

SURVIVAL STRATEGIES OF THE MEDIOPHYCEAN DIATOM DITYLUM BRIGHTWELLII (T.
WEST) GRUNOW.

BY

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Abstract

This project is aimed at building on the less known life history strategies in *Ditylum brightwellii*, by focusing examination on the production and morphology of various cell types. Vegetative cell enlargement was induced by allowing cultures of cells to deplete their medium of nutrients and subsequently introducing nutrient replete medium. Enlargement was examined by light microscope, epifluorescence microscope with DAPI and PDMPO (2-(4-pyridyl)-5-((4-(2dimethylaminoethylaminocarbamoyl)methoxy)phenyl)oxalole) stain. Attempts at observation enlarged cells under the scanning electron microscope were unsuccessful due to fragility of enlarged valves. Vegetative cell enlargement was confirmed to occur as cells enlarged and their non-enlarged valves remained on the cell. *D. brightwellii* was observed to vegetatively enlarge their cells through two methods: one-step enlargement, and multi-step enlargement. During one-step enlargement, a single *D. brightwellii* cell will enlarge without producing supernumerary valves. Multi-step enlargement is the opposite, where *D. brightwellii* cells produce progressively larger supernumerary valves. Resting cells were observed to occur in all samples and were not induced through any experimental methods. Resting cells were observed to exit the valves (naked cells) and produce pseudopod-like structures. The pseudopod structures were observed to move freely on a microscope slide and could extend or retract. Resting spores were induced by inoculating *D. brightwellii* into a nitrogen depleted medium, also known as T medium. Resting spores were induced after 2 weeks of being inoculated in the nitrogen depleted medium. *D. brightwellii* cells plasmolysed in the T medium and remained plasmolysed until they became resting spores. The plasmolysed cells lost the colour within their chloroplasts and retracted to a portion of the cell.

Acknowledgements

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Introduction:

I. Diatoms

Diatoms are unicellular, photosynthetic, micro-eukaryotic organisms. (Round et al. 1990). *Ditylum brightwellii* (T. West) Grunow is a diatom that belongs to the phylogenetic division Bacillariophyta, belonging to the class Mediophyceae, or non-polar centric diatoms (Medlin and Kaczmarska 2004). Diatoms are abundant in most water habitats from sea water to freshwater and can even be found on damp soils. Although they are single celled organisms, they often form colonies or filaments that can be floating or attached to a substrate. Their protective shell, the frustule, makes them unique as it is highly differentiated among taxa and is composed of silica (Round et al. 2009). Diatoms are the main aquatic producers of biogenic silica and they contribute heavily to the global silica cycle (Nelson et al. 1995). Due to silica being more dense than water, diatoms sink to the bottom of the ocean after their death and their cell walls may remain on the seafloor for thousands of years as fossils and can be used to monitor long term changes in the aquatic environment (Mannion 1982). Many species have requirements for specific salinity or water temperatures which makes them reliable bioindicators for environment assessments.

Diatoms are responsible for approximately 40% of marine phytoplankton productivity, arguably making them the most important eukaryotic phytoplankton (Falciatore and Bowler 2002). They are globally major primary producers and they form a significant portion of the food web. They are eaten by all members of the food web such as zooplankton, aquatic insects, fish, and whales. Additionally, they are one of the most species-rich algal groups (Martino et al. 2009).

II. Diatom cell cycle

Diatoms reproduce asexually through mitotic cell division, resulting in two daughter cells. The diatom frustule is made of two valves or thecae, one being slightly smaller than the other. The larger theca is known as the epitheca and the smaller theca is known as the hypotheca. Due to the differing size of diatom thecae, when the cell divides it results in one daughter cell being the same size as the parent and the other is slightly smaller (Round et al. 1990). This is a result of the mechanisms of cell division, each theca from the parent cell will become the epitheca for the new daughter cells. The siliceous valve is a biological constraint for diatoms because the valve cannot expand. Over time, this process of cell division causes the average cell size of diatom populations to decrease until it is no longer biologically viable for a cell to divide.

When a diatom cell is too small to continue cell division, most species must sexually reproduce to form an auxospore. Auxospores are the result of sexual reproduction in diatoms and they reintroduce a large cell size to the population. Each species of diatom has a threshold size where vegetative cells will become gametangia and undergo meiosis to produce gametes (D'Alelio et al. 2009). Diatom auxosporulation is highly specific and in some cases, environmental conditions may not allow for successful sexual reproduction. In these cases, diatoms are faced with the option of dying because they can no longer divide into a biologically viable cell or they can go through a process known as vegetative cell enlargement.

III. Vegetative cell enlargement

Vegetative cell enlargement (VCE) can be defined as the size restoration process by which a vegetative diatom will partially or completely release its protoplast from the frustule, expand its protoplast, and form a new frustule (Kaczmarek et al. 2013). VCE is an alternative to sexual reproduction and allows a diatom to extend its ability to divide mitotically. When diatoms are faced with unfavourable environmental conditions or the inability to find a mate, VCE is an option for some species to enlarge asexually. In addition to prolonging a diatom's ability to continue to divide, VCE gives individual diatoms a second opportunity to reproduce sexually at another time when conditions become favourable. This second chance at sexual reproduction makes VCE an important part of a diatom life cycle. An important difference between auxosporulation and vegetative enlargement is that cells produced through enlargement are not the same maximum size as the auxospores of the same species and they may differ in frustule morphology (Kaczmarek et al. 2013). Additionally, cells that result from VCE are genetically the same as parent cells whereas auxosporulation results in genetically distinct progeny.

IV. Resting cells

Resting stages are an important aspect of diatom life cycles and allow them to survive poor conditions. Resting cells appear morphologically similar to vegetative cells although they have undergone physiological and cytoplasmic changes (Ishii et al. 2012). Resting cells of *Actinopterychus senarius* have many granulated plastids that are unevenly distributed and darker in color than vegetative cells (Ishii et al. 2012). Alternatively, the plastids in the resting cells of *Biddulphia alternans* are darker in color than those in vegetative state and gather in a sphere at the center of the cell (Ishii et al. 2012). The plastids of resting cells of *Odontella longicruris* always attach to one side of the cell (Ishii et al. 2012). The diatom *Melosira granulata* has also been observed to produce resting

cells and they also contain condensed, dark plastids that expand upon the depletion of nutrients (Sicko-Goad and Stoermer 1986).

V. Resting spores

Resting spores in diatoms are also known as hypospores or endospores (Hargraves 1976). Resting spores are formed asexually and are an important aspect of the diatom life cycle (Hargraves 1976). Resting spores may be advantageous due to their tolerance of severe environmental conditions, such as the depletion of nutrients, predation, and darkness (Oku and Kamatani 1997). There are many factors that could induce spore formation in the ocean, and it is of interest to determine which factors can induce resting spore formation. Oku and Kamatani (1997) induced resting spores in the diatom *Chaetoceros anastomosans* through nitrogen depletion and high salinity conditions. Sugie et al. (2010) induced resting spores in the diatom species *Thalassiosira nordenskiöldii* through Fe- and N-limitation. French and Hargraves (1985) induced resting spores in the marine centric diatoms *Chaetoceros diadema* and *Leptocylindrus danicus* by limiting nitrogen. Nitrogen limitation is a common factor in the formation of resting spores across many species of diatoms. Spore formation can be enhanced by unfavourable conditions such as low temperature and darkness (Oku and Kamatani 1997). The resting spores of *Chaetoceros anastomosans* have been observed to be partly surrounded by bacteria but they were not harmed, this suggested that resting spores have a mechanism to protect themselves from bacterial infection (Oku and Kamatani 1997).

Resting spores are formed through two sequential acytokinetic mitoses within a vegetative cell, each mitosis produces a valve of the resting spore (French and Hargraves 1985). Acytokinetic mitosis occurs when a nucleus divides and becomes pyknotic in the absence of a cytoplasmic division, but the formation of one valve (French and Hargraves 1985). The vegetative cell diameter required for the formation of resting spores is species specific although many can form within a wide range of sizes (French and Hargraves 1985). In most species, when the resting spore has germinated it will produce a vegetative cell of the same diameter as the cell that formed the spore (French and Hargraves 1985).

VI. Pseudopodal cells in diatoms

There are virtually no reports of centric diatom cells producing pseudopods. It is known that raphe diatom species are capable of locomotion on underwater surfaces due to their paired slit systems (the raphe) (Wang et al. 2013). The diatom *Navicula* sp. has been observed to protrude two or more pseudopods or stalks out of their frustules for locomotion (Wang et al. 2013). Locomotion is realized

when the pseudopods or stalks of *Navicula* sp. pull off from the substratum to generate positive pressure while pushing down another pseudopod or stalk (Wang et al. 2013).

VII. *Ditylum brightwellii*

Ditylum brightwellii is a cosmopolitan, planktonic, marine diatom that can be found in coastal waters and has been used in various studies concerning sexual reproduction, diatom physiology and cytology (Koester et al. 2007). The alternate life stages of *D. brightwellii* such as the extrusion of protoplast into the medium and vegetative cell enlargement have often been confused with sexual reproduction (Koester et al. 2007). Sexual reproduction in *D. brightwellii* is homothallic and oogamous, eggs or sperm are produced by each cell through meiosis (Koester et al. 2007). Single vegetative cells can either produce two eggs or sixty-four sperm (Koester et al. 2007). Each sperm produced by *D. brightwellii* cells have a single anterior flagellum that moves in a sinusoidal pattern while swimming (Koester et al. 2007). The process of auxosporulation in *D. brightwellii* lasts approximately two days (Koester et al. 2007). Koester et al. (2007) observed enlarged cells in their study and concluded that their morphology was irregular or had rounded valves in comparison to the sexually derived cells.

The diatom *D. brightwellii* has very high uptakes of nitrate when it is exposed to strong temporal fluctuations in nutrients which allow it to dominate competitions for nitrate with other algae species (Brussaard et al. 1997). *D. brightwellii* cells adapted to grow in nitrogen limited conditions have been observed to survive periods of starvations more efficiently than fast growing cells (Brussaard et al. 1997). An explanation for this advantage may be that cultures that are preconditioned to low nitrogen concentrations often produce resting cells that are capable of surviving starvation (Brussaard et al. 1997). The production of resting cells allows *D. brightwellii* to survive conditions with very low nitrate concentrations and is an essential adaptation for the survival of the species (Brussaard et al. 1997).

Aims of Study

The aim of this study is to add to the knowledge of alternate life history strategies in diatoms, specifically *Ditylum brightwellii*. I will observe and describe vegetative cell enlargement, resting cells, resting spores and pseudopodal cells. Alternate life history strategies in *D. brightwellii* are often confused with auxosporulation or other cellular processes and their morphological differences must be described. Clearing up the differences among the life history strategies of *D. brightwellii* is an

important step in identifying similar life forms in other diatom species. Vegetative cell enlargement, resting cells, resting spores and pseudopodal cells may be significant life history strategies and should be further investigated to determine their importance.

