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AUTHOR: Christina Fuller

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Dr. Robert Sheath  
THESIS COMMITTEE CHAIR  
SIGNATURE:  
DATE: 12/6/13

Dr. Betsy Read  
THESIS COMMITTEE MEMBER  
SIGNATURE:  
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Dr. William Kristan  
THESIS COMMITTEE MEMBER  
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Dr. Betty Fetscher  
THESIS COMMITTEE MEMBER  
SIGNATURE:  
DATE: 12/9/13
Examining morphological and physiological changes in *Zygnema irregulare* during a desiccation and recovery period

Christina L. Fuller

Master’s Thesis

Department of Biological Sciences

Dr. Robert Sheath, advisor

California State University San Marcos
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Abstract

The Mediterranean climate in southern California results in the existence of temporary streams which present challenges to fresh-water algae since they must persist through long, dry summers to regrow once water returns. This study focused on a green-algal species, *Zygnema irregulare*, in an attempt to better understand the methods by which it tolerates desiccation and recovers once conditions are favorable. *Z. irregulare* was desiccated for two-months and then provided with full moisture for another month under laboratory conditions to observe any morphological and physiological changes. By the end of desiccation, there was a significant increase in the proportions of pre-akinetes and akinetes cells, 56% and 19% of the filaments respectively. Increases in the thickness of cell wall and pectin layers were also observed, with a 220% increase in the secondary cell wall and an increase of 470% for the secondary pectin layer. The decreases in the proportions of pre-akinetes and akinetes and the thickness of the cell wall and pectin layers after moisture was reintroduced suggests this change is triggered in part by desiccation. During desiccation, an increase of 34% in moisture content within *Z. irregulare* filaments was observed, supporting the theory that pectin layers trap in moisture to potentially slow desiccation in *Zygnema* species until mature akinetes are formed. Germination of akinetes and recovery of pre-akinetes was observed to occur within 7-14 days. Intracellular modification combined with structural changes which may delay the rate of moisture loss provides *Z. irregulare* with the ability to survive and reproduce after desiccation.

*Keywords*: Desiccation strategies, Recovery from desiccation, *Zygnema irregulare*, Akinete formation
Introduction

Mediterranean climates are generally characterized by their latitude, located between 30º to 40º north or south of the equator, and dominated by small, woody plants with leathery evergreen leaves (Gasith and Resh 1999). The year is broken into two seasons with a long, warm, dry season following a short, cool, wet season in which most of the rain for the year falls in a few major storms. This seasonal pattern of precipitation is predictable, but amounts vary from year to year. It is common for average rainfall levels to vary 30% or more, ranging between 27.5 and 90.0 cm. This situation results in a naturally water-stressed dry period and an environment in which streams may fluctuate between flooding and drying to semi-arid conditions.

Southern California coastal regions, with an annual rainfall between 25 and 65 cm, fall into this category of Mediterranean-type climate (Inman and Jenkins 1999, Lake 2003, Busse et al. 2006). Nearly 90% of this rain falls during a short winter period of several months, (November-March) while summers are long and dry (Lake 2003, Busse et al. 2006). Therefore, temporary streams are common in the region as they would be in other Mediterranean climates. Surface water may be present throughout the year, the stream may dry to form a series of isolated pools or dry to completion (Gasith and Resh 1999). Despite these harsh conditions, algal species are present year after year.

The hardest challenge for the fresh-water algae in such temporary streams is surviving these long, dry periods to inhabit the stream yearly. Dehydration affects the photosynthesis and growth of algal species and may lead to eventual cell death. The most common method to survive desiccation is by producing various resting stages such as zygospores, aplanospores and akinetes to persist through unfavorable conditions (Jane and Woodhead 1941, Evans 1958, Evans
Zygospores are formed sexually and the fertilized zygote is encapsulated in a thickened cell wall along with an accumulation of food reserves and oil (Lee 1999). Aplanospores are formed asexually; the ovoid spore is formed by a contraction of cell contents and enclosed by a cell wall separate from the parental cell. Lastly, the akinete is derived from a vegetative, or actively growing, non-reproductive cell which develops into a spore-like stage with food reserves and is surrounded by a thick, three-layered wall (Lee 1999). Generally akinetes are easy to identify since they differ in size from the rest of the vegetative cells, may vary in color and are granulated in appearance. All three spores have the ability to withstand prolonged desiccation and to germinate directly from the resting cell once conditions become favorable again.

Some species of algae have short life cycles to avoid extreme water stress and produce such resistant cells in just a few days with the ability to survive several years of desiccation (Moreno et al. 2001). Alterations in the vegetative cells themselves have also been observed and provide some resistance to desiccation. These modifications include thicker cell walls (Jane and Woodhead 1941, Morison and Sheath 1983), changes in cell wall composition (Gupta and Agrawal 2006), a reduction in chlorophyll $a$ (chl $a$) activity with increases in carotene production (Kawecka 2003), the production of envelopes of mucilage (Coleman 1983) and the modification of the lipid and protein content in the interior of the cell (Evans 1958, Morison and Sheath 1983).

Desiccation avoidance strategies also exist, such as taking refuge in various regions of a stream ecosystem. Evans (1958) reported that cells might migrate downward into leaf litter layers or soil with a decrease in water levels, but his focus was on diatomaceous species, not soft-bodied algae. Dry biofilms, macrophytes and permanent pools have been identified as
possible refuges from drought for varying algal species (Moreno et al. 2001, Kawecka 2003, Robson and Matthews 2004). Algal mats are particularly interesting avoidance structures in that they afford protection with upper layers exposed to the sun but multiple layers of filaments shielded below. In addition to playing a role in trapping moisture, these upper layers have sometimes been reported to produce UV-protective pigmentation in some green filamentous species and therefore not only shield algal filaments below from over-drying but also from UV damage (Holzinger et al. 2009a).

Whatever strategy is used, recovery to pre-desiccation states once water returns occurs quickly (O’Neal and Lembi 1983, Morison and Sheath 1983, Agrawal 2009). Algal biomass from dried creeks is known to increase significantly in the first few weeks of rewetting (Ledger and Hildrew 2001). Akinetes in the green algal species *Pithophora oedogonia* begin to respond and prepare for growth within a day of moisture reintroduction (O’Neal and Lembi 1983). Germination from the akinete is initially supported via respiration, with germination tubes appearing within 2-6 days and photosynthetic processes drive growth after 1-2 weeks (O’Neal and Lembi 1983). Within about 25 days, most akinetes have finished germinating (Agrawal and Singh 2000, Agrawal and Misra 2002). This kind of quick recovery upon rehydration occurs not only with *P. oedogonia* but with several other algal species. After a month of drying, several desert algal species, green and blue-green, begin photosynthesizing within an hour of rehydration (Gray et al. 2007). Studies with the green algal species *Cladophora glomerata* and *Rhizoclonium hieroglyphicum* report germination of zoospores at 15 and 5 days after rehydration, respectively (Agrawal and Misra 2002).
While some studies have examined various species and strategies used to tolerate desiccation, few studies have focused on species located in the Southern California region. *Zygnema* species are widespread, having been collected from every continent, and are often found in temporary freshwater areas, such as in ditches or ephemeral ponds (Transeau 1951). Therefore, it was expected that at least one species of *Zygema* would be present in California’s short-lived streams. The charophyta species *Z. irregulare* is of particular interest because it has been observed in several temporary streams in the region, and is often one of the dominant species. Its reoccurrence year after year confirms that whatever strategies the alga is using to persist through the long, harsh conditions with little to no water are effective.

