Holzinger 2015). In the present study, we found clear differences between the two examined genera concerning the range of the RV$_{RED}$-RH relationship. Most important, RV$_{RED}$ values below ~80% RH are considerably higher in *Zygnema* sp. than *K. crenulatum*. This means that the protoplasts in *Zygnema* sp. experience a clearly stronger reduction in volume under similar RH levels. This correlates well with the observations by Kaplan et al. (2012, 2013), and may be attributed to the higher vacuolization status of young *Zygnema* sp. protoplasts (Kaplan et al. 2013, Herburger & Holzinger 2015). Moreover, this could also explain the weaker recovery of YII in *Zygnema* sp. (Herburger and Holzinger 2015). In *Zygnema* sp., older cultures form pre-akinetes, which are in general more stress tolerant (e.g. Pichrtová et al. 2014), and likely have different osmotic potentials (e.g. Fuller 2013, Kaplan et al. 2013). In the present study, we used young cultures, to exclude this phenomenon. However, the measurement of the *Zygnema* sp. protoplasts was more difficult to perform due to the shape of the desiccated cytoplasm.

In both genera, only individual cells from solitary filaments were measured. Bundles of filaments showed a slower desiccation process and so a prolonged moistness was observed. This is a well-described strategy for filamentous, soil crust inhabiting organisms to enhance the water holding capacities in nature (e.g. Holzinger and Karsten 2014). In the experiment, however this leads to complications with the measurements (overlapping of filaments, protoplast shape) and was therefore avoided. Moreover, we used for the calculation only cells where the protoplast remained attached to the cross walls, assuring that the cells were not damaged too badly had the potential to recover (Herburger and Holzinger 2015).

Summarising the results of this study we here introduce a functional test chamber suitable to follow the desiccation process during microscopic observation. We demonstrated that the test chamber could create and maintain a constant RH during the desiccation experiments. These conditions allowed to subsequently calculate the volume reduction of the test organism *in situ*. The more rapid water loss observed in *K. crenulatum*, might in nature be compensated by their habitat in soil crusts, giving protection against desiccation (Karsten and Holzinger 2014). In contrast, *Zygenma* sp., collected from the sandy litoral zone of a river (Herburger et al. 2015) might be naturally exposed more frequently to higher RHs, which they are capable to tolerate. The stronger water loss at lower RHs resulted in severe damage, and it has been shown that particularly young vegetative *Zygnema* sp. cells cannot recover from desiccation over silica gel, while they tolerate desiccation at ~83% RH with good recovery success according to measurements of the effective quantum yield (Pichrtová et al. 2014). We are aware, that comparing genera after investigating only one strain per genus might lead to misinterpretations; therefore, future studies should include more strains.

The here presented chamber will allow to monitor desiccation kinetics under defined RHs. Repeated desiccation and rehydration cycles could be created in order to study possible legacy effects. Future studies should elucidate which osmotic compounds are responsible for water holding capacities that enable aeroterrestrial green algae to withstand desiccation. Additionally, this chamber might also be valuable for further studies measuring the photosynthetic activity of single cells during desiccation, which is possible with new generation chlorophyll fluorimeters or the observation of other physiological responses employing fluorescent probes, e.g. H2DCF-DA to detect the formation of reactive oxygen species during the desiccation process.

