RESTING SPORE FORMATION AND REVIVAL FOR THE DIATOM, *THALASSIOSIRA PSEUDONANA*

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Abstract: In this study, the model diatom *Thalassiosira pseudonana* was examined to determine whether it would form a unique resting spore morphology in response to nitrogen deprivation. Results demonstrate that substantial spore formation only occurs under low nitrogen conditions when kept at a room temperature. When kept at 5–10 °C, spores formed in both control and low nitrogen conditions, although the low nitrogen culture exhibited a higher conversion rate to the resting spore state. Additionally, a second diatom, *Phaeodactylum tricornutum*, only formed a substantial number of spores under low nitrogen conditions. While unable to revive freeze-dried and air-dried spores, *T. pseudonana* spores from a 2-year-old culture were able to be returned to a vegetative state and established a new culture. The potential to revive spores could provide for methods to ship these useful organisms to space colonies without taking up large amounts of spacecraft payload, such as by removing the necessity to keep them alive in large, water filled photobioreactors.

Introduction

Diatoms are microscopic algae that live all over Earth, both in freshwater and ocean habitats.[1] They are estimated to contribute to the global carbon cycle as much as all rainforests combined.[1] There are numerous different genera and species of diatoms, all with unique morphologies.[2] A distinctive feature of most diatoms is their silica frustule, which is a glass enclosure that surrounds the diatom and protects it.[1] These frustules have an intricate nanostructure, which makes them of interest to nanoengineers.[3] Among diatoms, there are two distinct classes: centric diatoms, which are radially symmetrical, and pennate diatoms, which exhibit bilateral symmetry.[4] The main diatom of focus in this study, *Thalassiosira pseudonana*, is a centric diatom. The diatom, *Phaeodactylum tricornutum*, is pennate and is briefly considered to act as a control but is not the focus of the study. Because of their rigid silica frustule, diatoms decrease in size when they asexually divide via mitosis.[5] To prevent becoming too small, at a certain minimum size, diatoms will engage in sexual reproduction, which results in a new diatom with the original (largest) cell size to be formed.[5] *T. pseudonana* is unique in that it avoids the shrinking that most diatoms experience, which leads it to be primarily asexual, but it does have a sexual form.[6,7] When diatoms are under conditions of environmental stress, they may enter what is called a resting spore state.[8] Conditions that constitute environmental stress are lack of nutrients, changes in salinity or water temperature, and drops in light intensity.[8] Resting spores tend to have a distinct morphology from the vegetative cells they arise from.[8] Their silica frustule may undergo increasing silicification and their cytoplasmic components become more condensed, which leads to a darker appearance of cytoplasmic matter.[8] This change in morphology helps to

facilitate sinking out of the water column to the bottom of the water body, which decreases the risk of grazing by potential predators.[9] Once at the bottom, the resting spore cell waits until conditions improve and it is whisked back into the water column by perturbations in the water and exposed yet again to sunlight.[10] Only then will it potentially reemerge from the resting state and return to the vegetative state.[10] It is hypothesized that resting spores are what allow for the seasonal blooms in diatoms each year.[9]

Establishing colonies of diatoms may be of interest to space travel and colonization endeavors. Diatoms are small, but efficient producers of oxygen, which can help provide livable conditions in space colonies.[1] Diatoms are also an integral food source for many organisms, so the establishment of diatoms could potentially be the basis for the farming of other species, such as fish.[2] The silica frustule of the diatoms may also prove of use. Research is already being done into the use of diatom frustules to create hierarchically structured porous ceramics that can withstand up to 800℃ with strength close to that of concrete with only half the density.[11] The special silica nanostructure have many uses, such as in medicinal applications[12,13] and chemical/engineering applications.[14] *T. pseudonana* was examined specifically in this study because it is a model diatom currently being studied in many research projects.[6] Resting spores are of particular interest because they could facilitate transport of diatom species to space stations and extra-terrestrial colonies. Shipping diatoms into space in their vegetative state would require incubators filled with water. This would dramatically increase shipment costs as well as take up precious space on a spaceship. If diatoms can be encouraged to enter a resting spore state, it may be possible to store them in a more travel-efficient way, such as in a dry-powder form, and then coax them back into a vegetative state at their destination.