*Z. irregulare* is not known to reproduce sexually (Stancheva et al. 2012) but rather it produces akinetes, which are non-flagellate “resting cells” (Graham and Wilcox 2000). Its presence each year is most likely due to the presence of these cells. Transeau (1951) and others refer to akinetes as the simplest, most primitive propagating cell. Due to this, akinetes are one of the most common types of resting cells that freshwater algal species use to survive desiccation and other unfavorable conditions (Transeau 1951, Evans 1958, Evans 1959, Coleman 1983, Agrawal and Misra 2002). As stated earlier, these thick-walled cells are derived asexually from vegetative (non-reproductive) cells on the algal filament, undergoing a variety of changes to become more tolerant to the difficult extracellular conditions over time (McLean and Pessoney 1971). The appearances of akinetes in *Z. irregulare* are distinctly different from vegetative cells. They tend to be slightly smaller (3-7µm in diameter), are brown-gold in color and have thick, lamellate cell walls. They are irregularly granulated with small pits on the mesospore layer and
contain the remnants of two green chloroplasts that are barely visible in the cytoplasm (Stancheva et al. 2012).

In terms of its recovery after drying, there have been accounts of pools of water dried for several years supporting high *Zygnema* growth in a short span of weeks after water is reintroduced (Transeau 1951, McLean and Pessoney 1971). Moreover the quick recovering species Transeau described was *Zygnema sterile*, which is known to only reproduce by akinete, similar to *Z. irregulare*. Although akinete formation in *Zygnema* species has been studied previously (McLean and Pessoney 1971), the germination of akinetes and the recovery of modified cells have only briefly been examined in green algal species.

*On akinetes*

Akinetes have the ability to directly germinate into new plants once conditions are favorable (Smith 1950) and it has been suggested that for *Zygnema*, survival is increased when akinetes are produced as opposed to zygospores (Coleman 1983). Akinetes withstand unfavorable conditions by reducing photosynthesis and respiration to a level that is below detection, inducing a “resting state,” and forming protective structures (Coleman 1983, Graham and Wilcox 2000).

The most informative observations of *Zygnema* akinete formation were made by McLean and Pessoney (1971). At 4 weeks, cell wall thickness, pectic layer thickness, pyrenoid visibility and chloroplast organization are already morphologically different from healthy vegetative cells. Bulges also begin to appear at transverse walls with the increased thickness of the secondary wall. At 5 weeks, the thick sheath begins to separate from around the akinete, releasing mature
akinetes into the surrounding environment roughly one week later. Enough changes had been made in a little over a month for vegetative cells to become functional resting cells.

For some species of *Zygnema*, the structure and ornamentation of an akinete is hypothesized to be similar to that of their sexual spores (Stancheva et al. 2012). Since *Zygnema* zygospores (sexually produced spores) are tri-layered with the mesospore, or median layer, being colored and ornamented, it is presumed to be similar in *Zygnema* akinetes (Smith 1950, Stancheva et al. 2012). Several authors have suggested the presence of sporopollenin-like or phenolic-like compounds in green algal spores, such as in *Spirogyra* (Hull et al. 1985, Delwiche et al. 1989, VanWinkle-Swift and Rickoll 1997). In *Spirogyra* zygospores, the mesospore layer is known to contain sporopollenin (Hull et al. 1985, Lee 1999). The other two layers, the exospore, or outside layer, and endospore, or inside layer, contain cellulose and pectin (Lee 1999). Autofluorescence at 395nm is indicative of sporopollenin-like compounds, and has been recorded in several charophyte species together with resistance to acetyolic-procedures, which are often used to clear everything but sporopollenin-containing compounds in spores (Kroken et al. 1996). Furthermore, HPLC analysis has been used to show that an arctic *Zygnema* strain has protective phenolic compounds (Pichrtova et al. 2012).

Reports of akinete recovery are often limited to the observations that old, air-dried akinetes are still viable or focus on cyanobacterial and other green algal species. In *Pithophora oedogonia*, akinete germination begins with an increase in respiration, the protrusion and extension of a germination tube, a decrease in respiration coupled to increased photosynthesis and finally declining starch and lipid levels as they are used to support initial growth (O’Neal and Lembi 1983). *Klebsormidium rivulare* akinetes germinate similarly; once rehydrated they
swell initially, vacuoles begin to appear, and lipid and starch reserves begin to disappear to aid initial filament growth (Morison and Sheath 1983). Eventually the chloroplast recovers to its original shape and the cells return to the vegetative form. A few observations have been done on the germination process in *Zygnema* species. Akinetes germinate in a manner similar to that of the *Zygnema* zygospore, whereby the two outer layers rupture at a suture and the protoplast escapes with the inner-most zoospore layer, the endospore, as its new cell wall (Smith 1950, Ogawa 1982). In akinetes, it is also the opening of the suture in the thick cell wall that is usually a first step towards germination which finishes with the germling fully emerging from the akinete (Agrawal 2009). These sutures are not only important for cell germination but are often times unique characteristics for species identification. Stancheva (2012) recorded the presence of an irregular or oblique-like suture in *Z. subcylindricum* which opens to release the first germinating cell, a process similar to the germination of zygospores.

The objective of this study was to examine morphological and life-stage changes over a two-month desiccation period and a one-month recovery period in *Z. irregulare*. More specifically, the goal was to examine: 1) the differences between filaments undergoing desiccation stress and filaments under normal growth conditions, 2) drying survival strategies, and 3) recovery in liquid media.

During desiccation, it was hypothesized that *Z. irregulare* filaments would have higher percentages of akinete and pre-akinetes cells per filament and lower percentages of vegetative cells as compared to well-watered controls. The inverse was expected for the period of recovery,
whereby akinete and pre-akinetes cell percentages would decrease and vegetative cell percentages would increase.

Cell wall layers and sheath (pectin) layers were hypothesized to increase in thickness as desiccation time progressed and decrease with recovery time progression. The increase in cell wall layers and pectin layers was also expected to cause an increase in dry weight, since carbohydrates would be produced to build up the cell wall (Morison and Sheath 1983). Therefore, it was hypothesized that the experimental, desiccated group would have lower relative fresh:dry weight ratios at the end of the two-month drying period than the control, with the inverse expected by the end of the recovery. Chl $a$ levels were hypothesized to decrease with increased desiccation time as an increasing number of cells began to differentiate into akinetes.

Concerning germination, Morison and Sheath (1983) noted an obvious disappearance of the starch and lipid bodies during the first 10 days of akinete rehydration, probably due to their breakdown and use to restore respiration and growth. A similar outcome was expected for this study. Akinete and pre-akinete cells were expected to initially swell when moisture was reintroduced, before undergoing reversing changes to regain the pre-desiccation state.

This is the first study to examine a *Zygnema* species from a site in the Mediterranean climate of southern California and on strategies used by *Zygnema irregulare* to tolerate desiccation. In addition, although thick pectic layers are known to occur in *Zygnema* species, this study would be one of the first to quantify said layers.
Materials and Methods

Experimental design

A two-month desiccation period was chosen since it has been reported that mature akinetes only begin to separate from the mother filament at approximately the sixth week (McLean and Pessoney 1971). Eight weeks was thought to provide ample time for any lagging filaments to complete the akinete formation process. One-month recovery was selected because several articles reported that akinete germination may fall within a week to one month after water reintroduction (O’Neal and Lembi 1983, Morison and Sheath 1983, Agrawal and Misra 2002). Therefore, filaments were sampled the first seven days of recovery for examination.

Two treatment groups, an experimental, desiccating group grown on BBM agar plates, and a control group in liquid BBM in Erlenmeyer flasks were used for this study. Triplicate samples for each analysis were collected on 14 sampling days. During desiccation, samples were taken at 0, 2, 4, 6 and 8 weeks (0, 14, 28, 42, 56 days). The 8 week desiccation mark also coincided with the first day liquid BBM was reintroduced, which begins the recovery period. During recovery, samples were taken the first 7 days and at 2 and 4 weeks (days 56-63, 70 and 84).

Fig 1. Visual overview of sampling dates. Pink denotes the desiccation period and blue the recovery. Filaments were sampled the first seven days of recovery since it
has been recorded for another green algal species, *P. oedogonia* (O’Neal and Lembi 1983) and seen by personal observation in *Z. irregulare* that germination may occur within the first week.