Acknowledgments

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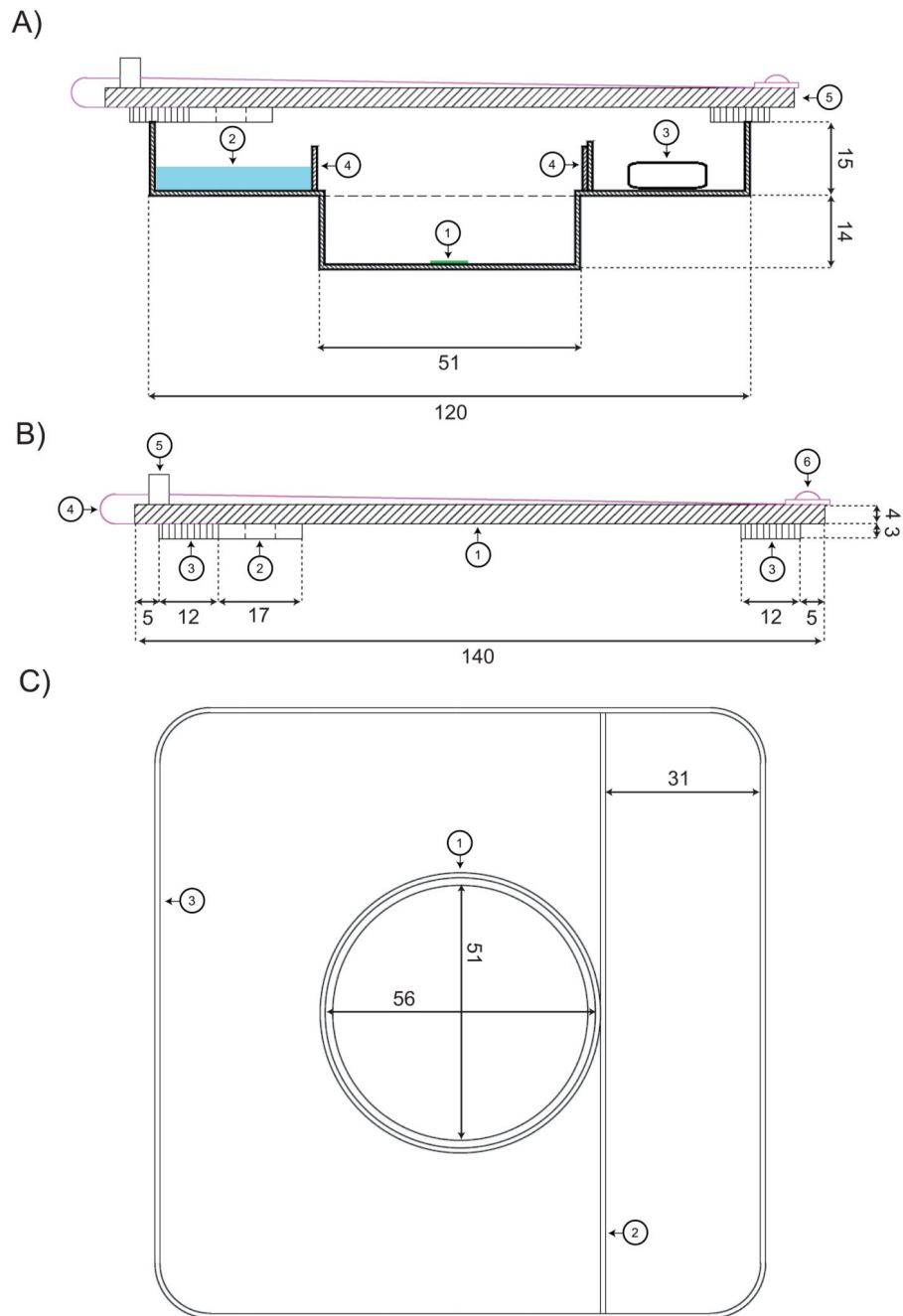
Fig. 1.
Two-storied test chamber. A) Two-dimensional, cross-sectional view of the test chamber during a desiccation experiment. Bottom storey with (1) algal sample; top storey with (2) salt solution; (3) air humidity and temperature sensor; (4) plastic ring of 9 mm height, preventing the contact of the salt solution with the investigated specimen; (5) Perspex lid. B) Detailed description of the Perspex lid. (1) square panel of Perspex, forming the central body of the lid; (2) cross-section of one of the two motorised mini fans; (3) sponge rubber seal; (4) power supply cable of one of the two motorised mini fans; (5) power adapter; (6) screw

fixing the power supply cable. C) Top view of the test chamber (1) plastic ring, (4) in A); (2) linear plastic delimitation of 10 mm height, preventing a contact of the salt solution with the sensor; (3) outer margin of the petri dish. All dimensions in mm.