Materials and Methods

I. Diatom culture

D. brightwellii was isolated from a sample collected in the English Channel (50° 01N and 4° 13W) by RK Pipe in March 2003. The clone of *D. brightwellii* was part of the Plymouth Culture Collection and its code is PLY 609. An additional clone, CCMP1810, was used for size distribution measurements. Cultures were grown in 125 ml Erlenmeyer flasks and kept at 14-16°C in f/2 medium (Guillard and Ryther 1962; Guillard 1975) with a photoperiod of 12:12 and a light irradiance of 14.7 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ measured with a LI-COR LI-250 light meter (LI-COR) within the cold room in the Flemington building. Cultures of *D. brightwellii* were transferred to fresh growth media biweekly to maintain growth. Experimental cultures were left to accumulate biomass for approximately 4-6 weeks.

II. Inducing cell enlargement

Vegetative cell enlargement was induced by allowing the culture to increase in biomass to deplete the medium of nutrients (Sutcliff 2013). The culture was grown in a 125 ml Erlenmeyer flask containing approximately 50 ml of f/2 medium and is kept at 14-16 °C with a photoperiod of 12:12 and an average light intensity of 14.7 $\mu\text{mol photons m}^{-1} \text{ s}^{-1}$. The cells were then transferred to a flask containing nutrient replete medium.

III. Inducing sporulation

Resting spores were induced by transferring exponentially growing cells into nitrogen-deplete “T” medium, and after two days transferred again to fresh “T” medium. Presence and abundance of resting spores were enumerated after 5, 10, 20 and 30 days. The nitrogen depleted modified f/2 medium, known as “T” medium, is made by replacing nitrate with 15 μM of ammonia and 7 μM of phosphate (French and Hargraves 1980). Throughout the experiment the cultures were grown in the cold room within the Flemington building at a temperature of 14-16 °C, a photoperiod of 12:12 or 15:9 light:dark and a light intensity of 14.7 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$.

IV. Light microscopy observations (vegetative cell enlargement and sporulation)

Cells were observed using light microscopy (Zeiss Axioskop 2 and Zeiss Axiolab Reflected-Light) and images were captured using a digital camera (Zeiss AxioCam Icc3). Using the light microscopy images, the valve lengths, and diameters (μm) of cells were measured using a measuring software (Digital Microscopy Facility Ehrman).

V. Fixation

Subsamples were fixed using 10 ml of glutaraldehyde in 90 ml of f/2 medium, resulting in a concentration of 2.5% glutaraldehyde. To fix the diatoms, 1ml of concentrated culture was transferred to a vial containing 10 ml of fixation solution. The vial was placed in the refrigerator at 4°C for at least 1 hour prior to examination.

VI. DAPI and PDMPO Staining Technique

The progression of valve silicification during vegetative cell enlargement was visualized by observing cells incorporated with PDMPO (2-(4-pyridyl)-5-((4-(2-dimethylaminoethylaminocarbonyl)methoxy)phenyl)oxazole); Thermo Fisher Scientific, Waltham, Massachusetts, USA). Diluted PDMPO was obtained by diluting 50 μL of 1mM PDMPO in 450 μL of MilliQ filtered water. 250 μL of the diluted PDMPO solution was added to the experimental culture and samples were subsequently taken after every hour and fixed. The final concentration of PDMPO in the culture flask was 0.125 μM (Leblanc and Hutchins 2005). When PDMPO was incorporated in silica it fluoresces green (550 nm) when excited by violet (405 nm) light. The nuclear behaviour was observed by staining cells with Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, California, USA) as per manufacturers instructions.

VII. SEM Cleaning and Observation

Cells of *D. brightwellii* intended for observation of valve morphogenesis were cleaned and prepared for observation using the scanning electron microscope (SEM). Fixed cells were rinsed with filtered water to remove the fixation solution. Organic material was removed from the cell by washing them with 15 ml of a 1:1 solution of concentrated sulfuric and nitric acids within a glass tube that was subsequently placed in a hot water bath. The length of time in the hot water bath varied from 10-20 minutes, depending on the fragility of the structures intended for observation. After boiling, the tube of culture and hot acid were poured in a beaker containing filtered water, it was next poured onto a 25 mm diameter, 1 μm pore size polycarbonate filter (Sterlitech Corporation, Kent, Washington, USA). The

acid treated cells were washed with 250 ml of filtered water onto the filter and left to air dry. Filters that had specimens on them were mounted on aluminum stubs with double-sided tape, rimmed with colloidal carbon, and coated with ~15 nm of gold using a Hummer 6.2 sputtering unit (Anatech Ltd, Union City, California, USA). Specimens were observed and imaged using a Hitachi SU3500 SEM (Hitachi High Technologies, Toronto, Canada) at a working distance of 5 mm and 10 kV accelerating voltage. Images were taken of valves that were at early, middle, and late stages of formation.

VIII. Cell types within flask population over time

The cell types were monitored daily within a flask population from November 20, 2019 until December 21, 2019. Each day a droplet of the culture sample was observed under a microscope to determine the cell types present. I counted 200 cells and classified them into 11 different categories: dividing cells, vegetative (healthy), vegetative (stressed/abnormal), extra valves, resting cells, enlarged cells, vestigial cells, cells with pseudopods, round cells, and balloon cells. The percentage of each cell type within the population was calculated for each day that the experiment was conducted.

IX. Statistics

Length and diameter measurements of cells were used to calculate the size classes of cells within the *D. brightwellii* culture population. The sizes classes were binned in increments of 5 μm such as: 0-10 μm , 11-15 μm , 16-20 μm , 21-25 μm , 26-30 μm , 31-35 μm , 36-30 μm , 41-45 μm , 46-50 μm , 51-55 μm , 56-60 μm and will be used to determine if cell enlargement occurs continuously or rarely. A regression analysis was conducted on the perivalvar length and valve diameters of both PLY-609 and CCMP-1810.

The diameters of valves within cells with supernumerary valves are measured to calculate the relative size change per cell. The oldest valves will be compared to the newest valve using a paired t-test to determine if there is a size increase or decrease.

Results:

I. Lab population of PLY-609

Both cultures of *D. brightwellii* frustule diameters remained within a range of 60-15 μm . The lab population PLY-609 had more measurements than CCMP1810. The lab population of CCMP1810 perished prior to July 2019 due to issues with culture medium.

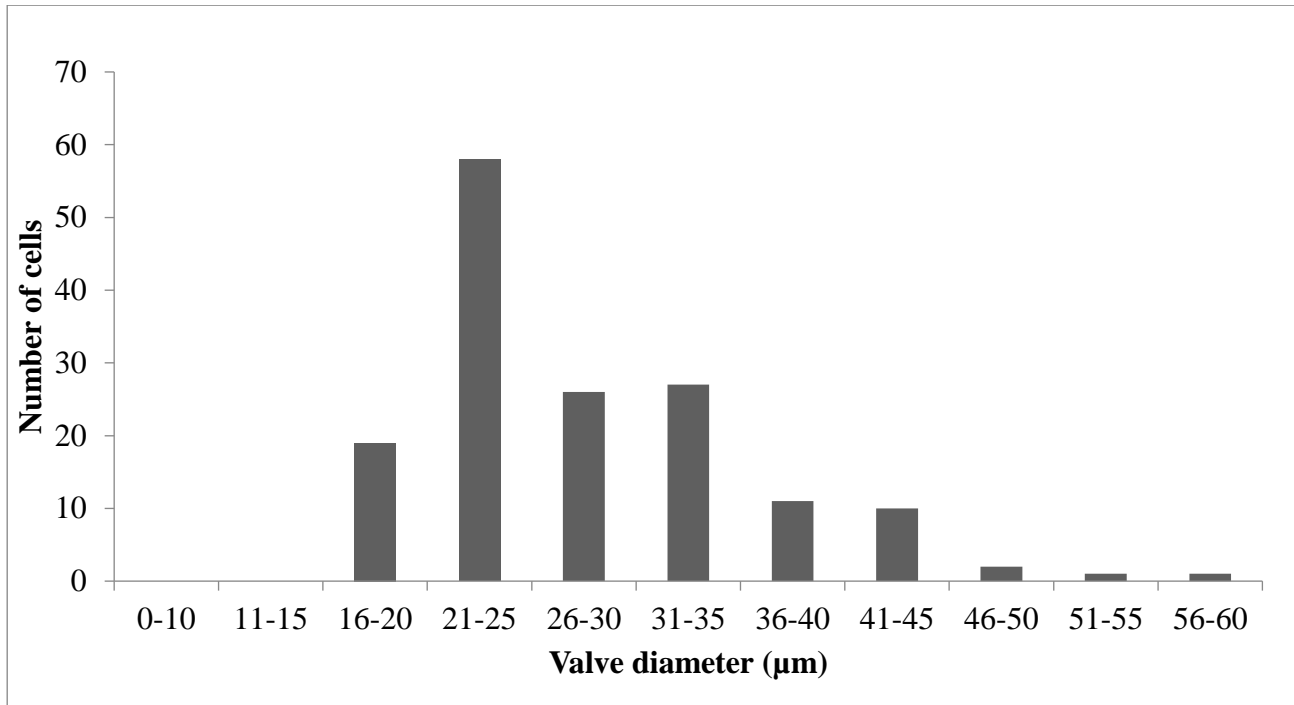


Figure 1 – Diameter of valves (µm) of a lab population of *D. brightwellii* (PLY-609)