**Species under study**

*Zygnema irregulare* H. Krieg in the active growth phase has a diameter of 36-40µm, reproduction is done via akinetes and conjugation is unknown. Mature akinetes are 33-40 x 20-54 µm, with thick, brown walls, irregularly spaced granules and pits 3-7 µm in diameter on the mesospore. The strain used in this study (RS012) was collected from Indian Creek in the Tijuana River watershed in San Diego County (32°90’ N, 116°49’ W) (Stancheva et al. 2012). Wet material of the species had been collected during a single collection event from a single reach. A filament from a culture grown from this field collection was mechanically isolated and recultured. This was grown for several months in BBM to provide enough biomass of the clonal culture before use in this study.

In order to enumerate cell types to document the timing of akinete formation, three different cell types, vegetative, pre-akinete and akinete, were used because they represented benchmark stages in the vegetative cell to mature akinete differentiation process. Dead cells were also counted since they would be encountered, although there were no specific hypotheses made with regards to this cell type. Their names and descriptions are based on the observations of McLean and Pessoney (1971), as well as from personal observation in preliminary research.

Characteristics of vegetative cells (Fig 2) include two stellate chloroplasts with projections radiating outwards (C), a visible nucleus (N) seen between the chloroplasts, a non-
extensive sheath (Sh), vacuolated (vacuolated area denoted by V), little evidence of lipid bodies, visible pyrenoids (P), and a cell wall (CW) with no significant thickening. The chloroplasts are grass-green in color.

Fig 2. Light micrograph of vegetative cells of *Z. irregulare* showing vacuoles (V), cell wall (CW), sheath (Sh), nucleus (N), chloroplasts (C) with arm extensions and pyrenoids (P).

Pre-akinetes (Fig 3) have thickened cell walls (CW), sometimes with bulges appearing at lateral walls, thicker sheath layers (Sh), condensed chloroplasts (C) in which chloroplast arms are no longer visible, non-visible pyrenoids, a decrease in the visibility of the nucleus (N) due to the accumulation of starch and lipid bodies, less vacuolization and are beginning to acquire the brownish-yellow color of mature akinetes.
Fig 3. Light micrograph of pre- akinetes cells of _Z. irregulare_ showing thickening cell wall (CW) and sheath layers (Sh), condensed chloroplast (C) and location of a barely visible nucleus (N).

In mature akinetes (Fig 4), starch is no longer confined around the pyrenoids, having dispersed throughout the cell along with lipid bodies, contents appear homogenous and granular, remnant chloroplasts (C) are visible, and the akinete wall is lamellate, thickened, and brown in color. The mature akinete lacks the thick sheath layer of the pre- akinetes and its width is slightly smaller than that of vegetative cells.
Fig 4. Light micrograph of a mature akinete of *Z. irregulare* before separation from the parental filament. Notice the outer cell wall (CW) still keeping the akinete attached but the lack of a sheath (Sh) layer around the akinete. Remnant chloroplasts (C) are still visible inside the akinete walls (AW).

Dead cells are cells whose contents have become colorless and are disintegrating but still have intact cell walls and pectin layers. Preliminary studies had determined that only pre- akinetes which maintained a golden-brown, granulated protoplast which lies closely to the secondary cell wall would be able to recover. For akinetes, cells which maintained a cylindrical appearance and non-collapsed, concentric lamellations of cell wall with the remnants of chloroplasts still visible would also recover. Any differentiated cells, pre- akinete or akinete, which had plasmolyzed or were red-ochre in color with a large mass of conjoined lipid globules were considered terminated cells. Reintroduced to water, these cells never germinated and
therefore were not used for the above study although they are sometimes mistaken for resting cells.

**Desiccation experiment**

To test the effects of desiccation, the experimental group and control group were grown under similar conditions, except for moisture levels. Both were grown in a 12h:12h light-dark cycle, an average lighting of 97.5 µmol s⁻¹ m⁻² (with a range of 73.9 µmol s⁻¹ m⁻² to 128.8 µmol s⁻¹ m⁻²), and temperature set at 15°C in a Biochemical Oxygen Demand (BOD) low temperature incubator (Model L115, Cornelius, OH, USA). Light intensity was measured using a Li-Cor Photometer (Model LI-189, Lincoln, NE, USA) sensor. The media used was BBM (Stein 1973), either 50mL liquid in 250mL flasks for the control group or 20mL BBM mixed with 1.5% agar in 100x15mm petri dishes for the experimental group. The experimental group was sprayed every 3 days with 0.1mL BBM so that the plates dried slowly. Control flasks were recultured monthly to avoid morphological changes due to a lack of nutrients (Andersen 2005). There were 18 flasks for the control group and 18 plates for the experimental to be able to provide enough biomass for the duration of the study.

Agar plates with no algae were included to measure agar moisture content. Plates were weighed every sampling date after any excess water was shaken from the lid of the plate, usually minimal, and water loss was calculated as a percentage of the initial plate weight.

On each sampling date, algal filaments were collected from the control flasks and experimental plates. After the samples of each treatment group were pooled and then separated into triplicates for analyses, clinging agar and bacterial pieces were manually removed.
Triplicates for chlorophyll \( a \) analysis were blotted 5x on a paper towel, weighed, and then stored frozen at -20ºC in 1.5mL eppendorf tubes until needed, up to one month. Chlorophyll extractions were performed with slight modifications to the methods of Hellebust and Craigie (1978, Chapter 6). The frozen samples were ground to a fine powder in a mortar with liquid nitrogen. 10mL of 90% spectrophotometric-grade acetone was added and the sample ground again briefly before centrifuging 5 min at 4ºC at 2500 rpm. The supernatant was extracted, serial dilutions were made and fluorescence measured using a Turner Designs Trilogy fluorometer (Model 7200-000, Sunnyvale, CA). Chlorophyll \( a \) standards were purchased from Fisher Scientific. The high chl \( a \) standard, 185µg/mL, was serially diluted and raw fluorescence measurements were plotted against known concentrations to create a standard curve. The chlorophyll fluorescence values of the samples were calculated using the previously generated standard curve. Chlorophyll \( a \) concentration per mg sample was then estimated with the following (Sterman NT, 1988): 1) chlorophyll fluorescence value = (fluorescence value of pigment – fluorescence value of blank), 2) \( \mu g \) chl \( a \) = (chlorophyll fluorescence value) x (reciprocal of the dilution factor) and 3) \( \mu g \) chl \( a \) mg\(^{-1}\) = (\( \mu g \) chl \( a \))/(dry weight in mg of sample).

The type of cell (vegetative, pre-akinete, mature akinete or dead) of 10 non-adjacent cells on 30 haphazardly encountered filaments was recorded and the average percentages of each cell type were calculated for each triplicate. The same number of cells were also photographed using an Olympus MicroFire S99809 digital camera (Olympus Imaging America, Center Valley, PA, USA) and the width of the cell walls (the cellulosic portions that stained with Calcofluor White) and surrounding pectin layers (the portions that stained with Ruthenium Red) were measured using the SPOT basic 4.7 imaging software (Diagnostic Instruments, Inc, Sterling Heights, MI).
Two cell wall layers were measured in this study; that which surrounded the individual cell and that which encompassed the entirety of the *Z. irregulare* filament. The pectin and cell wall layer measurements were done on vegetative cells and pre-akinetecells only, because during preliminary studies, mature akinetes were observed to be too opaque to be able to distinguish where the cell wall begins and ends. Additionally, mature akinetes do not have a pectin layer surrounding the cell. Cell wall and pectic layer thickness would be most important in vegetative cells, considering that it is hypothesized to be one of the characteristic changes that allow the cells to tolerate desiccation. The samples were then stored in 2.5% glutaraldehyde at 4°C for later reference.

Relative fresh:dry weight in the filaments was calculated by blotting the samples 5x on a paper towel, weighing them, drying them at 65°C for 24 h in a L-C Labline drying oven (model 3510, Melrose Park, IL, USA) and then reweighing to obtain the fresh:dry ratio. Moisture content of the filaments was calculated by subtracting the fresh weight of each sample by their dried weight and converting it to a percentage of the original fresh weight.