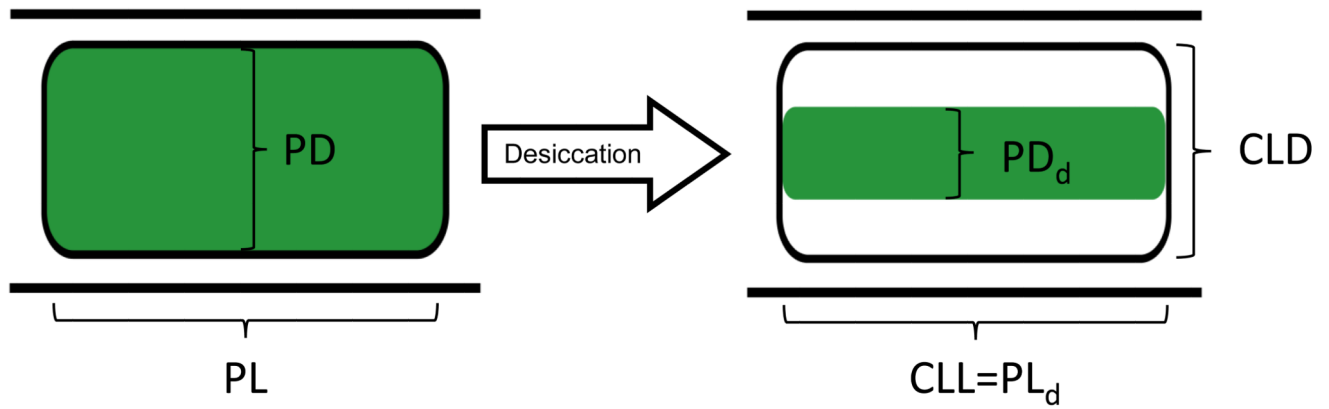
Fig. 2.
Schematic drawing of the change of protoplast shape during the desiccation process. The parameters, which were used for determining the protoplast volume before (volume in the saturated state, V_s) and after (volume in the desiccated state, V_d) desiccation, are depicted. Abbreviations: PD = protoplast diameter, PL = protoplast length, CLD = cell lumen diameter after desiccation, CLL = cell lumen length after desiccation; PD_d = protoplast diameter after desiccation; PL_d = protoplast length after desiccation.
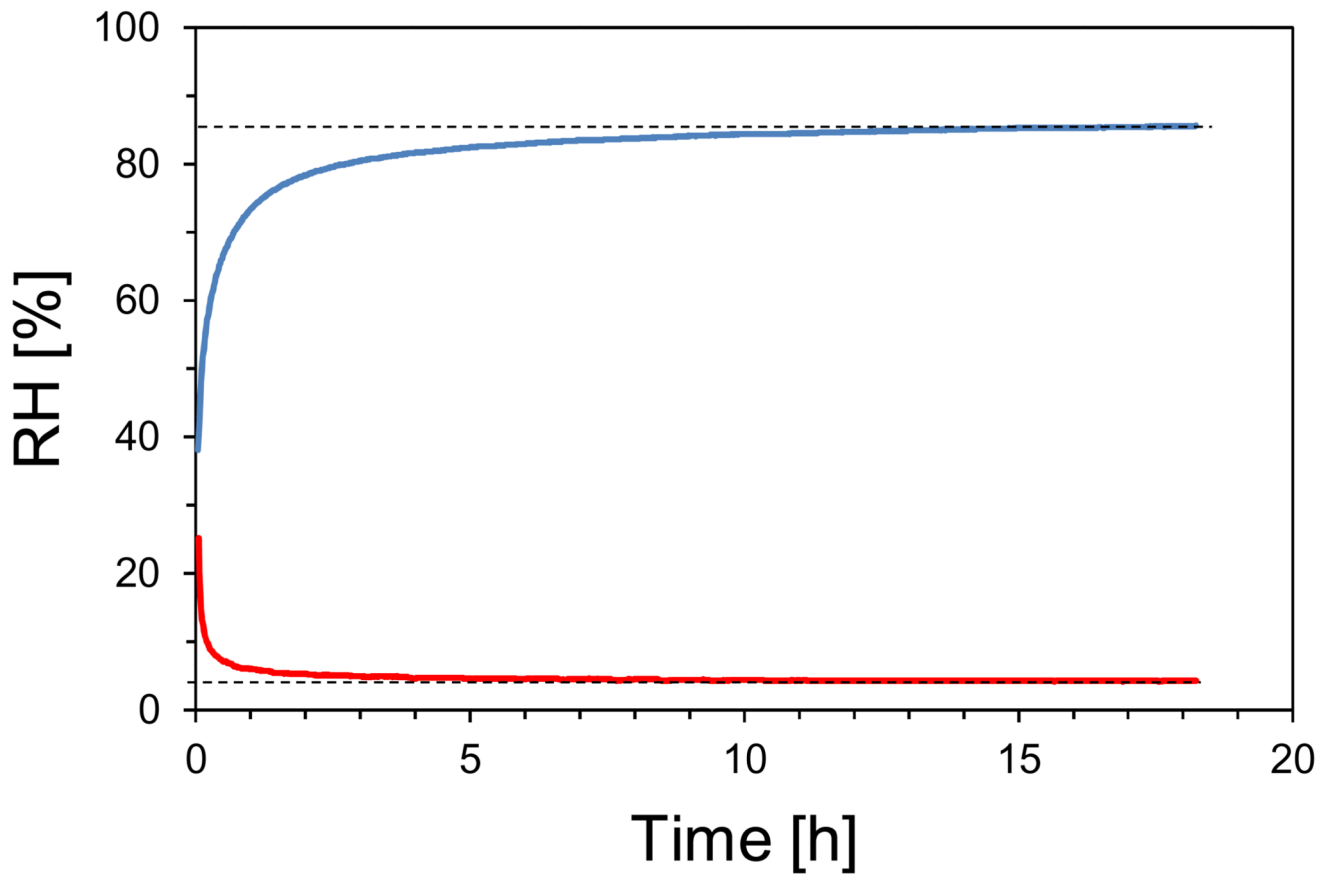
Fig. 3.
Exemplary presentation of time courses of the relative air humidity (RH) inside the test chamber using a 13 g/100 ml LiCl-solution (blue line) and silica gel (red line). The