Methods

Regular Temperature Conditions

T. pseudonana diatoms were obtained via sampling from the photobioreactor culture maintained in the laboratory. This culture is a 23 L culture stored in a 130 cm tall x 15 cm wide tube that is sealed from the outside, contains an air stone for air circulation, and receives an 18-hour light/6-hour dark cycle from aquarium-grade lights. The culture is harvested every 7 days, and the reactor is filled with new F/2 media, which is a high nutrient medium used for algal growth, every 7 days. Five mL of media containing *T. pseudonana* was removed from the photobioreactor and placed in a beaker. The beaker was stirred to evenly disperse the *T. pseudonana* throughout the solution. Ten µL was removed using a P10 micropipette and placed onto a hemocytometer. The hemocytometer sample was then examined using an Olympus IX71 inverted light microscope and photographs at a 20x magnification were taken for analysis using FIJI (ImageJ). Utilizing the FIJI cell counter plugin, the concentration of *T. pseudonana* per mL was calculated. Using this concentration, the amount of µL needed to achieve 800,000 cells was calculated. Ten cell culture flasks were divided into two groups: a control group with standard F/2 media, and a low nitrogen group, which contained a nutrient medium with the same composition as the F/2 medium, but without the nitrogen added in. Into the five control flasks, 14 mL of standard F/2 media was added. Into the five low nitrogen flasks, 14 mL of the low nitrogen F/2 media was added. Following this, the previously calculated amount of µL necessary to achieve an initial amount of 800,000 *T. pseudonana* was added to all ten flasks. Each flask was then topped off with its respective medium to reach a total volume of 15 mL. All ten culture

flasks were then placed in a sealed incubator. At the top of this incubator was a Hygger HG075 planted+24/7 aquarium LED light placed 12.5–15 cm away from the cell culture flasks that was set to a 16-hour light/8-hour dark cycle. A constant humidity was maintained via a water dish placed at the bottom of the incubator to prevent evaporation from the cell culture flasks. Except for days 2 and 4, the cultures were examined every 3 days until the 19th day after inoculation. After being shaken, 10 µL from each of the ten cultures was extracted and examined on a hemocytometer using the Olympus IX71 light microscope. Photographs were taken of six of the small squares within one large square on the hemocytometer grid. Photographs were taken at a 20x magnification to capture all six small squares. Then, to properly determine the total concentration of spores, the magnification was increased to 40x and each of the six small squares was photographed individually at different focuses until all *T. pseudonana* present in that square were sharply resolved in at least one photograph. The number of vegetative and spore cells in each photograph were counted manually.

Low Temperature Conditions

Due to the inability to convert the original incubator to an incubator that could consistently maintain temperatures of 5–10 ℃, a Frigidaire 6-Can Retro Mini Fridge was utilized to act as the incubator for the low temperature samples. A Rodinto plant-growth light was placed inside the Frigidaire on the top rack and set to 3500k white light. The temperature within the fridge was monitored via a thermometer and was consistently between 5–10 ℃. The light setting was set for 12 hours of luminance and 12 hours of dark. Unlike the regular temperature group, a second diatom species, *Phaeodactylum tricornutum*, was also examined under cold conditions. The purpose was to determine if the process of spore formation that worked for *T. pseudonana* also worked for another diatom species. To minimize the risk of nitrogen from stock solutions contaminating the low nitrogen cultures and to increase the number of cells inoculating the cultures, 5 mL of culture media from the *P. tricornutum* culture was split and placed into two centrifuge tubes and spun down using a Hettich Universal 320 centrifuge set at 7,000 rpm for 10 minutes. The same was performed on the *T. pseudonana* culture. The supernatant was poured off, leaving only a pellet of the diatoms from each culture. These four pellets allowed for the creation of four groups: control *P. tricornutum* and control *T. pseudonana* which had their pellets inoculated in 15 mL of the regular F/2 media; and low nitrogen *P. tricornutum* and low nitrogen *T. pseudonana*, which had their pellets inoculated in the low nitrogen modified F/2 media. After inoculation, the four culture flasks were placed into the sealed fridge and were left to incubate for 14 days.