Histochemical staining was also done to visually gauge lipid and protein content, as well as to distinguish cellulose-containing cell walls and pectic layers. All of the histochemical stains were obtained from Fisher Scientific, USA. Staining for lipids using Sudan Black B was done according to Ruzin (1999). Several drops of 50% EtOH were dropped onto algal filaments on a slide, stained in a saturated and filtered solution of Sudan Black B in 70% EtOH for 3 minutes and then differentiated in 50% EtOH for a minute before viewing under regular bright field. Sudan black B stains all lipids blue-black, including phospholipids (Jensen 1962). Proteins were stained according to the protocols by Gantt (1980) in which a drop of 0.1% solution of Fast
Green (aq) adjusted with 1M HCl to pH 2.0 was placed on algal filaments on a slide, heated gently on a hot plate until the edges started to appear metallic, rinsed and then viewed. Cellulose (β-linked glucans) was stained with Calcofluor White M2R stain in which the filaments were placed on a clean slide with a drop of stain and a drop of 10% KOH, covered with a coverslip and viewed under UV light after one minute. Calcofluor White binds to cellulose and chitin, fluorescing blue-white (Ruzin 1999). Pectin layers were stained with a drop of 0.005% Ruthenium Red (aq) over filaments on a slide. Pectic substances (specifically acidic polysaccharides) stained pink to red (Ruzin 1999).

Fig 5. Light micrograph of a desiccated filament of *Z. irregulare* (A) and a fluorescent microscope image (B) showing the primary cell wall (CW1) and the secondary cell wall (CW2) layers after staining with Calcofluor White M2R.
Fig 6. Light micrograph of a desiccated filament of *Z. irregulare* showing the primary pectic (P1) and secondary pectic layers (P2).

**Recovery experiment**

Recovery growth conditions were identical to conditions used for the desiccation experiment except that liquid BBM was added to each experimental group plate and a pool maintained throughout the one-month recovery. Subsamples were collected and analyzed in the same manner as described in the desiccation experiment section except for chlorophyll *a* analysis, which was not done for the recovery portion of this study.

Mature akinetes were further examined for the presence of phenolic-like, or sporopollenin-like compounds. Filaments with mature akinetes were treated with 3% HCl (1h, 90°C), 2% KOH (20 min, 80°C) and 65% H₂SO₄ (5-7 min, room temp). This method is used to clear away the exospore (outer) layer in Zygematales zygospores and leave the layer underneath for viewing (Hull et al. 1985). It is similar to acetolysis in that it may clear a pollen grain surface,
which contains sporopollenin, (Hesse and Waha 1989) but is gentler as not all pollen grain structure is destroyed in the process (Hull et al. 1985). Even sporopollenin pre-cursors may be destroyed by acetolysis.

After treatment, the wall remainder was examined for autofluorescence at 395nm according to Kroken et al. (2012). To determine if there is any cellulosic content in the remaining layer, it was also stained with Calcofluor White.

Statistics

Statistical analyses were performed using Minitab 16 (Version 16.2.4, Minitab Inc., State College PA, USA) and R (Version 2.15.1, The R Foundation for statistical computing). All graphs were generated using Microsoft Excel 2007 (Microsoft Corporation, Redmond, WA, USA). Standard error, or the variability of sample estimates, was used for all of the graphs. Duplicate plate weights and fresh:dry weight ratios were analyzed in Minitab using Generalized Linear Models, having met all homogeneity of variance and normality assumptions. Post-hoc 2-sample t-tests were conducted in Minitab to find the specific days of significance. Cell type composition was statistically analyzed using loglinear analyses in R since the proportions lacked normal distribution. For loglinear analyses, models were fitted to the observed frequencies of cell counts according to a cross-tabulation of date sampled (day), treatment and cell type. The idea was to design a model to best fit the observed data by either omitting or adding terms. The goodness-of-fit of a model was assessed by comparing the expected frequencies of the model to the observed frequencies from the study and the least complicated model that fits the data well is sought. Significance was assessed using likelihood ratio tests in which the saturated model and a model without the three-way interaction between day, treatment and cell type were compared to
calculate a p-value. If a significant lack of fit is observed when a term is dropped, it indicates that the term is needed and is thus significant. Post-hoc analyses were conducted with the same analytic procedure, comparing each day to the initial day of the desiccation and recovery periods respectively.

The remaining analyses were done using a Generalized Linear Model and a permutation test (lmPerm package in R, Wheeler 2010), since cell walls and pectic layers did not have normal distribution even with log-transformation. Post-hoc tests for cell walls and pectin layers were done using ANOVA, since statistical power was lacking for effective post-hoc permutation tests. Spearman’s rank-order correlations were used for trend analyses. A Bonferroni-adjusted alpha of 0.01 was used for the desiccation period and 0.0056 for the recovery period. Bonferonni correction is used to correct for the increased possibility of showing significance when there is none (a type 1 error) while examining multiple hypothesis in a test, such as when conducting t-tests on each of the individual days during the desiccation and recovery periods.

**Results**

*Desiccation period*

By the end of the desiccation period, average duplicate plate weight in the experimental group had dropped from 91.4g to 77.4g, a decrease of 15% in moisture content. This represents a significant decrease when compared to the control plates ($F_{(4,20)}=8.536$, $p<0.05$) (Fig. 7). This difference had already become significant between the two groups by the 4th week ($t_{(4)}=4.99$, $p<0.05$) with a 7.8% decrease in average experimental plate weight (from 91.4g-84.3g) was observed.
In response to desiccation in the agar environment, relative fresh:dry weight ratios in the experimental group increased 57%, from a ratio of 1.1 to 1.7 by the end of the desiccation period \( (F(4,20)=59.98, p<0.001) \) (Fig. 8). Significant differences were apparent by the 2\textsuperscript{nd} week at which time there was an increase of 7.3%, with a ratio increase from 1.1 to 1.2. This is two weeks earlier prior to when the moisture loss in the petri dish environments had become statistically significant \( (t(4)=17.32, p<0.001) \).

Relative moisture content of the filaments in each treatment group was also calculated throughout the desiccation period (Fig. 9). By the end of the desiccation period, a 34% increase in moisture content was recorded for filaments in the experimental group (an increase from 9.6%-44%) \( (F(4,20)=263.9, p<0.001) \). Similar to the relative fresh:dry weight ratios, significant water content differences were noted by the 2\textsuperscript{nd} week \( (t(4)=16.48, p<0.001) \), with a 6.1% increase in moisture content, from the initial 9.6% to 16% in the experimental filaments.

The morphology of the control filaments remained unchanged throughout the desiccation period with cell type percentages ranging between 96-99% for vegetative cells, 0-0.9% for pre-akinetes, 0% for akinetes and 0.9%-4.0% for dead cells (Fig. 10). Desiccation did affect the cell type composition in the experimental filaments \( (\chi^2_{(12)}=282.7, p<0.05) \). By the end of the desiccation period, the experimental group contained 87% fewer vegetative cells (from an initial 96% to 8.9%), 56% more pre-akinetes (from an initial 0% in filaments to 56%), 19% more akinetes (from an initial 0% to 19%), and 11% more dead cells (from an initial 4.0%-15%), (Fig. 11). The shift in cell type proportions became significantly different \( (\chi^2_{(3)}=151.8, p<0.05) \) at week 4 when there were 76% fewer vegetative cells (an initial 96% in filaments to 20%) and 55% more pre-akinetes (from 0%-55%) in the experimental group. Dead cells were 19% higher (from
4.0%-23%), but this percentage fluctuated; 9.4% by the 6\textsuperscript{th} week and then the 11% reported by the end of desiccation. Mature akinetes were recorded for the first time at 6 weeks, with 15% (from an initial 0%) more akinetes present in experimental group filaments.