target moisture content of 85.6 % and 4.2 %, respectively, is indicated by horizontal dotted lines.
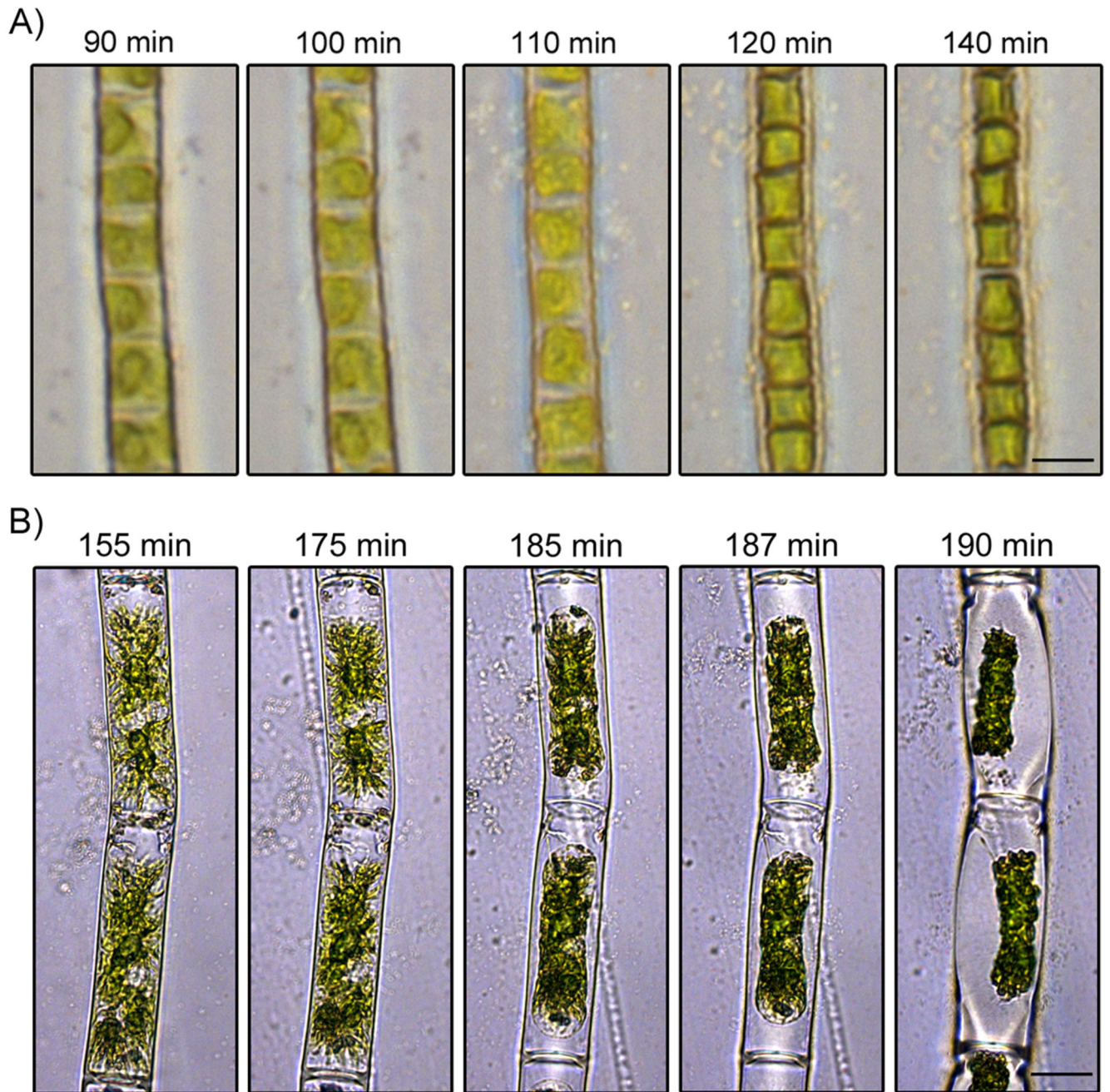
Fig. 4.
Light microscopic image series of the desiccation process in A) *Klebsormidium crenulatum* at 78.3% RH, scale bar 5 μm and B) in *Zygnema* sp. at 56.4% RH, Time in minutes (min) after the start of the desiccation experiment. Scale bars 20 μm.