Revival of Spores - 3 Methods

The goal of spore revival was not just to return spores from their resting spore state to their vegetative state, but also to try to remove liquid from the culture to mimic conditions ideal for space shipment. Three methods were utilized and will be discussed below in their sections: 1.) Simple revival from a liquid culture; 2.) Revival of Freeze-Dried Diatoms; 3.) Revival of Air-Dried Diatoms. Methods 2 and 3 were used for both the control and low nitrogen conditions. The reason was to determine whether spores were necessary in the first place. If the control cultures, which had no spores, were able to be revived, then the spore-formation process may not be necessary to establish a colony. Method 1 does not have a control and low nitrogen group because there is no two-year-old culture of diatoms that was stored in a fridge that is not in spore form.

Revival of Spores - Method 1

The first method of spore revival was simply to examine whether *T. pseudonana* spores could return to their vegetative state and establish a new and growing culture after long-term storage. If this was unable to occur, then there would be potentially no evidence of *T. pseudonana* being a viable diatom for spore usage. This method involved the attempt to revive a *T. pseudonana* culture that had been stored in the laboratory fridge at 37 °C for around two years. 10 µL was extracted from the two-year-old culture to look for the presence of spores under the Olympus microscope. A small number of *T. pseudonana* spores were present in the culture. Following this, 1/3rd of the original fridge culture (about 3–4 mL) was removed from the original vial and put in a new cell culture flask. The cell culture flask was then topped up to 15 mL total by adding regular F/2 media. The flask was then stored for 14 days in the regular temperature incubator (see conditions described in "Regular Temperature Conditions" above).

Revival of Spores - Method 2

The second method for reviving spores involved the process of removing liquid from the culture via freeze-drying. This step utilized practice-run cultures created in a method the same as that described in the "Regular Temperature Conditions" section. There were four cultures total - two control cultures and two low nitrogen cultures in cell culture flasks. The cultures were poured into 15 mL centrifuge tubes (divided into a low nitrogen tube and a regular tube), placed in the Hettich centrifuge, and centrifuged at 7,000 rpm for 10 minutes. The supernatant was poured off. Then, the second culture was poured into the same centrifuge tube for its respective culture and centrifuged again. The reason for a second centrifugation was due to the tube's capacity being half that of each group's total volume. The supernatant was again poured off. Then, each pellet was washed with DI water to eliminate salts before being flash-frozen with liquid nitrogen. Following this, the tubes were placed on the lyophilizer/freeze-dryer (to sublimate the water) overnight and removed the next day. The tube was stored in the fridge until 41 days later. When needed, the tube was removed from the fridge, refilled with 15 mL regular F/2 media, and shaken to resuspend the pellet. The liquid was poured into a cell culture flask and stored in the regular temperature incubator. The culture was imaged on the 1st, 7th, 14th, and 18th days of the revival attempt.

Revival of Spores - Method 3

This third method used simple air-drying to remove water. Yet again, practice-run cultures were utilized and were created in the same manner as that described in the "Regular Temperature Conditions" section. There were four cultures total - two control cultures and two low nitrogen cultures in cell culture flasks. The same centrifugation steps as in Method 2 were used. Once centrifuged down, the cap of the centrifuge tube was lightly placed on top of the tube, but not sealed tightly, to allow more evaporation. The tubes were placed in a beaker containing Drierite desiccant at the bottom. The top of the beaker was sealed with Parafilm and stored in the hood for a week. The centrifuge tubes were removed, and the pellet was resuspended using regular F/2 media. The culture was then transferred into cell culture flasks and placed into the regular temperature incubator for revival. Cultures were examined on the 1st, 3rd, 5th, 8th, 11th, and 21st days since the revival attempt.

Data Analysis

All cultures were examined by utilizing photographs taken using the Olympus microscope. These photos were stored in the cloud and examined manually later. When analyzed, the total amount of cells per culture

was counted manually by using FIJI (ImageJ)'s cell counter. Following this, the number of spores was counted by looking for distinct morphological differences between the resting spore cells and vegetative cells. The criteria to be a resting spore was to have cytoplasmic contents clustered into a single circle that tended to be at the edge of the frustule (See Figure 2D). Data was managed and graphed in Microsoft Excel and statistical testing was performed using R.