Experimental group filament morphology also changed during desiccation. Cell walls thickened throughout the desiccation period as follows: primary cell walls significantly thickened by 66% (from an average of 0.6µm to 1.0µm) (df=4,20, Iter=5000, p<0.001) (Fig. 12) and secondary cell walls by 220% (from an average of 0.5µm to 1.7µm) in the experimental filaments (df=4,20, Iter=5000, p<0.001) (Fig. 13). Pectin layers also thickened throughout the desiccation period: primary pectin layers significantly thickened by 96% (from an average of 0.7µm to 1.4µm) (df=4,20, Iter=5000, p<0.001) (Fig. 14) while secondary pectin layers significantly thickened by 470% (from an average of 1.2µm to 7.0µm) (df=4,20, Iter=5000, p<0.001) (Fig. 15).

The primary cell wall ($F_{(1,4)}=40.18$, $p<0.05$), primary pectin layer ($F_{(1,4)}=33.94$, $p<0.05$) and secondary pectin layer ($F_{(1,4)}=168.9$, $p<0.001$) of the experimental group were significantly thicker at the 4\textsuperscript{th} week of desiccation, a 63% (from 0.6µm-1.0µm), 60% (from 0.7µm-1.2µm) and 290% (from 1.2µm-4.8µm) increase in thickness respectively. However, the secondary cell wall was already significantly thicker by the 2\textsuperscript{nd} week with a 120% increase (from 0.5µm-1.2µm) ($F_{(1,4)}=24.15$, $p<0.05$). Increases in the percentage of pre-akinetes positively correlated with the increases in thickness of the primary cell wall ($r_s(5)=1$, $p<0.001$), the secondary cell wall ($r_s(5)=0.7$, $p=0.188$), the primary pectin layer ($r_s(5)=0.9$, $p<0.05$) and the secondary pectin layer ($r_s(5)=0.9$, $p<0.05$) using a Spearman rank correlation. Visually, cells that had not differentiated
into akinetes appeared denser with granulated cell contents now hiding the previously visible nucleus, condensed chloroplasts, and brownish-yellow coloration of pre-akinetes (Fig. 3).

All of these morphological changes in the experimental filaments were coupled with a significant decrease in chl $a$ concentration, which had decreased by 78% (from 232 chl $a$ $\mu$g mg$^{-1}$ to 47.1 chl $a$ $\mu$g mg$^{-1}$) as compared to the controls at the 8th week, (df=4, 16, Iter=5000, p<0.001) (Fig. 16). To note, there was an initial increase in chl $a$ concentration at the 2nd week for the experimental group but was calculated to be statistically insignificant. Filaments at this time were growing to spread out on the plate and once established, the concentration level fell as expected and was significantly less in the experimental group than in the controls by the last day of the desiccation period ($F_{(1,2)}=403.7$, p<0.05). Even though variations existed within the control group from date to date, there was no significant difference in chl $a$ concentration per mg algal weight (df=4, 6, Iter=2886, p=0.0683).
Fig. 7. Duplicate plate weight during desiccation. Shown as a percentage of the initial plate weight. Bars represent \( \pm 1 \) SE about the mean. Exp = Experimental group.
Fig 8. Relative fresh:dry weight ratio of filaments during desiccation. Bars represent ±1 SE about the mean. Exp = Experimental group.
Fig. 9. Relative percentages of moisture content from filaments during desiccation. Bars represent ±1 SE about the mean. Exp = Experimental group.
Fig. 10. Proportion of cell types in control group during desiccation. Bars represent ±1 SE about the mean.

Exp = Experimental group.
Fig. 11. Proportion of cell types in experimental group during desiccation. Bars represent ±1 SE about the mean.

Exp = Experimental group.
Fig. 12. Primary cell wall width during desiccation. Bars represent ±1 SE about the mean. Exp = Experimental group. Thickness of the first cell wall layer in experimental group filaments ranged from 0.2-2.4μm.
Fig. 13. Secondary cell wall width during desiccation. Bars represent ±1 SE about the mean. Exp = Experimental group. Thickness of the second cell wall layer in experimental group filaments ranged from 0.1-2.8µm.
Fig. 14. Primary pectin layer width during desiccation. Bars represent ±1 SE about the mean. Exp = Experimental group. Thickness of the first pectin layer in experimental group filaments ranged from 0.3-3.0µm.
Fig. 15. Secondary pectin layer width during desiccation. Bars represent ±1 SE about the mean. Exp = Experimental group. Thickness of the second pectin layer in experimental group filaments ranged from 0.1-31.2µm.
Recovery period

The agar of the duplicate experimental plates readily took up the liquid media that was added during the recovery period to increase 13% in weight (from 77.4g-87.3g by the end of the recovery period) \( (F_{(1,40)}=48.70, \ p<0.001) \) (Fig. 17). Similarity in weight between the controls and experimental group was observed the first day after moisture reintroduction \( (F_{(9,40)}=0.6015, \ p=0.7881) \), after an average weight increase of 8.5% (from 77.4g-84.0g in the experimental plates).
In response to the reintroduction of media, relative fresh:dry weight ratios in the experimental group filaments significantly decreased by 31% (from a ratio of 1.7 to 1.2) by the end of the recovery period ($F_{(9,38)}=96.91$, $p<0.001$) (Fig. 18). Similarity in the relative fresh:dry weight ratios was observed in the two treatment groups on the 3rd day ($t_{(4)}=-3.099$, $p=0.0362$). Relative fresh:dry weight was 33% lower (from a ratio of 1.7 to 1.2) in the experimental group compared to the desiccation period. This similarity in weight observed in the experimental and control group continued throughout the rest of the desiccation period, despite small variations. Slight variations also occurred in the control group during the first 7 days, but none were statistically significant.

Relative moisture content in experimental group filaments also significantly decreased by 27% (from 44%-16%) ($F_{(9,36)}=153.8$, $p<0.001$) over the recovery period (Fig. 19). By the 3rd day of recovery, there was no longer any significant difference in moisture content between the two treatment groups with Bonferonni correction ($t_{(4)}=-3.0769$, $p=0.0370$). At this time, the moisture content had already decreased by 30% in the experimental group (from 44% to 14%).

During recovery, in the control group, percentages of vegetative cells throughout the study ranged between 94-99%, pre-akinetes ranged between 0-4.6%, akinetes ranged between 0-0.3% and dead cells ranged between 1.0-5.4% (Fig. 20). In the experimental group, rehydration altered the relative proportions of the different cell types ($\chi^2_{(27)}=602.1$, $p<0.05$). By the end of recovery, the proportion of vegetative cells had increased by 47% (from an initial 8.9% to 56%), pre-akinetes had decreased by 25% (from an initial 56% to 31%), akinetes had decreased by 19% (from an initial 19% to 0.8%) and dead cells had decreased by 11% (from an initial 15% to 4.6%) (Fig. 21). The most dramatic changes in the proportions of vegetative and pre-akinetete cells was
evident at 2 weeks into the recovery period when vegetative cells had increased by 69% (from an initial 8.9% to 78%) and the preakinetes had decreased by 52% (from an initial 56% to 4.2%). However, significant differences between the control and experimental group persisted throughout the recovery period, an indication that the distribution of cell types in the experimental group was unlike that of the control group.

The morphology of the experimental filaments within the first week began to change in the direction of their initial appearance before desiccation. By the end of the recovery period, primary (df=9,40, Iter=5000, p<0.001) (Fig. 22) and secondary cell walls (df=9,40, Iter=5000, p<0.001) (Fig. 23) in the experimental filaments decreased in thickness by 40% (from 1.0µm to 0.6µ) and 69% (from 1.7µm-0.5µm) respectively. Pectin layers also decreased in thickness throughout the recovery period. The primary (df=9,40, Iter=5000, p<0.001) (Fig. 24) and secondary pectin layers (df=9,40, Iter=5000, p<0.001) (Fig. 25) decreased by 49% (from 1.4µm to 0.7µm) and 83% (from 7.0µm-1.2µm) respectively. With the exception of the primary pectin layer, rapid statistically significant changes occurred during the first three days of recovery. By the 3rd day, primary walls in the experimental group had decreased by 21% (from 1.0µm-0.8µm) in thickness (F(1,3)=22.04, p=0.0093), secondary cell walls had decreased by 55% (from 1.7µm-0.8µm) (F(1,4)=18.98, p<0.05) and secondary pectin walls had decreased by 48% (from 7.0µm-3.6µm) (F(1,4)=15.38, p=0.0172). For the primary pectin layer, statistical difference was observed up until the 5th day after there was an average decrease in thickness by 48% (from 1.4µm to 0.8µm) (F(1,4)=13.08, p=0.2242). Decreases in pre-akinete percentages positively correlated with decreases in the thickness of the primary cell wall (r_s(10)=0.806, p<0.001), secondary cell wall
(rs(10)=0.806, p<0.05), primary pectin layer (rs(10)=0.903, p<0.001) and secondary pectin layer (rs(10)=0.915, p<0.001) using a Spearman’s rank correlation.