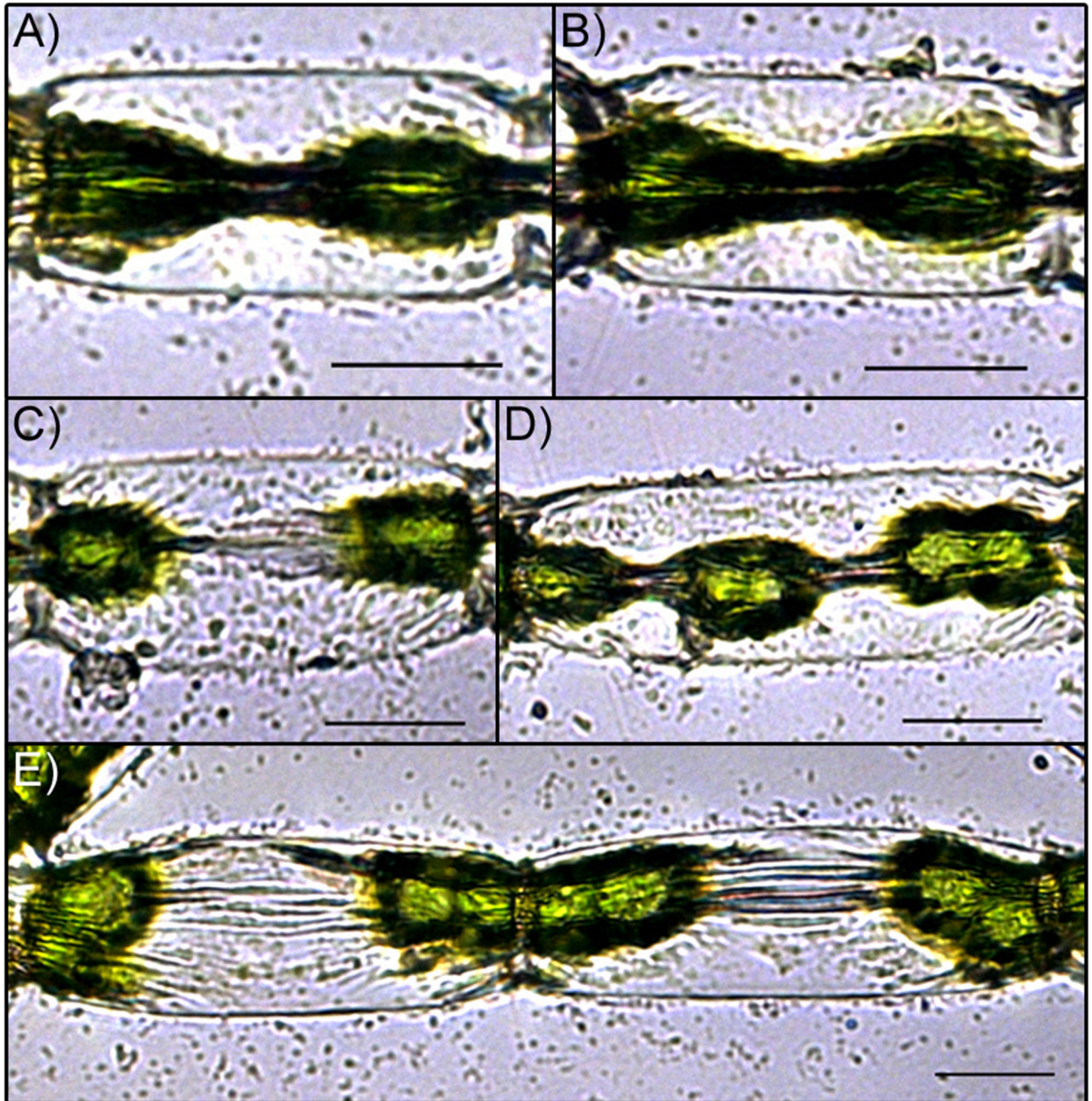
Fig. 5.
Light microscopic images of *Zygnema* sp. cells after 3h 20 min desiccation at 56.4% RH.
Images A) & B) represent examples of 'hourglass' shape. Images C) – E) show cases where
the protoplast of *Zygnema* sp. disintegrated as an effect of the desiccation. Scale bars 20 µm.