Results

Regular Temperature - Overall Cell Density

Both the control *T. pseudonana* and low nitrogen *T. pseudonana* began with an initial concentration of about 0.05 million cells per mL (800,000 cells placed into a culture flask of 15 mL culture solution). Both increased in cell density at roughly the same rate until the control surpassed the low nitrogen group at day 4 with a concentration of about 4.1 million cells per mL compared to the low nitrogen group with 3.5 million cells per mL. However, at day 10 and beyond, the control *T. pseudonana* culture began to rapidly decline in cell concentration. By day 12, the concentration was at 1.4 million cells per mL and stayed relatively stable. When examined later the 30th day, both cultures had an extremely low number of cells. Rather than *T. pseudonana* cells being the main organism present, a new organism had taken their place. This organism was roughly the same size as *T. pseudonana*. It had a flagellum and could move throughout the medium. It is speculated this organism may be a species of dinoflagellate and will be referred to henceforth as such. The cultures were also examined again at day 44 and these new organisms were still dominant and surviving in the culture, although another alga or fungal strain appeared to be growing as well. No remaining *T. pseudonana* were found at 44 days after inoculation.

Regular Temperature - Proportion Spores

Regarding the formation of resting spores in the two treatment groups, the control *T. pseudonana* group failed to form a substantial number of resting spores until day 19 with a maximum of about 11% resting spores. On the other hand, the low nitrogen *T. pseudonana* group began to form resting spores on the 4th day of incubation and then rapidly increased to a percentage of 39% spores by day 7. By the final day measured, the average percentage of spores present was 75%. However, on day 30th, the low nitrogen *T. pseudonana* culture was also dead.

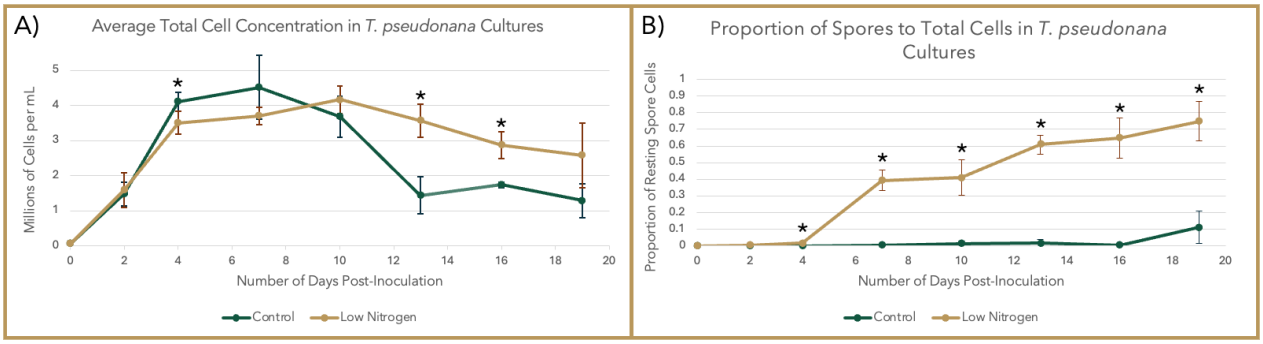

Figure 1: (A) The average growth rate of the two *T. pseudonana* cultures (n = 5) are depicted. Not depicted on the graph is the entire death of both cultures by day 30th and the emergence of a new predominant organism, presumed to be a dinoflagellate. Error bars represent +/- 2SE. Asterisks represent a statistically significant difference ($p < 0.05$) between the control and low-nitrogen treatments on each day as determined via a Mann-Whitney U test. **(B)** The average

proportion of *T. pseudonana* cells in a resting spore state relative to the total number of cells in each of the two cultures (n = 5) is depicted. Error bars represent +/- 2SE. Asterisks represent a statistically significant difference ($p < 0.05$) between the control and low-nitrogen treatments as determined via a Mann-Whitney U test.