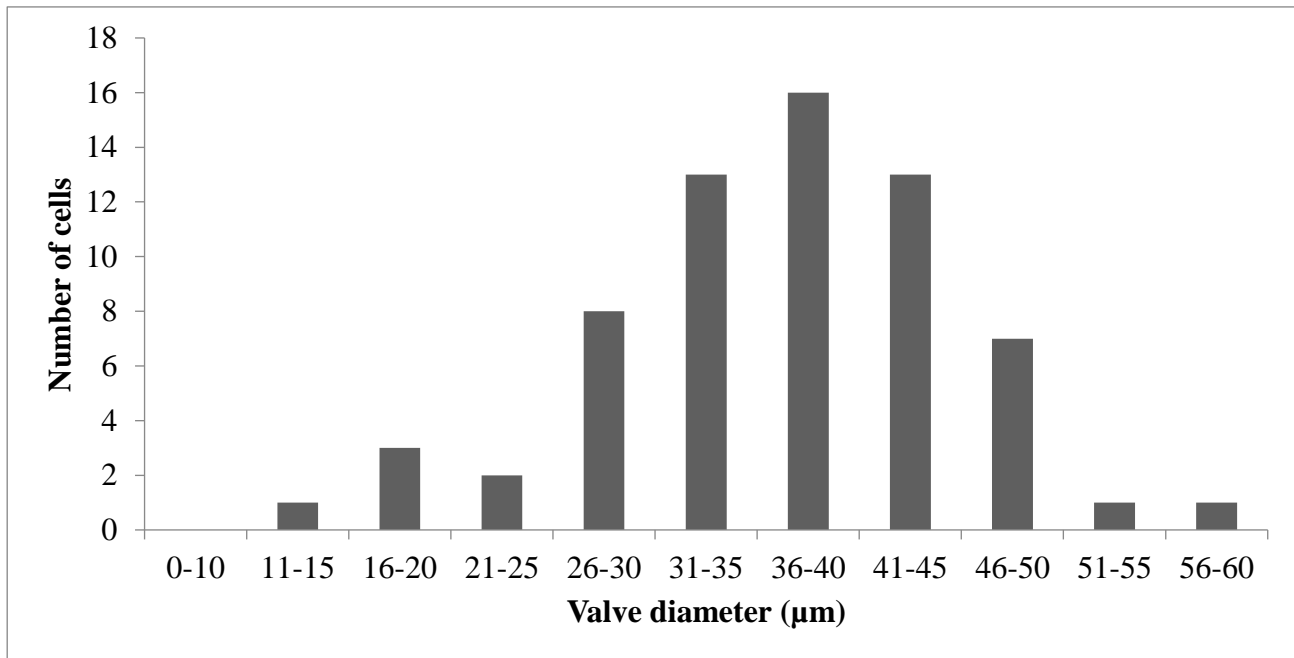


Figure 2 – Diameter of valves (µm) of a lab population of *D. brightwellii* (CCMP1810)

The mean diameter of valves in the lab population PLY-609 was 27.4 ± 7.5 µm (n=58). The smallest valve diameters were 20 µm (n=19) and the largest were 60 µm (n=1). The occurrence of large valves was highly infrequent in comparison to small valves. Cells with intermediate valve diameters, 30 – 40 µm, occurred frequently and made up for 64 of the 155 observations.

The mean diameter of valves in the lab population CCMP-1810 was $35.9 \pm 8.9 \mu\text{m}$ ($n=16$). The smallest valve diameter was $15 \mu\text{m}$ ($n=1$) and the largest was $60 \mu\text{m}$ ($n=1$). Large and small valves occurred infrequently while cells with medium sized valve diameters were observed most often.

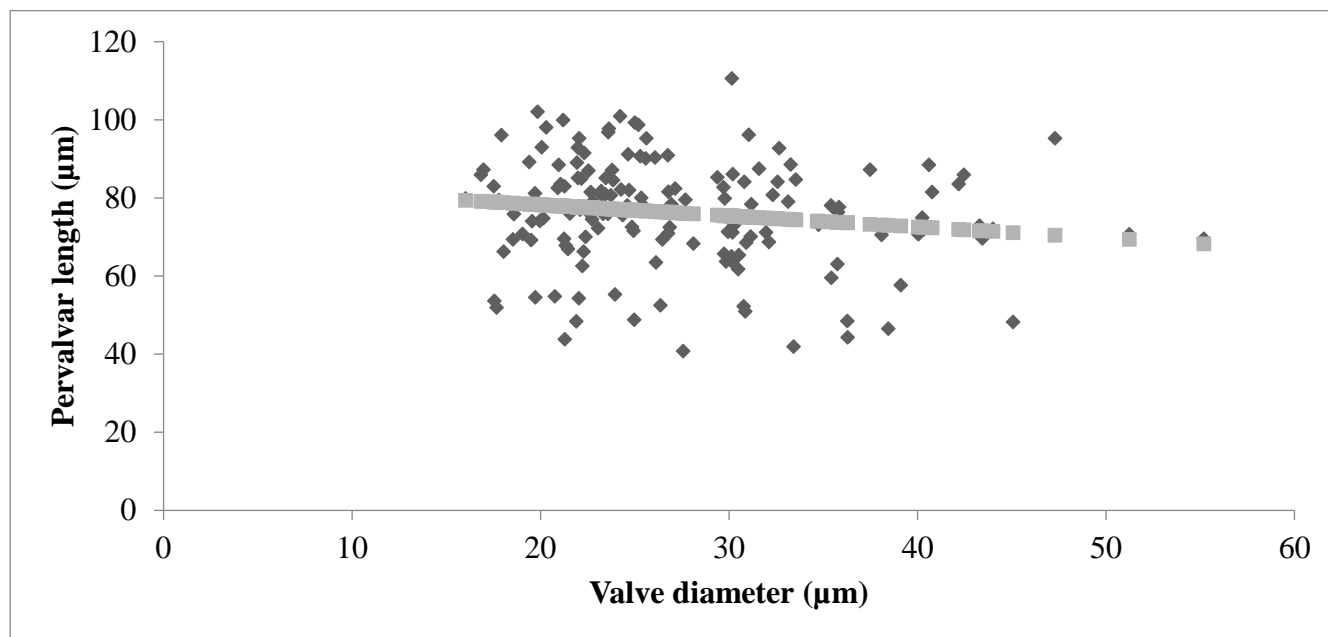


Figure 3 – Relationship between perivalvar length and valve diameter in the PLY-609 clone of *D. brightwellii* ($n = 155$)

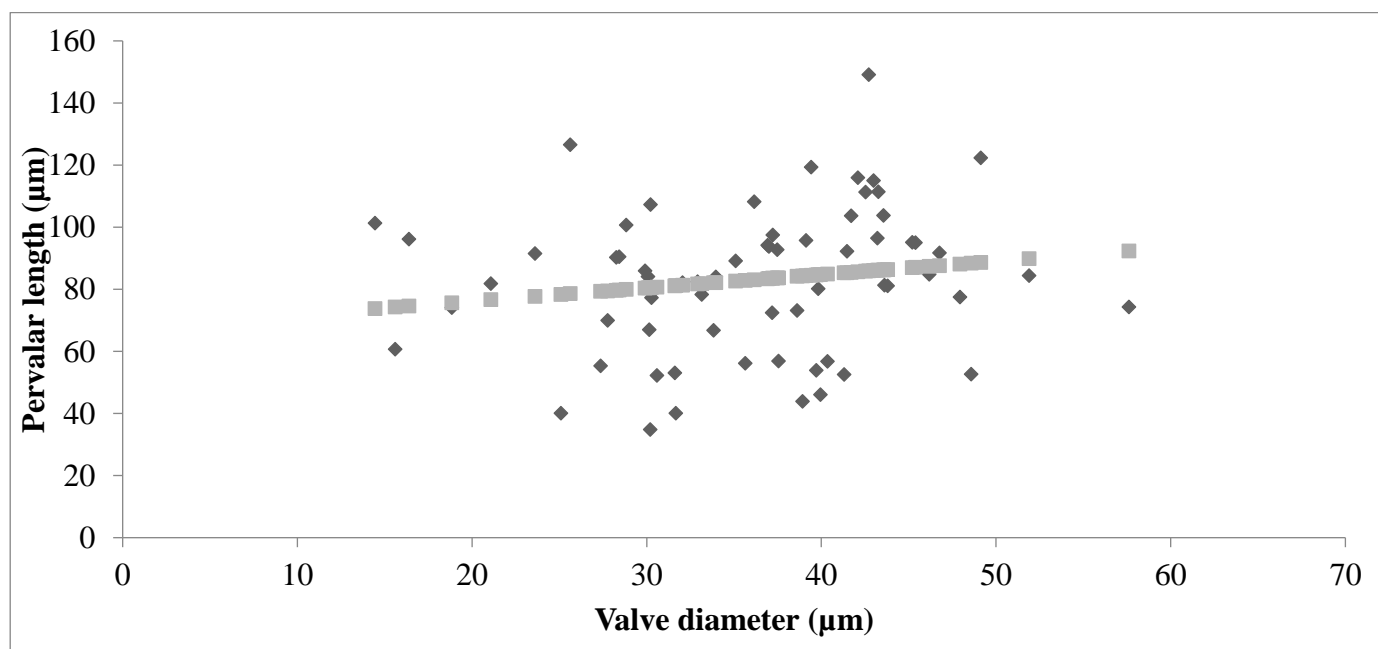


Figure 4 – Relationship between perivalvar length and valve diameter in the CCMP-1810 clone of *D. brightwellii* (n= 65)

Figures 3 and 4 depict a regression analysis of the perivalvar lengths (μm) and valve diameters (μm) of both lab populations of *D. brightwellii*. The interrupted line represents the predicted perivalvar length for a given valve diameter.

II. Flask population of *D. brightwellii*

Various cell types were observed over a period of 30 days of classifying 200 cells. From November 20 until December 3 the lab population of *D. brightwellii* cells remained healthy and vegetative with no abnormalities (Figure 5). Cells that have produced extra valves increased within the population on December 4, coinciding with the decrease in healthy vegetative cells. Resting cells were observed at low levels constantly and peaked on December 5.