![Graph showing duplicate plate weight during recovery.](image)

**Fig. 17.** Duplicate plate weight during recovery. Shown as a percentage of the initial plate weight. Bars represent ±1 SE about the mean.
Fig. 18. Relative fresh:dry weight ratio of filaments during recovery. Bars represent \( \pm 1 \) SE about the mean.

Exp = Experimental group.
Fig. 19. Relative percentage of moisture content in filaments during recovery. Bars represent ±1 SE about the mean. Exp = Experimental group.
Fig. 20. Proportion of cell types in control group during recovery. Bars represent ±1 SE about the mean. Exp = Experimental group.
Fig. 21. Proportion of cell types in experimental group during recovery. Bars represent ±1 SE about the mean. Exp = Experimental group.
Fig. 22. Primary cell wall width during recovery. Bars represent ±1 SE about the mean. Exp = Experimental group.

Thickness of the primary cell wall layer in experimental group filaments ranged from 0.4-1.5µm.
Fig. 23. Secondary cell wall width during recovery. Bars represent ±1 SE about the mean. Exp = Experimental group. Thickness of the secondary cell wall layer in experimental group filaments ranged from 0.1-1.6µm.
Fig. 24. Primary pectin layer width during recovery. Bars represent ±1 SE about the mean. Exp = Experimental group. Thickness of the primary pectin layer in experimental group filaments ranged from 0.2-2.1µm.
Fig. 25. Secondary pectin layer width during recovery. Bars represent ±1 SE about the mean. Exp = Experimental group. Thickness of the secondary pectin layer in experimental group filaments ranged from 0.1-3.0µm.

**Histochemical staining**

Staining was used to gain a better understanding of lipid, protein, cellulose and pectic content in the different cell types which were observed during desiccation. Calcofluor White was used to visualize presence or absence of cellulose in the filaments. In vegetative cells (Fig. 5), the walls are comprised of two cellulosic layers, while in mature akinetes, staining only revealed one outer layer of cellulose which could be the exospore wall (Fig 26e-f). The akinete mesospore wall itself had no reaction to Calcofluor White (Fig. 26a-b), even when cut to expose it completely in case dye would not penetrate the wall (Fig. 26g-h). It was observed, however, that
when the akinete was mechanically broken, positive staining for cellulose was observed for the inner endospore layer of the akinete (Fig. 26c-d).

Mature akinete cells treated with the acid-base-acid treatment did have their cellulosic-containing wall layers removed (Fig. 27a), but autofluorescence in the remaining layer was not visible after treatment. Any attempt at Calcofluor staining was inconclusive. The KOH used with the Calcofluor dye as per protocol often burst these treated akinetes and the dye itself would often coagulate if there was any residual H₂SO₄ on the akinetes.

As expected, lipid contents were observed to increase, at least visually, with desiccation as cells differentiated into pre-akinetes. Some scattered lipid droplets were visible in vegetative cells (Fig. 28a) while in pre-akinetes, lipid droplets comprised a large part of the protoplast (Fig. 28b). Although akinetes are rich in lipids, staining does not appear to penetrate the akinete cell wall since mechanical rupturing of the akinete will result in positive staining of its contents (Fig. 28c).

Fast Green stain was used to localize protein content in the cell. The vegetative cells positively reacted with the Fast Green stain to a large extent with the densest staining occurring around the pyrenoids (Fig. 29a). These pyrenoid regions are no longer the most densely stained objects in the pre-akinete but rather the nucleus, a visual support for the reducing of proteins involved in the photosynthetic processes concentrated in the pyrenoids as the cells differentiate towards a resting state (Fig. 29b). Fast green stain put into prominence lipid globules, visible at the periphery of pre-akinete cells. No positive reaction for proteins was observed for the pre-akinete wall (Fig. 29d) or akinete mesospore (Fig. 29c) even when the akinete was mechanically ruptured (Fig. 29e).
The pectic layer underwent the largest morphological change along the vegetative to akinete pathway as shown by Ruthenium Red staining. Vegetative cells originally were enveloped in a thin pectin layer (Fig. 30a) which rapidly thickened in the pre-akinetes (Fig. 30b). A heavily positively stained layer lies against the surface of the filament with a lighter stained region above. As desiccation continued, this sheath thickened and became opaque, the two layers becoming harder to distinguish. Pre-akinetes, when matured fully into akinetes, lose this opaque pectic layer, the layer eventually thinning to nothing to release the mature akinete (Fig. 30c).

Germination and Pre-akinete recovery

To better examine how *Z. irregulare* recovers from this desiccation stress and grow once more, *Z. irregular* filaments from the experimental group were placed in separate petri dishes with BBM to monitor germination. Both processes of germination and recovery of pre-akinetes happened within one to two weeks.

Mature akinetes were observed to have one or two horizontal or slightly oblique sutures in *Z. irregulare* (Fig. 31a-b). These akinetes may either remain in the parental filament or be released into the surrounding environment. Some swelling was seen initially in the akinetes (Fig. 31b) but at two days of water reintroduced, many of the akinetes still remained similar in appearance aside from the convergence of lipid globules often towards the center of the cell (Fig. 31c). Akinetes ready to germinate often shifted to one side of the parental filament if still attached, visible slightly in Fig 31c. At around four days, the new germling bursts open the akinete at the suture (Fig. 31d); several cells in length are seen at approximately day 5 (Fig. 31e) and these cells emerge by around the 6th day (Fig. 31f). A new young filament of up to 10 cells
in length is then clearly visible by the 7th-14th day, sometimes still attached to the akinete by a fine pectic layer (Fig. 31h) or with a small cap at one end (Fig. 31g) from the bursting open of the akinete cell. During the first couple days of germination, the chloroplasts still seem disorganized as they would have been inside the akinete in resting state. Chloroplast projections and the highly vacuolated appearance are usually restored within a week of the germling emerging from the akinete.

The granulated, dense pre-akinetes (Fig. 32a) do not undergo much change by the 2nd day except some filaments which will begin to show a breakdown of the pectin layers (Fig. 32b). The sheath becomes increasingly diffuent and is often gone by day 4-6. By around the 4th day, cells show increasingly more vacuolization (Fig. 32c) and are coming less granulated in appearance. By around day 5 (Fig. 32d), chloroplast reorganization is beginning to show and the central part of the cell is opened by approximately the 7th day (Fig. 32e). Pyrenoids have also become quite visible by this time. Chloroplast arms have begun to form and between 7-14 days the cells have regained their initial appearance.
Fig. 26. Light and fluorescent micrographs of *Z. irregulare* akinetes stained with Calcofluor White. (a-b) Mature akinete with lipid exuding due to mechanical disruption (arrow) does not positively stain with Calcofluor White; (c-d) Endospore contents positively react (arrows); (e-f) Calcofluor staining shows the thinning cell wall around the mature akinete, the akinete exospore; (g-h) Cut akinete, unstained. Scale bars: a-h 50µm
Fig. 27. *Z. irregulare* akinetes post HCl-KOH-H$_2$SO$_4$ treatment. (a) Mesospore layer left after treatment (b) with sometimes visible lipid globules. Scale bars: a-e 50µm
Fig. 28. Light micrographs of *Z. irregulare* stained with Sudan Black B. (a) Vegetative cells with scattered lipid globules (arrows); (b) pre-akinetes and akinetes; (c) Mechanically disrupted akinete, note exuding lipids. Scale bars: a-c 50µm

Fig. 29. Light micrographs of *Z. irregulare* stained with Fast Green. (a) Vegetative cells with defined staining around pyrenoid region (arrow); (b) Pre-akinetes with heaviest staining at the nuclear region and with visible lipid globules (arrows); (c) Akinete and (d) filament cell wall have not stained (e) contents of a mechanically-ruptured akinete will positively stain, note release of lipid globules (arrow).