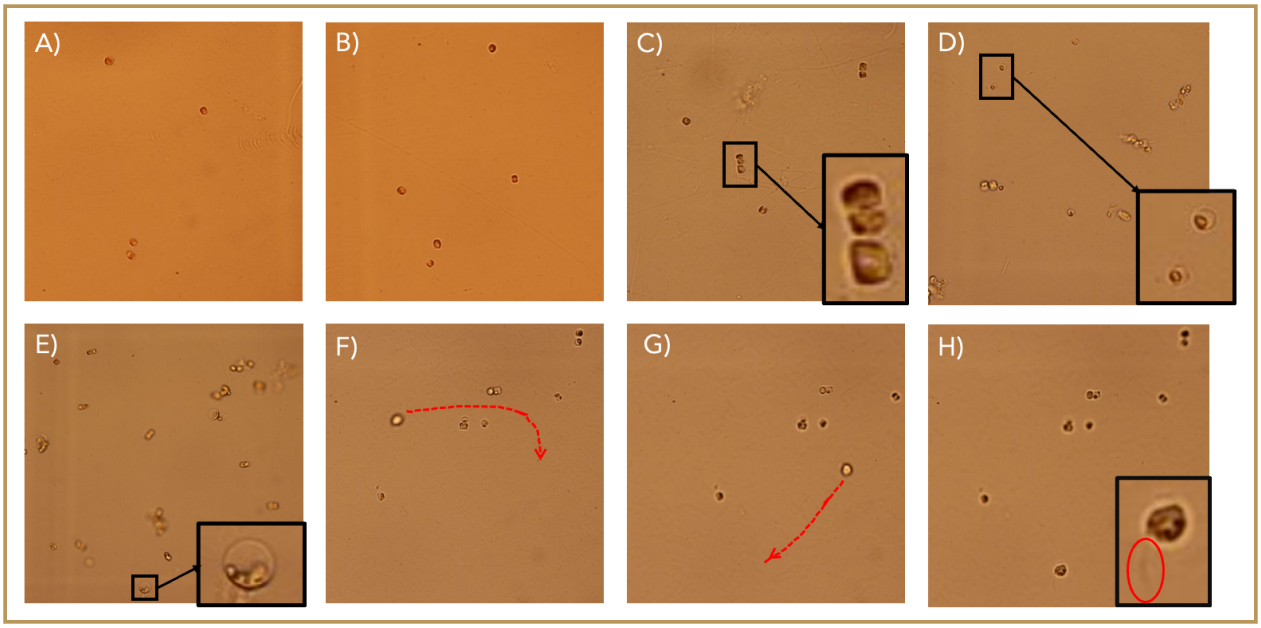

Figure 2: (A) Control *T. pseudonana* culture (2 days post); **(B)** Low Nitrogen *T. pseudonana* culture (2 days post); **(C)** Control *T. pseudonana* culture (19 days post) with inset of vegetative cell; **(D)** Low Nitrogen *T. pseudonana* culture (19 days post) with inset of resting spore cell; **(E)** Control *T. pseudonana* (13 days post) with inset of sexually-reproductive oogonium; **(F, G, H)** Movement pattern of potential dinoflagellate with Inset of Organism

Low Temperature - Overall Cell Density

For the *T. pseudonana* cultures, both cultures grew at roughly the same rate with the low nitrogen culture only be somewhat behind the control culture in average total cell concentration. Interestingly, the cultures had not died by the 32nd day, unlike what was seen in the regular temperature cultures. In addition, unlike previously seen in the regular temperature culture, the low nitrogen *T. pseudonana* formed spores at a much lower rate. When visually comparing the two samples, the low nitrogen culture had the same turbidity of the control culture but was milky in color rather than a yellow brown. It is possible that a new strain of *T. pseudonana* had formed that was more resistant to low nitrogen treatments, but still would eventually form spores due to being deprived essential nutrients. For the *P. tricornutum* cultures, the control culture continuously grew, even beyond the 32nd day. In contrast, the low nitrogen culture was never able to grow very much at all, which is very different from the *T. pseudonana* low nitrogen culture which tended to perform somewhat similarly to the control culture.

Low Temperature – Proportion of Spores

Regarding the formation of resting spores, unlike in the regular temperature group, the control *T. pseudonana* was able to form

some spores when in the low temperature conditions, although still less than the low nitrogen group. In contrast, the control *P. tricornutum* did form some spores, but not many whereas the low nitrogen culture got up to 87.5% spores, although this is with a very small number of cells.