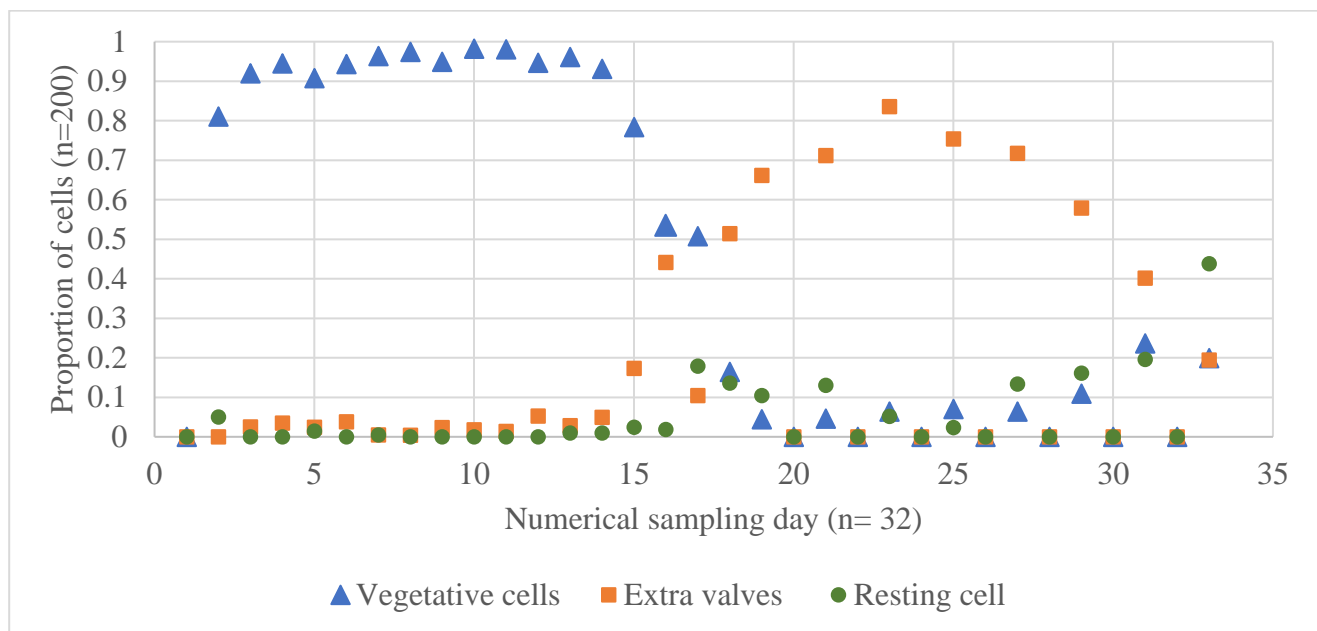


Figure 5 – Proportions of various cell types within a flask population of *D. brightwellii* (PLY-609) over the period of 32 days (November 20, 2019 – December 21, 2019)

Vegetative or healthy cells can be distinguished by their plastids that evenly spread out through the cell and the nucleus can be observed in the center (Figure 6A). Cells that had more than one valve connected to them were classified as cells with extra valves, and the extra valves often curved around the cell (Figure 6B). The resting cells were distinguished by their aggregations of dark plastids, spherical cytoplasm, and are often found in one half of the cell (Figure 6C). Cells that were classified

as dividing were visually evidently in the process of cell division as in Figure 6D. Cells undergoing mitotic division could also be distinguished by having their nucleus appear dense and in the center and the plastids are separated into the two halves of the cell.

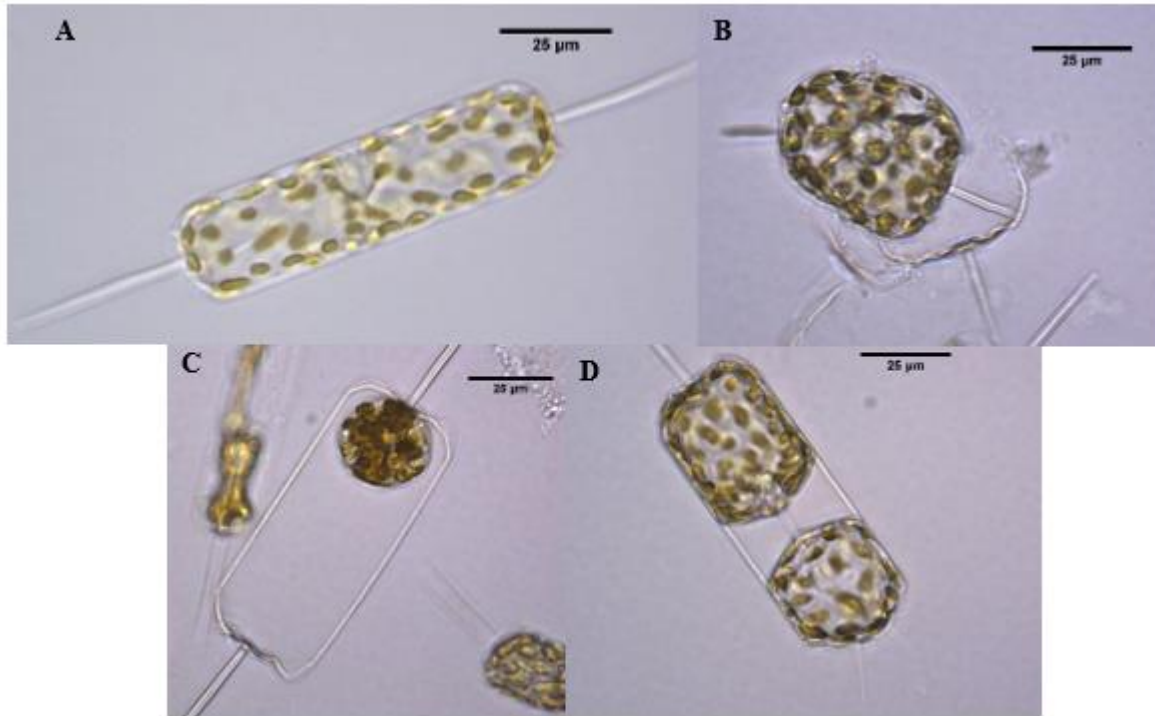


Figure 6 – Representatives of cell types observed within a flask population of *D. brightwellii* (A = vegetative healthy , B = extra valve , C = resting cell (centre cell), D= dividing cell)

Resting cells that produced pseudopods were first observed on December 5 and subsequently remained in the culture (Figure 7). Cells that have undergone vegetative enlargement occurred at a very low rate throughout the experiment and peaked on December 5th. Vestigial cells, or the remnants of cells after cell divisions were observed at a low but constant rate throughout the experiment and did not peak. Free, vegetative cells (classified as round cells) were present from December 4 until December 14 and were infrequent. Cells classified as balloon cells appeared on one day of the experiment (December 6), and were not observed after that date.

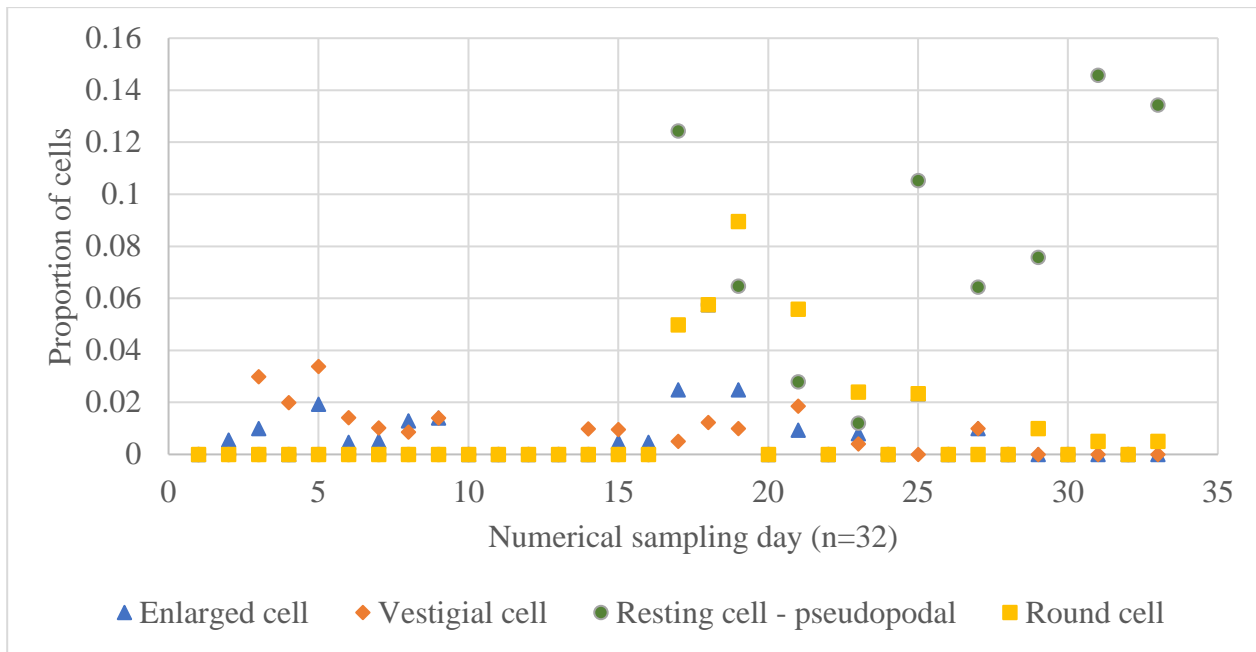


Figure 7 – Proportions of infrequent cell types within a lab population of *D. brightwellii* (PLY-609) over the period of 32 days (November 20, 2019 – December 21, 2019)

Enlarged cells were distinguished from other cell types due to their unique morphology. The valves of enlarged cells of *D. brightwellii* appear abnormal and slightly rounded. Some enlarged cells will also contain supernumerary valves (Figure 8A). Vestigial cells are remnants of the cytoplasm that remain within the valves of cells that have divided. They often do not appear to have a nucleus, but their plastids are of normal color. The size of vestigial cells is much smaller than vegetative or resting cells (Figure 8B). Resting cells sometimes possessed pseudopod-like structures when they were no longer within the cell valves. Pseudopodal resting cells are distinguished by their granular pseudopod-like structures and can exist as a naked cell (Figure 8C) or within valves. Round or naked cells are distinguished by their unique spherical appearance and the lack of siliceous valves (Figure 8D). The plastids of the naked cells are similar to the arrangement of vegetative cells. Balloon cells are distinguished solely by their morphology. The balloon cells are described by their appearance as it appears like the cell is being inflated although they are likely a cell undergoing enlargement or are developing into a free cell (Figure 8E).