Scale bars: a-e 50µm
Fig. 30. Light micrographs of *Z. irregulare* stained with Ruthenium Red. (a) Vegetative cells; (b) Pre-akinete cells with thickened pectic sheath layers; (c) Akinete cell with thinning pectin layer. Scale bars: a-e 50 µm
Fig. 31. Light micrographs of germination stages in Z. irregulare. (a) Mature akinete with visible suture (arrow); (b) Two vertical or slightly oblique sutures near transverse walls on mature akinetes (arrows); (c) After 2 days lipid globules converge in akinetes (arrow) (d) 4 days, first cell of germling emerges, akinete opening like an envelope (arrow); (e) 5 days, germling now a couple cells in length, lipid globules sometimes present (arrow); (f) Germling emerging from envelope on day 6; (h) Day seven, young filament still loosely attached by pectic layer (arrow) and (g) sometimes with (possibly) pectic “cap” from emersion from akinete (arrow). Scale bars a-h: 50µm
Fig. 32. Light micrographs of pre-akinete recovery in *Z. irregulare*. (a) Pre-akinete on day 0 of water reintroduction; (b) Day 2, greener in color with some pectin layers becoming more diffusent; (c) Day 4, cells more vacuolated (arrow) and greener (d) Day 5, chloroplast organization is beginning to reform, vacuolization continues (e) Day 7, chloroplast projections (arrow) and pyrenoids (p) are reformed (f) Between days 7-14 cells regain their original vegetative appearance with visible nucleus (arrow) between chloroplasts with pyrenoids (p). Scale bars a-f: 50µm.
Discussion

Southern California, with its seasonal rainfall pattern, presents challenges to many fresh water-inhabiting organisms. Temporary streams are common and algae present in these types of streams must be well adapted to desiccation stress. This study aimed to document several strategies used by one particular species to survive in such stressful conditions.

Despite some limitations, such as the difference of hard and soft media used between the treatment groups and fluctuations in the control group attributable to maintenance reculturing, morphological and physiological changes were evident. Bacteria present in either treatment group were minimal until the last week of the recovery period and therefore should not have confounded the findings seen here. However, the observations here were done using a clonal representative of *Z. irregulare* and future studies using filaments from other stream reaches would be beneficial to confirm the findings below are representative of the species as a whole.

*Z. irregulare* with its ability to modify its cell wall layers, pectic layers and rearrange cellular components to differentiate into akinetes tolerates such osmotic stress as mentioned above, maintaining the ability to reproduce successfully year after year. The observation of increased percentages of pre-akinetes and akinetes, in conjunction with decreased percentages of vegetative cells after 2 months of drying and its reversal after media reintroduction suggests that these changes are triggered in part by desiccation. During the desiccation period, moisture loss in the duplicate plates and the shift in the proportional relationship between cell types in the experimental group became significant the same week, further supporting that these changes were in response to desiccation. The percentage of dead cells was observed to increase by the 4\textsuperscript{th} week and then decrease by the 6\textsuperscript{th} week during desiccation. In preliminary studies, nicks were
observed to form on either side of dead cell walls, which would eventually cleave the cell into two sections. Fast Green staining had been observed only at these nicks in the cell walls, perhaps indicative of an enzymatic breakdown. It is presumed that this decreased observation of dead cells is due to the breaking of these cell walls, whose fragments would not be counted during examination of the different cell types since only whole cells were enumerated. The differentiation of cells into pre-akinetes during desiccation is rapid, although the observation of mature akinetes present only after the 6 week date of desiccation agrees with the observations on akinete formation according to McLean and Pessoney (1971).

Although cell type proportions in the control and experimental groups were still determined to be significantly different from each other throughout the recovery period, it is important to note that the proportional changes in cell types were observed in the directions expected until the fourth week. There may be a slight skew in the observation of increased proportions of vegetative cells during recovery however; during this time, not only were pre-akinetes recovering to a vegetative state but akinetes also would be germinating into new filaments. The unexpected increase in the percentage of pre-akinetes seen between 2-4 weeks recovery is most likely attributable to unstable plate conditions. During preliminary studies, *Z. irregulare* filaments grown on agar and provided with full moisture would differentiate into preakinetete cells after 2 months. McLean (1968) observed that aging agar media would result in an accumulation of lipids, thickened cell walls and decreases in chl *a* of the green alga *Spongiochloris typica*, most likely attributable to a limitation of nitrogen in the media. This may have been compounded by the minimal removal of condensation on the lids of the agar plates. It should be taken into consideration that the differentiation of vegetative cells into pre-akinetes
and akinetes in the desiccation group may also be partially due to decreasing nutrient content in aging media, not just desiccation.

During differentiation, vegetative cells had a marked visual increase in lipid content, decrease of protein, loss of vacuolization, a change of color to a brownish-green, an obscuring of the nucleus and chloroplasts as well as a contraction of the chloroplast projections. The obstruction of cellular contents from view was largely due to the accumulation of storage material. The accumulation of lipid globules in *Z. irregulare* pre-akinetes and akinetes during desiccation has also been observed for other *Zynema* species undergoing osmotic stress (McLean and Pessoney 1971, Holzinger et al. 2009b). Unexpectedly, lipid bodies were especially apparent with Fast Green staining, since the heat needed for the procedure caused the bodies to coalesce and become more visible.

McLean and Pessoney (1971) suggested that these lipid globules were not resistant structures, but only indicate a resting state. Instead, their accumulation may be from their displacement from membranes as seen in higher plants during desiccation (Gasulla et al. 2013b), a method which may reduce oxidative damage during osmotic stress. Additionally, lipid content may fuel the respiration processes reported during initial akinete germination (O’Neal and Lembi, 1983). The lipid content remains abundant well into the akinete stage and globules become largely visible usually just before germination.

Decreases of chl *a* content in desiccated *Z. irregulare* filaments were also reported, a well-established observation during osmotic stress in green algal species such as *Klebsormidium rivulare, Pithophora oedogonia* and in other algal assemblages (Morison and Sheath 1983, O’Neal and Lembi 1983, Angradi and Kubly 1993). An initial increase in chl *a* concentration
was attributed to the spreading growth of *Z. irregulare* on the newly inoculated plates. Larger biomass was observed to grow on the hard media in comparison to the control flasks until moisture levels became significantly different between the two groups. In conjunction with the decrease in chl *a*, chloroplasts were observed to reorganize with desiccation, contracting in pre-akinete, with the loss of chloroplast projections quite evident. This disappearance may be due to a collapsing of thylakoid membranes, theorized to disrupt the photosynthetic electron transport chain and reduce free radical production (Kirst 1989, Smirnoff 1993), protecting the osmotically-stressed cell from intracellular damage in the process.