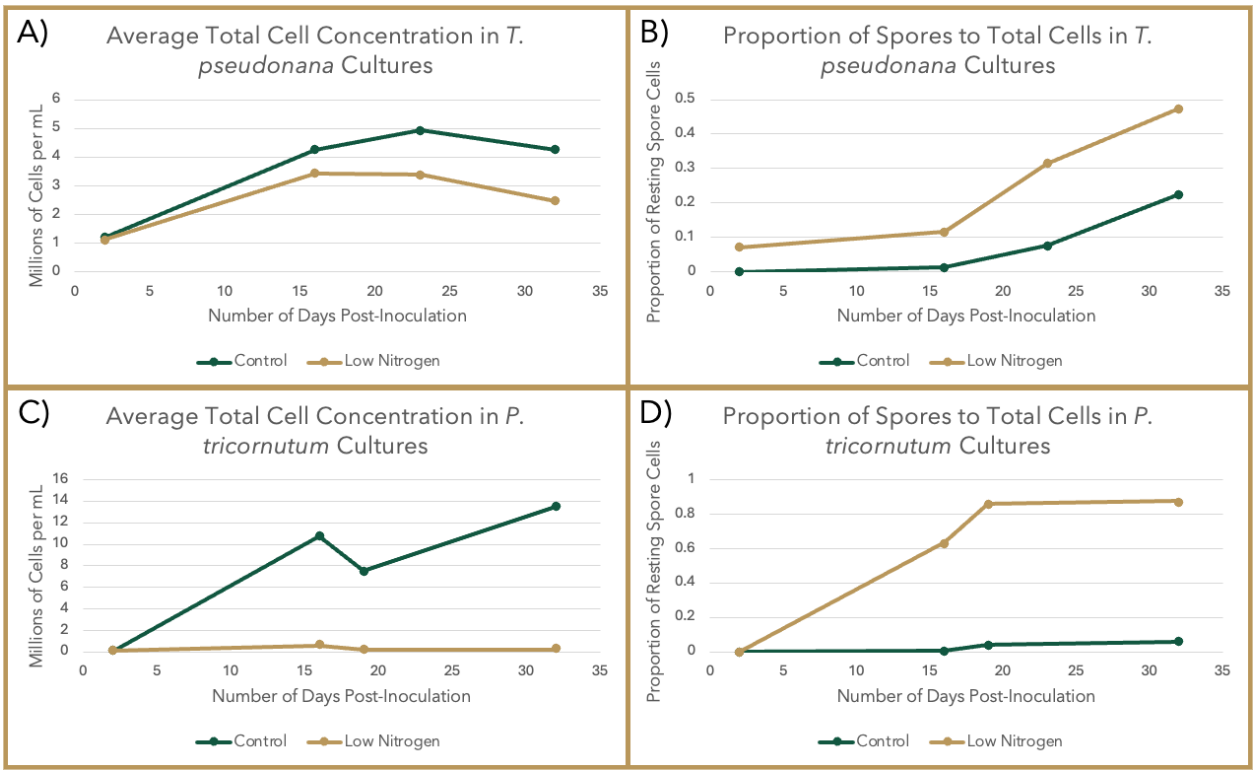

Figure 3: (A) The average growth rate of the two *T. pseudonana* cultures (n = 1); **(B)** The average proportion of *T. pseudonana* cells in a resting spore state relative to the total number of cells in each of the two cultures (n = 1); **(C)** The average growth rate of the two *P. tricornutum* cultures (n = 1); **(D)** The average proportion of *P. tricornutum* cells in a resting spore state relative to the total number of cells in each of the two cultures (n = 1); Due to only having one replicate for A-D, no error bars or statistical tests are shown.

Revival of Spores - Method 1

By day 14, the liquid culture, which had previously only consisted of a small number of spores, had grown to become an established colony. It appears that the revival of *T. pseudonana* from a spore state back to a growing, vegetative state is possible, at least in a liquid culture. By day 14, the newly established culture had a cell density of around 2.3 million cells per mL.

Revival of Spores - Freeze-Dry Procedure

Despite being placed into standard F/2 media and the confirmation of some, albeit a very small amount, of spores being present, the freeze-dried groups were unable to establish a new culture and were not able to be revived from the resting spore state.