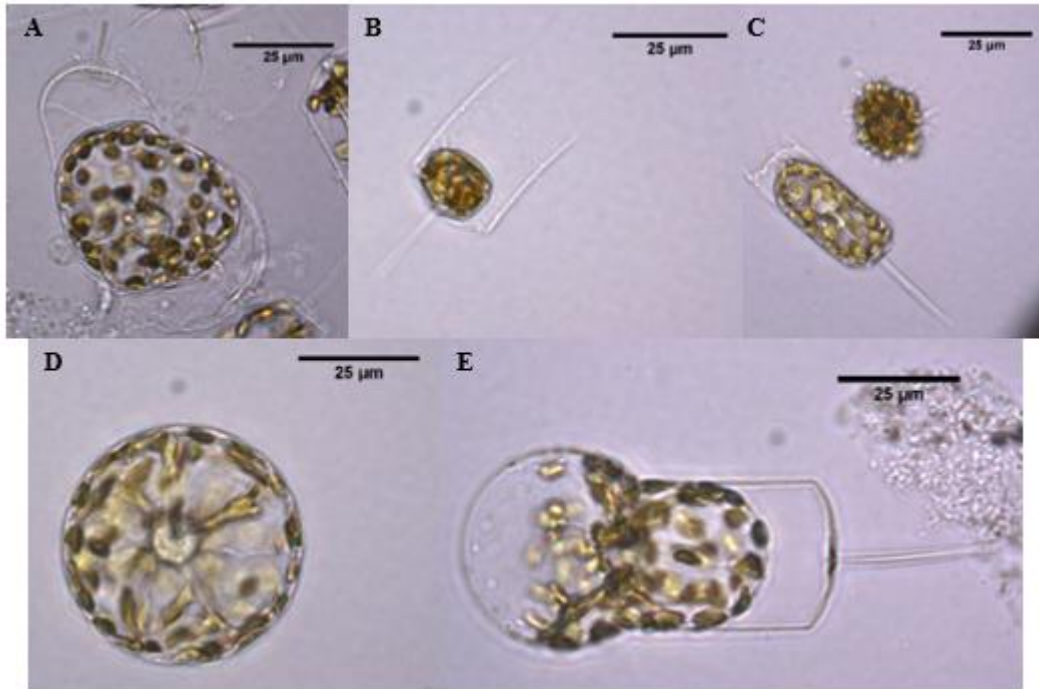


Figure 8 – Representatives of infrequent cell types found within a flask population of *D. brightwellii* (A= enlarged cell, B= vestigial cell, C= resting cell with pseudopods, D= round cell, balloon cell)

III. Supernumerary valves or multi-step enlargement

Supernumerary valves as a form of enlargement can occur in two ways: progressively with multiple valves (Figure 9A) or at once (Figure 9B) In image B, the supposed ends of the valves are bent facing the same direction and a new valve is being produced at the corner. Cells producing supernumerary valves were frequently observed daily and they did not always indicate enlargement. Cells with supernumerary valves sometimes did not progressively become larger and instead were always the same size as the first. Cells that did produce progressively larger valves were found to be significantly larger (Table 1). The mean valve diameter of the oldest valves was $26.4 \pm 7.6 \mu\text{m}$ and the mean diameter of the newer valves was $32.1 \pm 7.6 \mu\text{m}$.

Table 1 – T-test of cell enlargement via multiple valves

	<i>Oldest Valve</i>	<i>Newest Valve</i>
Mean	26.4	32.1
Variance	58.2	58.3
SD	7.6	7.6
Observations	39	39

df	38
t Stat	-5.13670641
P(T<=t) two-tail	8.6E-06
t Critical two-tail	2.024

Lab culture populations of PLY-609 frequently contain cells with supernumerary valves. Many of the supernumerary valves were larger than the parental valve and they became progressively larger. A t-test of the older valves and newer valves shows that the newer valves are significantly larger. (Oldest valve diameter size ranged from 15.8 - 47.8 μm and the newest valve diameter sizes ranged from 20 - 48.6 μm)

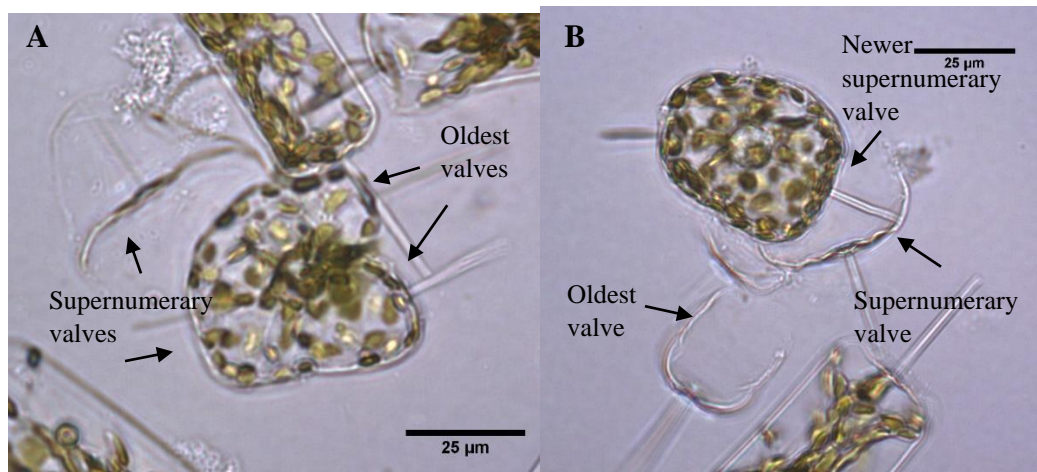


Figure 9 – Cells of *D. brightwellii* (PLY-609) producing progressively larger supernumerary valves (A&B)

Cells that produce supernumerary valves were also frequently observed to be multinucleated. The additional nucleus did not always degenerate, and it remained within the functioning cell. The pyknotic nucleus can be distinguished because it is smaller than the original nucleus (Figure 10B).

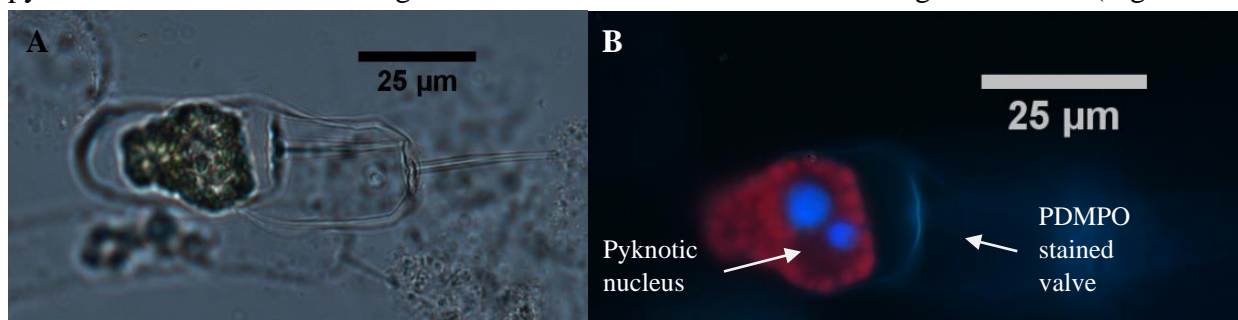


Figure 10 – A multinucleated *D. brightwellii* cell with an additional valve (A = Light microscope, B= PDMPO and DAPI stained cell under epifluorescence microscope)

IV. One Step Enlargement

One step enlargement was distinguished by the occurrence of an enlarged cell in the absence of progressively larger supernumerary valves (Figure 11A-C). One step enlargement typically occurred in two forms: the cell would bend in half and produce a new and larger valve at the corner (Figure 11A), or the cell contents would exit the original valves and produce new, larger valves (Figure 11B,C). This type of enlargement occurred less than the multi-step enlargement and was not observed daily. Additionally, one step enlargement typically resulted in a cell that was much larger than the original cell, as opposed to a progressively larger cell that is produced through multi-step enlargement.

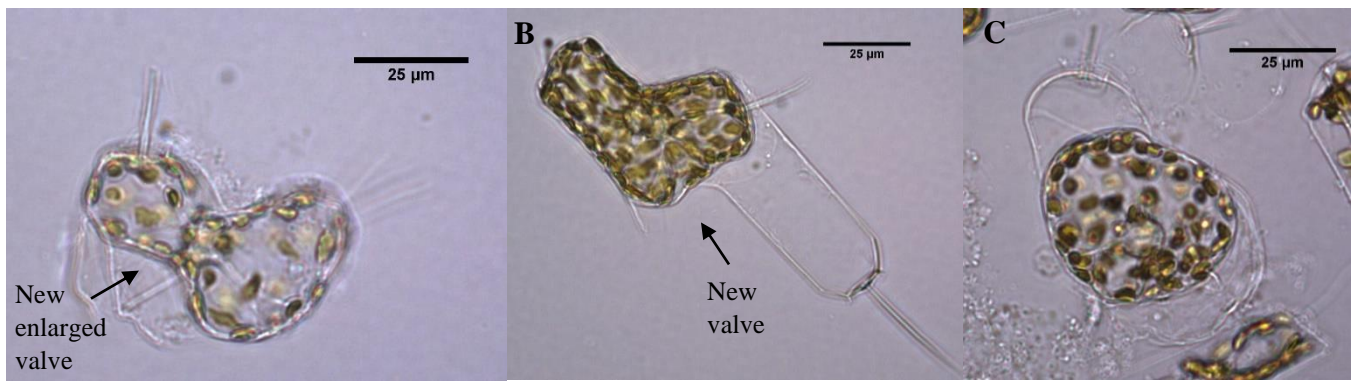


Figure 11– One step vegetative cell enlargement in *D. brightwellii* (A = cell has folded in half and is forming a new, larger valve at the corner, B= the protoplast has been partially exposed and is forming a new valve, C = the protoplast has exited the frustule and has formed a new, larger valve)

V. Resting cells

Lab populations of *D. brightwellii* produced cells in a resting state after approximately 2 weeks of being inoculated into fresh f/2 medium. The cells in an apparent resting state have a condensed cytoplasm and are circular in shape. The resting cells are found within the parental frustule and often have an incomplete supernumerary valve accompanying them (Figure 12).

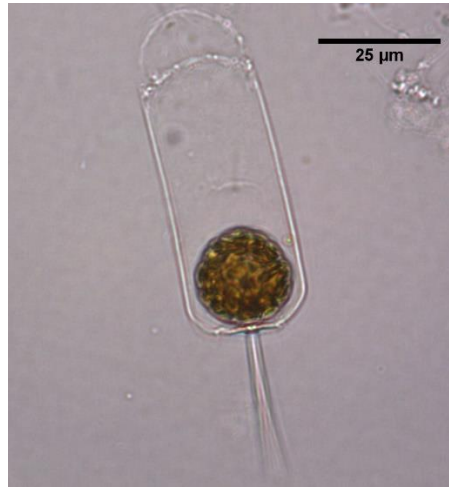


Figure 12 – Resting cell of *D. brightwellii* within the parental frustule accompanied by an incomplete supernumerary valve

VI. Free “resting” cells with pseudopods

Cells in resting state were observed to exit their parental frustule and live as a cell lacking siliceous cell walls while growing in f/2 medium. Such cells were observed to be contained within one valve and it appeared as though they preferentially exiting the valve (Figure 13A,B). The resting cells were frequently observed producing and extending glandular pseudopods that allowed them to move (Figure 13C-E). The pseudopods were observed to move freely on a microscope slide and were not associated with the water current under the microscope slide. Free resting cells were distinguished from free vegetative cells due to their aggregation of dark plastids (Figure 13F).