Fast green staining is dense around the chloroplasts and pyrenoids in vegetative cells, but greatly reduced in pre-akinete cells. The decrease of chloroplast staining is attributable to the decrease in chl *a* but contrary to the loss the decreased staining at the pyrenoid region may suggest, pyrenoids have shown to be still visible in electron microscopy with desiccated cells (McLean and Pessoney 1971, Morison and Sheath 1983). This loss of pyrenoid staining is probably less indicative of a loss in the complete pyrenoid structure but rather a decrease in Rubisco (ribulose-1,5-biphosphate carboxylase) production, its main component. A similar decrease in proteins associated with carbon fixation was found in the lichen green alga *Asterochloris erici* (Gasulla et al., 2013a). Also notable is the observation that the densest Fast Green staining occurs around the nucleus of pre-akinetes as opposed to around the pyrenoids in vegetative cells. While Fast green at lower pH is known to bind with acidic nuclear proteins, (Dhar and Shah 1982), this heavier staining in pre-akinetes may also indicate an increase of proteins associated with the differentiation process or proteins that may protect against the increased possibility of oxidative damage as the cell desiccates. It has already been reported that
higher amounts of the antioxidant enzymes superoxide dismutase (SOD), ascorbate peroxidase and catalase were present in the green alga *Cladophora* during desiccation stress (Choo et al. 2004). In addition, a Cu/Zn SOD in *Zygnema* has already been observed, resembling those in higher plants (Lee 1999). Another possibility may be the presence of proteins that are similar to LEA proteins (late embryogenesis abundant proteins), whose roles deal with protecting DNA and membrane structure. Although usually associated with their abundance in pollen and in seeds, a LEA protein has recently been characterized in a halotolerant green algal species, *Chlamydomonas* (Tanaka et al. 2004). In any case, this speculation of the possible existence of antioxidant enzymes, LEA proteins or other protective proteins in *Zygnema* would require future investigation.

The rapidity of these intracellular changes leading to the differentiation of vegetative cells is indicative of how quickly *Z. irregulare* may acclimate to harsh conditions. However, the observation that filaments remain largely comprised of pre-akinetes, even after ample time for complete maturation, indicates that the success of *Z. irregulare* may be reliant on this partial change. In contrast with *Z. irregulare*, other papers have reported majorities of akinetes formed during unfavorable conditions within a comparatively narrow time-span. (O’Neal and Lembi 1983, Agrawal and Misra 2002). In streams known to dry quickly, the presence of pre-akinetes provides immediate chances for survival until further differentiation, the maturation of the akinete being a slower process. This mix of stages is potentially advantageous during recovery as well. Isolated akinetes germinated into new plants quickly but in terms of biomass production would be potentially slower. A filament of approximately only 10 cells long was formed within the 7-14 day period in the akinete, while pre-akinetes may begin to divide once recovery of the
chloroplast and photosynthetic processes was complete. If longer chains of pre-akinetes were present, having recovered to vegetative state, they would inevitably produce larger biomass faster, but akinetes are better in prolonged dry spells. Additionally, it has been reported that in glacial areas with a limited time-span to available liquid water, only vegetative cells of several Zygnematophyceae species are found (Remias et al., 2009 and Remias et al., 2012).

The correlations between pre-akinete percent and cell wall and pectin layer thicknesses were evident and consistent with previous literature (Jane and Woodhead 1941, McLean and Pessoney 1971, Morison and Sheath 1983). The majority of cell wall thickening was due to deposition of the secondary wall, the distinctions of the primary and secondary wall made clear with the Calcofluor White staining. The increases in these walls provide possible protection by increasing the distance over which water would need to diffuse, and decreasing the surface to volume ratio, but may also be beneficial by maintaining the turgor pressure of the cell (Droomgoole 1982, Jacob et al. 1992).

Despite the increase in cell wall thickness, it was pectic layer thickening that was particularly remarkable for Z. irregulare, indicative of its role in protecting the alga from the extremes of desiccation stress. Although the range of pectin width in the haphazardly encountered cells only thickened up to an average of 35µm, it is common for Z. irregulare to produce wider sheaths along the filament in areas (personal observation). As pectic layer thickness increased, so did the relative fresh:dry weight ratio. This ratio, used to approximate cell wall carbon deposition and water content, was the inverse of what was expected in experimental filaments throughout both desiccation and recovery. Dry weight did not appear to increase as had been reported in previous literature in desiccated algae (Morison and Sheath 1983), but the
species previously studied is not known to form the extent of pectin present with *Z. irregulare*. The large magnitude of pectin formed by *Zygnema* had increased the fresh weight of the relative fresh: dry weight ratio to an extent to where any increase that may have occurred due to carbon deposition in the cell walls would not be noticeable. When taking into consideration the thickened pectic layers, an increase of roughly 118% of cell volume was calculated in desiccated cells. So it would be more appropriate to state that dry weight may have increased, but was undeterminable via this method. It is important to note, however, that the maintenance of the pectin layer around pre-akinetes until full maturation indicates a continuing production and excretion of polysaccharides until akinete maturation.

The homogalacturonic (β-1,4 glucan) backbone of algal pectin may form calcium cross linkages, affecting porosity and fluidity (Jarvis 1984, Knox 1997, Vinckin 2003). These cross-linkages form “egg-box structures” which not only strengthen the pectic matrix but may also entrap water molecules. This change in the structural characteristics of the pectin layers are visible in the shift from an initially watery pectic layer to a condensed, opaque structure and back from desiccation to recovery. The pectin layer is then comparable to a sponge, an idea supported by the increases and decreases of moisture content in the experimental filaments throughout the desiccation and recovery period respectively. Furthermore, the day on which the larger, secondary pectic layer was considered no longer statistically different from the control during the recovery period, relative fresh: dry weight and moisture content of experimental filaments also became similar to that measured in control filaments.

The ability of the pectin layer to act as a sponge offers some protection for *Z. irregulare* pre-akinetes during periods of osmotic stress. With no significant amounts of protective
osmolytes known to accumulate in Streptophyte species (Morison and Sheath 1983, Holzinger et al. 2013), *Z. irregulare* would be largely reliant on extracellular protection. This would explain the characteristically large sheaths observed in *Z. irregulare* up until mature akinete formation, when other resistant compounds were formed. Even without an observation of autofluorescence, most likely due to not having an optimal filter set for the purpose, the remainder of the akinete mesospore layer after acid-base-acid treatment suggests the presence of phenolic or lignin-like compounds, reported in other green-algal spores (Kroken et al. 1996, Van-Winkle-Swift and Rickoll 1997, Pichrtova et al. 2012). Sporopollenin and sporopollenin-like compounds are considered to be fairly impermeable to water and offer some dehydration tolerance (Delwiche et al. 1989, Gensel and Edwards 2001). The impermeability of this wall is evidenced by the multiple instances of dyes not crossing into the akinete without first rupturing the cell, and may also create a reduction in the transport of pectic material to the cell exterior. This possibility may help explain the breakdown of the pectic layer around mature akinetes, although this theory would need to be examined in a future study and the reduction of photosynthetic processes must be taken into consideration as well. Akinetes matured, the cells no longer appear to be reliant on the protective mucilage layer.

Post desiccation, *Z. irregulare* rapidly recovers to a growing, vegetative state. Similar to *Pithophora oedogonia* akinetes, a 2-day lag period in which there was little visual change in the akinetes aside from a converging of lipid globules and a greening of the chloroplasts was observed (O’Neal and Lembi 1983). Within a week, biomass of the germling forces open the *Z. irregulare* suture. The granulated appearance of the new germling, seen through the akinete wall, is rapidly lost as storage products are used for initial respiration until photosynthetic processes,
evidenced by the reappearance of chloroplast projections and pyrenoids at 7-14 days, drives cell growth (O’Neal and Lembi 1983). This brief lag was also seen in pre-akinete recovery, in which there was not an extensive visual change the first two days; cells became greener, with the sheaths sometimes becoming more diffufluent, but the nucleus and chloroplasts were still largely obstructed from view. By around the 4th day, vacuolization of the cells becomes apparent and within a few short days, recovery of photosynthetic activity is evidenced by the reappearance of the pyrenoid and chloroplast projections.

The intracellular modifications combined with the structural changes to delay loss of and capture moisture mentioned here provides *Z. irregulare* with the ability to survive and reproduce after desiccation. These results correlate with those noted in previous studies, but provide some emphasis on the crucial role the characteristic pectic layer in *Zygnema* has to play in its tolerance of desiccation. The filaments are protected from rapid dehydration in part due to this thickened pectic layer and the thickening of the walls, allowing the slower process of akinete formation to complete. Then when conditions become favorable again, the species is able to recover and germinate quickly, strategies that have allowed *Zygnema* to be distributed on every continent and in some of the harshest conditions.

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