Revival of Spores - Air-Dry Procedure

Yet again, despite placement into standard F/2 media and confirmation of some spores, no *T. pseudonana* spores were able to germinate and establish a colony. Unlike the freeze-drying procedure, however, a strain of green algae was able to establish itself within the culture flasks.

Discussion

This research shows that with only nitrogen limitation, *T. pseudonana* diatoms can reach a relatively high proportion of spores to vegetative cells (about 75%). It also demonstrates that *T. pseudonana* is capable of a resting spore state, which has previously not been established in this particular species, although it was established within the genus.[9] Interestingly, despite mimicking conditions of previous research in the same genus, cultures were unable to reach a 100% conversion rate to spores.[9] Despite previous research success with the conversion of resting spore states back into vegetative states, even when found in a desiccated state, this study was unable to achieve this conversion for *T. pseudonana*.[15] However, the study was able to revive *T. pseudonana* from a spore state to a vegetative state in a two-year-old liquid culture. Thus, revival may be possible, but other methods may need to be examined to allow for revival. Regarding the freeze-dried procedure, osmotic shock may have harmed the *T. pseudonana*. In theory, because their rigid frustule influences ion transport and provides structural support, they should be more resistant to osmotic pressure.[16] In regard to the air-drying method, a more pure culture or a process that prevents or limits other algal growth may be needed Because a different strain of algae grew in place of *T. pseudonana, T. pseudonana* could have potentially been revived if not outcompeted by the other algae.

This research also demonstrates the ability of *T. pseudonana* diatoms to achieve a mutation that allows for resistance to low nitrogen conditions, at least for a limited duration. This mutation does not seem to be frequently observed in the wild or laboratory cultures generally. It is possible that this mutation, while not inferring deleterious effects in low nitrogen environments, hinders other aspects of growth and metabolism that prevent it from being prevalent in the general *T. pseudonana* population. Future research is being conducted on this culture to examine if it is solely a quirk of being in low temperature conditions (which is unlikely to be the case due to past research) or if there is an actual mutation in this strain that causes higher resistance to nitrogen deprivation.[9]

This paper also demonstrates that having an axenic culture is essential for these experiments. The regular temperature cultures both died off by the 30th-day post-inoculation and were supplanted by an unknown organism of roughly the same size as the diatoms. Due to its morphology, it is speculated that this organism may be a kind of dinoflagellate. Certain species of dinoflagellates are known to prey upon diatoms, which could explain the disappearance of diatoms from their cultures.[17] In addition, unknown algae supplanted the diatoms in the air-dried *T. pseudonana* cultures and may have prevented their growth. While this alga may have come from their air, it is also possible that it was present in the culture initially, was spun down and stayed in the pellet with the *T. pseudonana* and was able to revive faster when placed back in liquid culture than the *T. pseudonana*. As such, it may have outcompeted them. With an axenic culture, these two problems could have been eliminated and allowed for better results and removal of potential confounds. However, it is interesting that the low temperature

cultures, while still having some amounts of these dinoflagellates, all survived past the 30th day. It is possible that these contaminant species dislike the cold temperatures and using a cold incubator may help prevent damage from them.

This research benefits future efforts to study diatom spore formation and revival by suggesting a standardized approach to inducing spore formation. Various methods need to be improved for this research to become more efficient and accurate. For one, a manual-visual identification of spores is incredibly difficult and introduces user-error. Different researchers may judge what is and is not a spore differently. As such, developing a quantitative method of determining what is or is not a spore is necessary. Because of differences in morphology among spores and vegetative cells, it may be possible to use molecular biology techniques, such as image-based sorting to separate the two.[18] Another aspect is that until the photography and analysis method is streamlined, this research is very time-intensive. Photographing all ten samples for the regular temperature section took three hours consistently, and this was before any image analysis. For this reason, the dates of sampling in this study were unusual and not as regular as desired. This research is best performed by those who have many hours to dedicate to the project and will be able to maintain a regular schedule.

Future avenues to consider are examining how diatoms are buried in the sediment in nature, such as in the ocean. By understanding this system, we may be able to understand how to mimic those conditions and thus allow for spore revival. Another future goal would be to image spore cells using a scanning electron microscope to get an understanding of the potentially different morphology of spore cells verses vegetative cells. This would provide insight into how to identify resting spore cells.

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