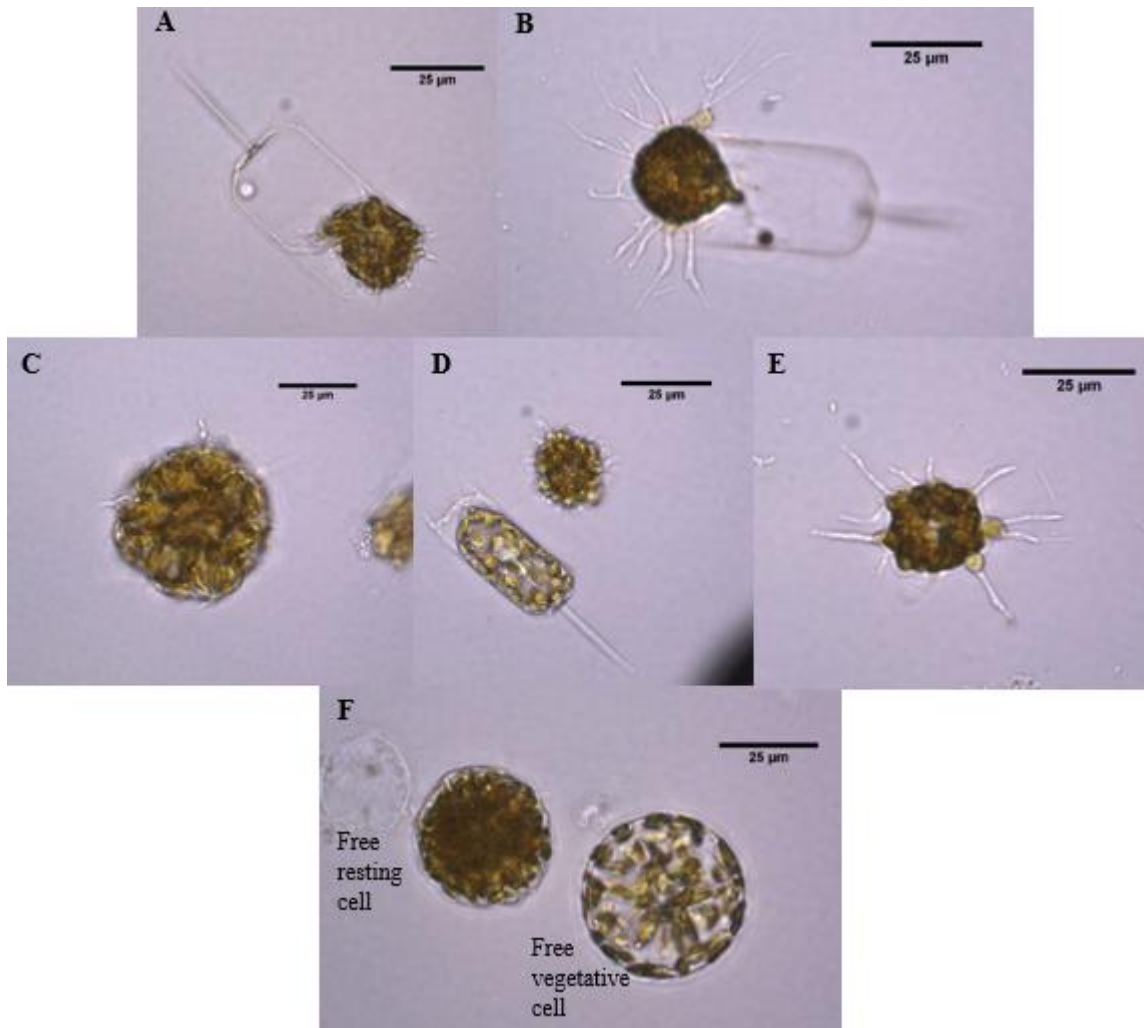


Figure 13 – Free resting cell of PLY-609. A resting cell that appears to be exiting the frustule (A & B), resting cell that is extending pseudopods (C, D, E), comparison of free resting cell with a vegetative free cell in the process of enlargement or becoming an egg cell) (F).

The resting cells that were capable of producing and extending pseudopods were also observed to be producing new siliceous structures (Figure 14A-D). The new siliceous structures produced by the pseudopods were circular in shape and appeared to be different than siliceous structures produced by vegetative cells. Figure 14B shows the first production of a siliceous structure on a free, resting cell although it is not visually obvious in Figure 14A.

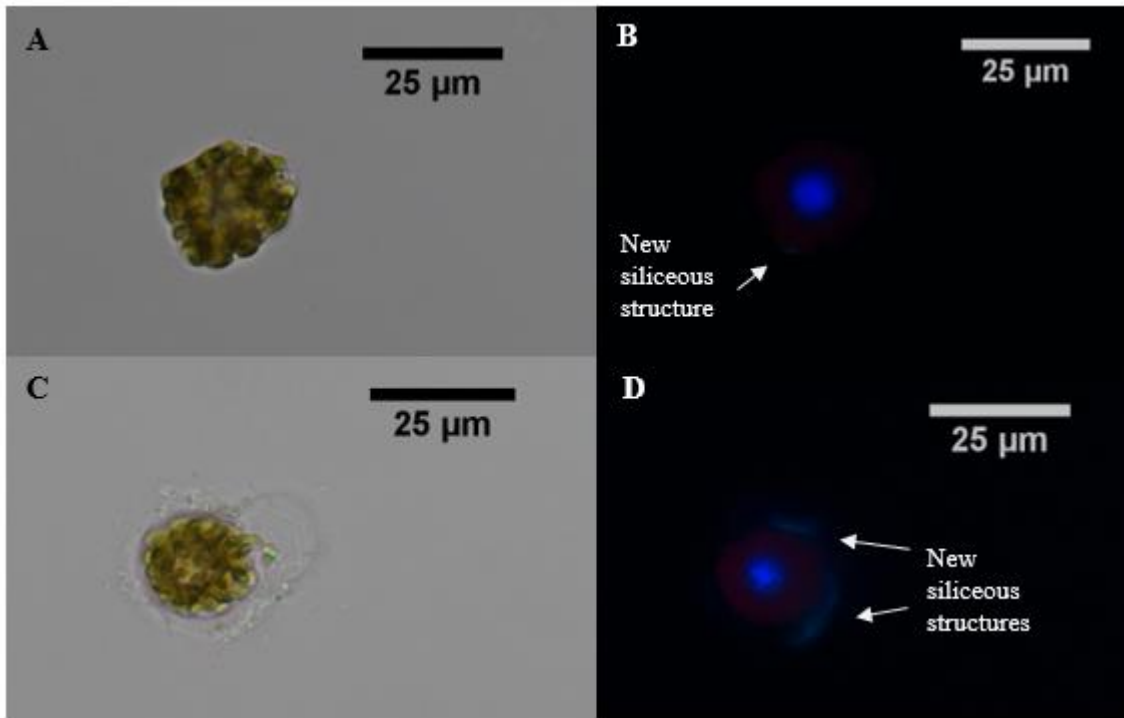


Figure 13 – Images of free “resting cell” producing a new siliceous structure shown by light microscope (A, C) and PDMPO (B,D). The earliest stage of the “resting cell” producing new siliceous structures is shown in B and a later stage in D.

VII. Resting spores

D. brightwellii cells being the process of sporulation by plasmolysing and by reducing the color of their plastids (Figure 15A-D). After spending approximately two weeks in a plasmolysed state, the cell contents will aggregate to one half of the cell body and appear spherical in shape (Figure 15E). A spore is produced after the cell contents have aggregated to one half of the cell. Plasmolysed cells were predominant in the culture flask for the duration of the experiment and resting spores occurred at low rates.

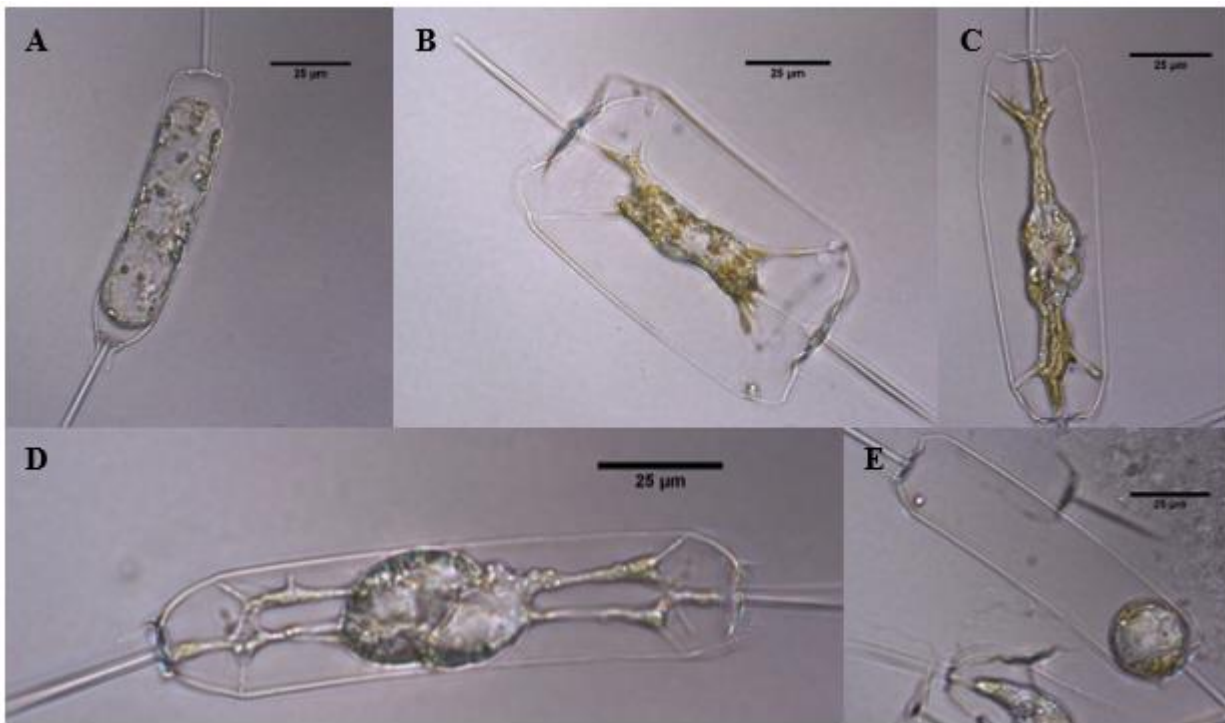


Figure 15 – Cells grown in nitrogen limited modified f/2 medium known as “T” medium induced to be resting spores (A= reduction in plastid colour, B= plasmolysed cell, C= plasmolysed cell, D= plasmolysed cell with no plastid colour, E= condensed plasmolysed cell)

The resting spores of *D. brightwellii* are different in morphology when compared to vegetative, and resting cells. The spore produced is the same diameter as the cell that produced it. Resting spores of *D. brightwellii* can be different in morphology among each other (Figure 16A-C). Some resting spores can appear thin or wide, some are free from the original valves, others can be contained within a cell body, some spores contain more than one process, others may occur in the center of the cell body rather than an edge. All spores produced by *D. brightwellii* had pyramidal shaped valves and were heavily silicified. The plastids within the spores have regained their colour and aggregate within the spore.