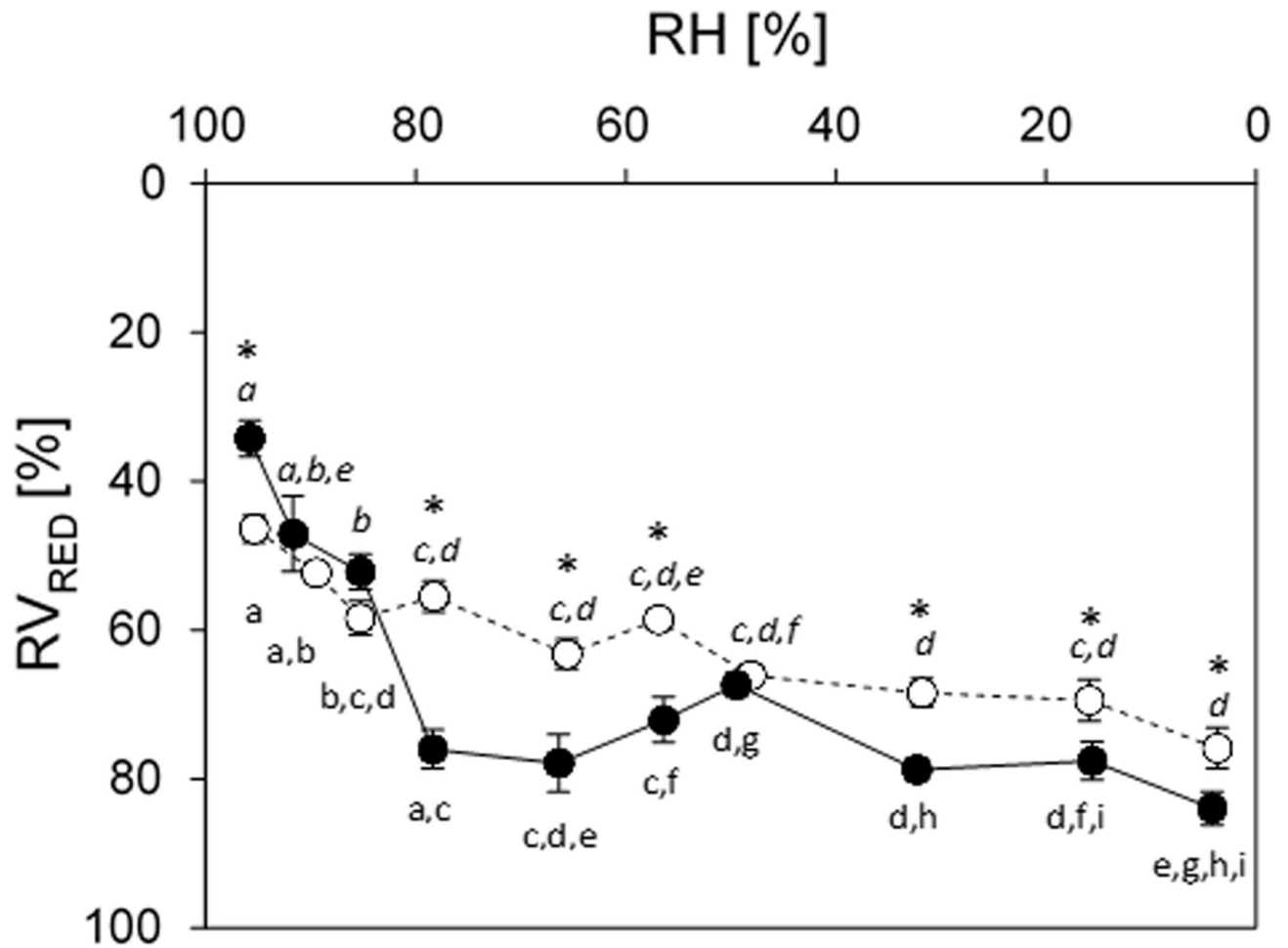
Fig. 6.
Relative volume reduction (RV$_{RED}$ [%]; mean values ± SE; standard error) in *Klebsormidium crenulatum* (open symbols) and *Zygnema* sp. (solid symbols) determined at certain relative air humidity RH levels. Significant differences between mean values of RV$_{RED}$ [%] for each investigated genus (*K. crenulatum*, *Zygnema* sp.) are indicated by different letters (normal font: *Zygnema* sp., italics: *K. crenulatum*; one-way ANOVA followed by Games-Howell's *post-hoc* test; P<0.01). Significant differences of RV$_{RED}$ [%] between the two genera at a certain RH level are indicated by asterisks (Student's t-test; P<0.05).