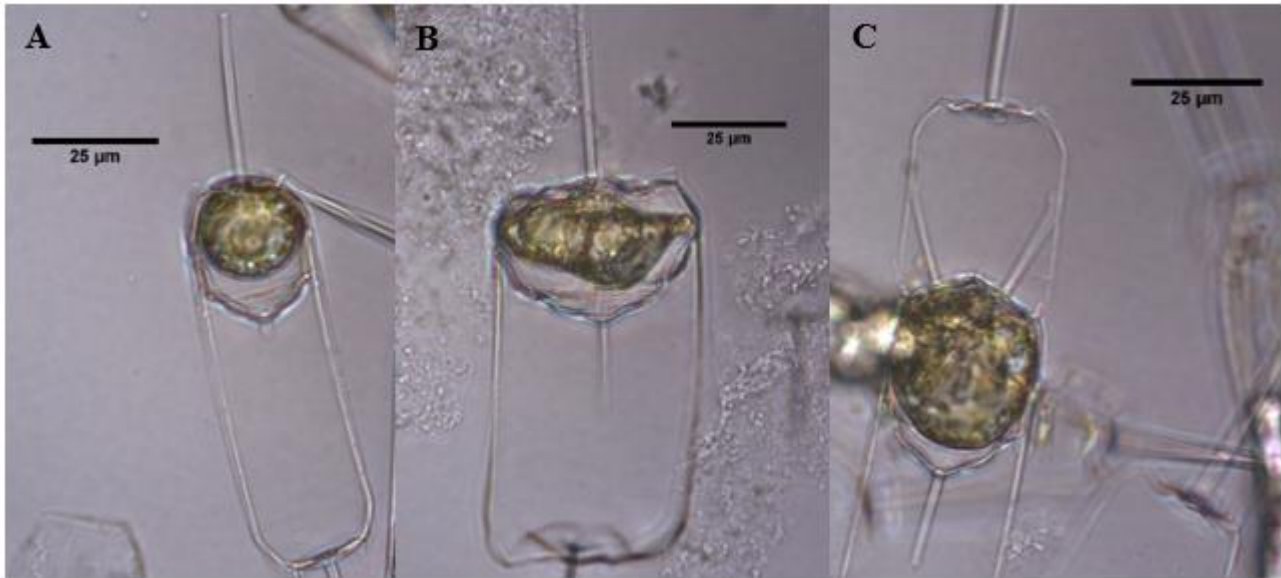


Figure 16 – Resting spore cells of *D. brightwellii* (A = resting spore, B= wide resting spore, C= resting spore that has a different orientation and two processes on one half of the cell)

Discussion

I. Cell enlargement

Size classes of *D. brightwellii* clone PLY-609 indicate that enlargement is an ongoing process and that there is not a large enlargement event occurring in the culture. Frequent light microscopy observations of PLY-609 indicate that there is a low level of enlargement occurring each day. The induction of enlargement was able to occur when a culture of stressed, nutrient deprived diatoms received an aliquot of nutrient replete medium. *D. brightwellii* was not induced for enlargement through inoculating a stressed, nutrient deprived aliquot of cells into a flask containing fresh, nutrient replete f/2 medium. When inoculated into fresh, nutrient replete medium, the stressed and starved cells did not undergo enlargement and had slower growth. This suggests that inoculating stressed cells into fresh, nutrient replete medium may shock them and hinder their ability to continue functioning properly for a period. Two types of enlargement were observed to occur in *D. brightwellii*: one-step enlargement and multi-step enlargement.

One step enlargement is the “classic” enlargement where a diatom cell will produce a new, larger siliceous structure outside of their cell body which allows them to expand and continue dividing. In *D. brightwellii*, one step enlargement had two forms, the first is the cell producing a new, larger valve after it has expelled or shed an older valve as in Figure 11B and C. The second form of one-step

enlargement in *D. brightwellii* is the cell appears to fold in half, and it produces a new valve at the open corner as in Figure 11A. Both forms of one step enlargement result in a larger, slightly rounded, abnormal valve and allow the cells to continue to survive and divide.

Multi-step enlargement is a novel form of enlargement found in *D. brightwellii*. Multi-step enlargement can be described as a single cell producing supernumerary valves that progressively become larger. *D. brightwellii* has been known to produce supernumerary valves and were observed by Sutcliff (2013). Previous studies of supernumerary valves in *D. brightwellii* did not show that they allowed the cell to enlarge (Sutcliff 2013). Cells with extra valves were a result of acytokinetic mitosis which signified a nuclear division and a new valve was produced in the absence of a cytoplasmic division (French and Hargraves 1985). Sutcliff (2013) described the nuclear behaviour of vegetative cell enlargement in *D. brightwellii* and found that two mitotic divisions occur with pyknotic nuclei. Due to the constant on-going process of enlargement in my culture flasks through the duration of my study, multi-step enlargement may be a variation of vegetative cell enlargement in *D. brightwellii* that enable it to remain at a certain size for a long period of time.

Cells that underwent enlargement in my study increased their average diameter size from 26.4 ± 7.6 μm to 32.1 ± 7.6 μm ($n = 39$). The size of valve diameter for the oldest valves ranged from $15.8 - 47.8$ μm and the size range for the newest valves was $20 - 48.6$ μm . *D. brightwellii* cells in the Koester et al. (2007) study that underwent enlargement had a size range of $27-53$ μm and a mean of 40 μm . Cells that are the result of sexual reproduction were found to be within the range of $47.5-71$ μm in diameter with a mean of 59 ± 5 μm (Koester et al. 2007). Pre-sexual cells of *D. brightwellii* were found to have valve diameters within the size range of $12-26$ μm and did not sexually reproduce when they were larger than 26 μm (Koester et al. 2007). The cells that enlarged in my study were similar in size to those in Koester et al. (2007) and they were much smaller than cells that are the result of sexual reproduction.

Cell enlargement is an adaptive life history strategy that *D. brightwellii* utilizes in order to introduce larger cell sizes to the population without sexually reproducing. Other species of diatoms use vegetative cell enlargement as an alternative way to introduce larger cell sizes to the population such as: *Skeletonema costatum*, *Eucampia zodiacus*, *Coscinodiscus wailesii* (Gallagher 1983; Nagai et al. 1995; Nishikawa 2017) Vegetative cell enlargement or asexual enlargement is an advantage because sexual reproduction in many species of diatoms is highly specific and requires certain environmental parameters that may not always be present. There are several hypotheses for the triggers of diatom sexual reproduction such as stressful conditions due to nutrient depletion, variation in photoperiod or

light intensity, or cell size reduction (Amato 2010). Species of diatoms that are capable of asexual enlargement have the advantage of remaining present in the water column until the environmental conditions allow them to sexually reproduce. In *D. brightwellii*, there appears to be a constant level of enlargement occurring within the population which may be an advantageous adaptation to an unpredictable life at sea. Vegetative cell enlargement does not allow for genetic recombination, a purpose for enlargement may be to keep certain species of diatoms, such as *D. brightwellii*, within a viable size range within the water column that will remain capable of sexual reproduction when the conditions are ideal.

II. Resting cells

Resting cells of *D. brightwellii* have frequently been investigated although the presence of pseudopods has not been observed or described. Gross described spherical bodied resting spores that extend pseudopods as a method of germinating (1937). The germination of resting spores begins by the spore extending protoplasmic filament-like pseudopods that connect the cell to the cell wall and allow the protoplast to expand until it appears as a vegetative cell (Gross 1937). Gross also observed resting spores that would develop a siliceous membrane that allow them to persist as spores for a long period of time (Gross 1937). Free resting cells of PLY-609 were observed to produce a new siliceous structure (Figure 14B,D).

Pseudopodal cells are not common in diatoms. The extension of pseudopods in vegetative cells by *D. brightwellii* is a novel concept in this case. Centric diatoms such as *D. brightwellii* are not known to be capable of locomotion although diatoms with raphes can move (Wang et al. 2013). The pseudopod-like structures of *D. brightwellii* were frequently observed to be freely moving on the microscope slides and not moving in association with the water current. It was unclear whether the pseudopods were produced for the purpose of locomotion, nutrition, or the production of new siliceous structures. The pseudopod-like structures were frequently observed to be freely moving on a microscope slide that also contained bacteria (cultures were not axenic). The pseudopods also appeared to move in association with the bacterial movement, as if the cell was attempting to consume the bacteria. On other occasions, the pseudopod-like structures enabled cells of *D. brightwellii* to shift and glide along a microscope slide. On further observations of naked resting cells with pseudopod structures under the epifluorescent microscope, it was apparent that the cells had recently produced a siliceous ring around the cell. Due to each of these vastly different instances of free resting cells with pseudopod structures, their purpose remains unclear. Similar structures have been observed in *D. brightwellii* previously by

Pollock and Pickett-Heaps (2005) during a cell recovering from plasmolysis. The resting cells and pseudopodal structures in my study are different from cells recovering from plasmolysis because the cells in my study remained a generally spherical shape while extending the pseudopodal structures. The cells recovery from plasmolysis first appear similar to those in my study as they are spherical in shape but when they begin to extend their filopodia and attach to the inside of the cell wall, they lose their spherical shape and are able to recover (Pollock and Pickett-Heaps 2005). The cells in my study that appeared resting and extended pseudopods remained in a spherical-shape state and often exited the valves. When the free resting cells were extending pseudopodal structures in the medium, they also retained a spherical-like structure. The cells that appear to be resting or plasmolysed in my study but seem to be vegetative are a different life stage of *D. brightwellii* than that shown Pollock and Pickett-Heaps (2005)'s study. Further investigation of resting cells in *D. brightwellii* and the fact that they are not "resting" will be required to determine why they occur and how they may be advantageous.

III. Resting spores

Resting spores are formed by *D. brightwellii* in during periods nitrogen limited conditions. This is an advantageous survival strategy as it allows the planktonic, oceanic diatom to survive unfavourable conditions for long periods of time. Resting spores may allow diatoms to survive adverse conditions for a long or short period of time, can act as a dispersal device, or allow certain species to pass through the guts of predators (French and Hargraves 1980). This advantage of being able to germinate despite passing long periods of time resting differs from the sexual process of auxosporulation because an auxospore must germinate within a few days or it becomes unviable (French and Hargraves 1980).

Prior to the formation of resting spores, the plastids in the cells of *D. brightwellii* lost their dark colour and the cells plasmolysed. For the period between inoculation and the formation of resting spores, the cells remained plasmolysed and had a reduction of colour in their plastids. French and Hargraves (1980) observed the loss of 75% of photosynthetic capacity in resting spores of *D. brightwellii* and suggested that there may be a breakdown of the spores' photosynthetic apparatus. The resting spores of *D. brightwellii* are often observed to remain inside the mother cell and the valves are heavily silicified. The resting spores were formed in both 12:12 and 15:9 light:dark cycles. Resting spores of *D. brightwellii* can vary in shape and size but they all possess a triangular shaped valve.

Conclusion:

In conclusion, *D. brightwellii* has a few alternate life history processes that may allow it to survive non-ideal, nutrient deprived conditions such as two methods of vegetative cell enlargement, the formation of resting cells, and resting spores. Advantageous survival strategies become apparent in a culture population of *D. brightwellii* after approximately 2 weeks and when inoculated in nutrient replete conditions can continue to survive. One-step cell enlargement allows a cell to increase in size immediately and multi-step enlargement allows a cell to slowly enlarge as it produces multiple progressively larger valves. Cells with multiple valves also had pyknotic nuclei which signified that a nucleus divided, a new valve was produced but the cytoplasm did not divide. The earliest stages of supernumerary valve production remain unclear, but the process does allow *D. brightwellii* to vegetatively enlarge. *D. brightwellii* is also capable of forming resting cells due to unfavourable conditions. Resting cells appear morphologically like vegetative cells although they contain dark plastids that are aggregated in a sphere that is often located at one edge of the cell. Resting cells were observed to extend pseudopod-like structures and to exit the cell valves they were contained in. It was unclear whether the pseudopod structures were intended for locomotion, nutrition, or the production of new siliceous structures. The resting cells with extended pseudopods were capable of movement on a microscope slide and were observed under the epifluorescent microscope to have produced new siliceous structures in a halo-like shape. The resting cells of *D. brightwellii* were capable of movement and producing new structures and it was clear that they were not “resting”. More information will be required on resting cells of *D. brightwellii* to determine how advantageous they are or why they occur. Resting spores were formed when *D. brightwellii* was exposed to nitrogen limited conditions, and they were capable of germination after being introduced to nitrogen replete medium. *D. brightwellii* has multiple, survival strategies that must be further investigated to determine the importance of each process in its life history. Each life history strategy may offer *D. brightwellii* different adaptive advantages through its life cycle and should be further investigated.

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Appendix

I. Culture medium

f/2 Medium:

NaNO ₃ (75.0 g/L dH ₂ O)	1.0 mL
NaH ₂ PO ₄ · H ₂ O (5.0 g/L dH ₂ O)	1.0 mL
Na ₂ SiO ₃ ·9H ₂ O (30.0 g/L dH ₂ O)	1.0 mL
f/2 Trace Metal Solution	1.0 mL
f/2 Vitamin Solution	0.5 mL
Double filtered seawater to	1.0 L

Autoclave with all contents except the f/2 Vitamin Solution and 0.5 mL of Na₂SiO₃·9H₂O , cool in cold room for 24 hours and add remaining contents before use

T Medium :

From French and Hargraves (1980)

“T” medium consists of a f/2 modification of ammonia (replacing nitrate) at 15 µM and phosphate at 7 µM, the trace metals, vitamins and silicate remained the same as f/2. The ammonia was added aseptically to the sterilized medium after being cooled for 24 hours.

II. Measurements of cell diameter and length of *Ditylum brightwellii* (PLY-609) cells (n = 155)

<u>Image code</u>	<u>Pervalvar length (µm)</u>	<u>Valve diameter (µm)</u>
07312019_0001	81.62	26.776
07312019_0002	78.123	35.404
07312019_0003	80.982	23.429
07312019_0004	74.122	19.541
07312019_0005	96.236	31.043
07312019_0006	80.739	23.729
07312019_0007	67.85	21.327
07312019_0008	79.137	22.763
07312019_0009	98.089	20.295
07312019_0010	84.75	33.528
07312019_0012	63.105	35.748
07312019_0013 left cell	75.884	22.615
07312019_0014 middle cell	57.737	39.096

07312019_0015 right cell	80.935	23.33
07312019_0018	92.75	32.647
07312019_0019	62.618	22.207
07312019_0021	95.288	47.294
07312019_0023	83.557	42.177
07312019_0024	82.072	24.687
07312019_0025	81.801	23.216
07312019_0026	92.959	21.968
07312019_0027	88.559	33.259
07312019_0029	52.568	26.347
07312019_0033	95.318	25.601
07312019_0034	69.412	26.46
07312019_0035	78.153	23.269
07312019_0036	110.637	30.134
07312019_0040	100.99	24.216
07312019_0041	87.193	23.795
08082019_0001	93.008	20.057
08082019_0002	69.647	43.436
08082019_0003	74.825	20.138
08082019_0004	84.136	30.796
08082019_0005	96.099	17.908
08082019_0006	89.072	21.924
08082019_0007	91.558	22.312
08082019_0008	90.704	25.282
08082019_0010	79.466	17.787
08082019_0011	73.803	34.667
08082019_0012	99.343	24.987
08082019_0013	84.572	23.845
08082019_0014	77.669	35.82
08082019_0015	54.324	22.031
08082019_0016	79.115	33.126
08082019_0017	75.985	23.319
08082019_0019	85.149	21.968
08082019_0020	70.781	19.041
08082019_0021	53.727	17.536
08082019_0022	95.329	22.045
08082019_0023 top cell	70.037	22.388
08082019_0023 bottom cell	85.335	29.381
08082019_0024	102.09	19.828
08082019_0025	76.909	27.103
08082019_0026	70.692	40.045
08082019_0027	85.949	42.448

08082019_0028	44.362	36.291
08082019_0030	96.825	23.587
08082019_0031	48.533	36.263
08082019_0032 top left	98.766	25.181
08082019_0032 bottom right	83.013	17.51
08082019_0033	55.358	23.942
08082019_0034	82.43	27.136
08082019_0035	84.192	32.57
08082019_0036	73.2	34.735
08082019_0037	84.996	22.155
08082019_0038	87.314	16.966
08082019_0039	80.804	32.32
08082019_0040 top left	75.004	40.247
08082019_0040 bottom right	85.151	23.442
08082019_0041 right	88.507	40.595
08082019_0041 left	52.287	30.768
08082019_0042	90.393	26.062
08082019_0043	90.932	26.744
08082019_0044 top	65.692	29.729
08082019_0044 bottom	79.893	29.766
08082019_0045 left	68.742	32.117
08082019_0045 right	89.274	19.394
08082019_0046 right	82.637	20.897
08082019_0047	82.775	29.698
08082019_0048	71.182	31.948
08082019_0049	48.312	45.061
08082019_0050	70.725	51.23
08082019_0051 top	69.59	55.188
08082019_0051 bottom	81.06	23.195
08082019_0052 top	46.597	38.448
08082019_0052 bottom	72.978	43.281
08192019_0001	81.174	19.691
08192019_0002	87.017	22.532
08192019_0003	99.96	21.187
08192019_0005	97.777	23.624
08192019_0006	78.129	24.584
08192019_0007	86.137	30.19
08192019_0008	88.485	20.953
08192019_0009	91.242	24.639
08192019_0010	76	23.577
08192019_0011	75.884	18.571

08192019_0013	81.564	22.652
08192019_0014	66.298	22.273
08192019_0015	70.585	38.085
08192019_0016	76.411	35.777
08192019_0017	68.564	30.895
08192019_0018	78.392	31.181
01242020_0009	71.366	29.93
01242020_0010	74.146	19.954
01242020_0011	72.511	26.853
01242020_0012	77.006	22.084
01242020_0013	63.786	29.833
01242020_0014	41.951	33.411
01242020_0015	87.312	37.482
01242020_0016	68.333	28.098
01242020_0017	74.58	22.725
01242020_0018	54.59	19.716
01242020_0020	80.051	25.341
01242020_0021	72.158	44.001
01242020_0022	69.257	19.492
01242020_0023- top cell	65.04	30.118
01242020_0024 - bottom cell	83.033	21.265
01242020_0025	76.077	21.552
01242020_0027	87.486	31.583
01242020_0028	77.654	21.875
01242020_0029	71.663	24.932
01242020_0030	66.292	18.028
01242020_0031	70.164	31.141
01242020_0032	82.207	24.264
01242020_0033	71.194	30.17
01242020_0034 top cell	72.263	23.047
01242020_0035 bottom cell	90.124	25.574
01242020_0036	63.492	26.114
01242020_0037	70.953	26.768
01242020_0038	54.835	20.754
01242020_0039	73.227	30.271
01242020_0040	69.599	21.246
01242020_0041	59.603	35.411
01242020_0043	61.782	30.487
01242020_0044	72.641	24.838
01242020_0045	85.96	16.836
01242020_0046	79.555	27.676

01242020_0047	78.394	26.924
01242020_0048	48.849	24.955
01242020_0049	40.821	27.556
01242020_0050	79.933	16.013
01242020_0051	78.231	25.404
01242020_0053	76.937	23.374
01242020_0054	83.579	21.048
01242020_0055	66.968	21.453
01242020_0056	69.379	18.522
01242020_0057	51.984	17.669
01242020_0058	48.409	21.89
01242020_0059	50.965	30.851
01242020_0060	65.381	30.521
01242020_0061	43.869	21.27
01242020_0062	75.667	24.347
01242020_0064	72.042	40.323
01242020_0066	81.524	40.764
01242020_0067	63.766	30.147

III. Supernumerary valve measurements

<u>Image code</u>	<u>Oldest valve (μm)</u>	<u>Newest valve (μm)</u>
11192019_0015	33.1	43.7
11192019_0018	31.9	33.9
11192019_0019	25.8	26.7
11192019_0022	16.0	35.3
11192019_0027	28.5	26.7
11192019_0030	28.7	29.4
11192019_0033	17.7	42.4
11192019_0037	25.4	28.5
11192019_0038	21.7	23.0
11212019_0002	26.1	24.7
11212019_0012	31.5	31.7
11212019_0021	29.0	30.4
11212019_0026	47.8	46.3
11212019_0038	25.1	22.9
11212019_0046	26.6	27.3
11222019_0013	46.4	48.6
11222019_0049	30.5	40.5
11232019_0001	20.4	24.4
11232019_0008	26.9	30.5
11232019_0031	26.2	28.5

11242019_0001	22.8	27.6
11242019_0004	25.0	29.0
11252019_0013	18.4	26.4
11262019_0003	16.1	26.4
11262019_0005	30.0	31.5
11272019_0009	21.3	24.0
11272019_0011	20.5	20.0
11272019_0057	15.8	37.6
11272019_0071	23.5	39.2
11282019_0043	31.3	36.7
11282019_0072	22.3	41.1
12012019_0005	26.8	32.1
12012019)0008	41.9	44.5
12012019_0012	30.2	37.0
12012019_0055	22.5	21.3
12012019_0059	17.9	28.6
12012019_0078	38.2	42.7
12022019_0009	22.8	36.9
12022019_0033	18.7	22.9