Table 1

Osmotic potential at full turgor (Ψ_{osat}) and relative water content at the turgor loss point = incipient plasmolysis (RWC_{TVP}) of different poikilohydric species of angiosperms, ferns, mosses and lichens. The references, from which the data were obtained, are also listed.

Species name	Ψ_{osat} (-MPa)	RWC_{TVP} (%)	References
Angiosperms			
DICOTYLEDONS			
Myrothamnus flabellifolia	1.92	90	Beckett (1997)
MONOCOTYLEDONS			
Xerophyta scabrida	0.55	–	Tuba et al. (1994)
Xerophyta viscosa	1.41	81	Beckett (1997)
Ferns			
Gymnopteris hispida	1.3	–	Walter & Stadelmann (1974)
Cheilanthes lindheimeri	2.4	–	–□–
Trichomanes melanotrichum	1.77	78	Beckett (1997)
Mosses			
Dumortiera hirsuta	0.38	85	Proctor et al. (1998)
Marchantia polymorpha	0.38	60	–□–
Conocephalum conicum	0.54	45	–□–
Dumortiera hirsuta	0.59	94	Beckett (1997)
Hookeria lucens	0.95	70	Proctor et al. (1998)
Sphagnum girgensohnii	0.98	36	Hájek & Beckett (2008)
Atrichum androgynum	1.05	77	–□–
Sphagnum cuspidatum	1.08	62	–□–
Sphagnum tenellum	1.09	41	–□–
Sphagnum magellanicum	1.12	61	–□–
Mnium hornum	1.21	70	Proctor et al. (1998)
Neckera crispa	1.27	65	–□–
Sphagnum fuscum	1.31	61	Hájek & Beckett (2008)
Rhytidiadelphus loreus	1.34	70	Proctor et al. (1998)
Tortula ruralis	1.36	75	–□–
Antitrichia curtipendula	1.47	65	–□–
Andreaea alpina	1.59	70	–□–
Anomodon viticulosus	1.65	65	–□–
Frullania tamarisci	1.78	60	–□–
Porella capensis	1.89	71	Beckett (1997)
Homalothecium lutescens	2.08	70	Proctor et al. (1998)
Polytrichum commune	2.09	75	–□–
Plagiomnium rhynchophorum	2.37	55	Beckett (1997)
Lichens			
Peltigera leucophlebia	0.62	70	Nardini et al. (2013)

Species name	Ψ_{osat} (-MPa)	RWC_{TVP} (%)	References
Peltigera canina	1.00	59	Beckett (1995)
Lobaria scrobiculata	1.43	54	–◻–
Cladonia convoluta	1.52	60	Proctor et al. (1998)
Peltigera rufescens	1.55	60	Nardini et al. (2013)
Sticta limbata	1.61	51	Beckett (1995)
Ramalina celastri	1.95	44	–◻–
Roccella hypomecha	2.19	42	Beckett (1997)
Usnea undulata	2.19	46	Beckett (1995)
Pseudocyphellaria aurata	2.23	49	–◻–
Parmotrema tinctorum	2.52	47	–◻–
Roccella montagnei	2.79	43	–◻–

Table 2

Salt solutions and silica gel used to generate different relative air humidity levels (RH) in the test chamber, slightly different values were measured after equlibartion of the test organisms *Klebsormidium crenulatum* and *Zygnema* sp.

Material	Quantity (g/100ml)	RH (%) *K. crenulatum*	RH (%) *Zygnema* sp.
Lithium chloride (LiCl)	4.8	95.4	95.8
	9.4	89.5	91.7
	13.0	85.3	85.3
	17.1	78.3	78.4
	24.1	65.5	66.4
	30.0	56.9	56.4
	43.5	31.8	32.3
	64.1	15.9	15.6
Potassium carbonate (K_2CO_3)	112	48.1	49.5
Silica gel	–	3.7	4.2