Temperature control and encystment of the toxin- producing dinoflagellate*, Alexandrium catenella,* in Puget Sound, Washington

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NON- TECHNICAL SUMMARY

Alexandrium catenella is a dinoflagellate that secretes a neurotoxin. The neurotoxin enters the water column and is concentrated in shellfish when they feed. When humans consume the toxin-concentrating shellfish, they experience Paralytic Shellfish Poisoning (PSP). *A. catenella* occurs in Puget Sound and causes PSP. The main objective of this study was to inventory the presence of the resting stage of this cell in Discovery Bay, Penn Cove, and the Main Basin of Puget Sound and to determine at which temperatures this organism goes into a resting stage in the sediment. The highest concentrations of *A. catenella* were found in central Penn Cove and the northern Main Basin near Triple Junction. In the laboratory, the organism formed a temporary resting stage but not a permanent resting stage at 8°C and 10° C. The active cells also formed chains with one another as a possible reaction to temperature stress. The cells adapt to temperature changes over time in the ranges tested.

ACKNOWLEDGEMENTS

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ABSTRACT

A. catenella cyst abundances were quantified in sediment samples from Discovery Bay, Penn Cove, and the Main Basin of Puget Sound in Washington after they were collected aboard the *R/V Thomas G. Thompson* from 19 March 2007 to 23 March 2007. Possible trigger temperatures to force active cells into cyst stage were measured at 8° C and 10° C. Penn Cove had the most concentrated numbers of cysts with an average of 52.5 cells/mL and the Main Basin at West Point with the least concentrated cysts at 5 cells/mL. The cells in the 8° C room formed temporary cysts after 6 days of exposure while the cysts in the 10° C room formed temporary cysts after 5 days of exposure. The 8° C cells formed chains after 4 days of exposure. The 10° C cells had no discernable pattern in chain formation. These data stress the importance of understanding the conditions that influence the life cycle of *A. catenella*.

INTRODUCTION

Alexandrium catenella is a dinoflagellate that produces saxitoxin, a neurotoxin that is bioaccumulated in shellfish and passed to humans when shellfish are consumed. *A. catenella* cells can be unpredictable with respect to blooms and distribution. This poses a looming threat for the shellfish industry in Puget Sound, Washington. The understanding of *A. catenella* in Puget Sound is far from complete, however, the overall goal of this study is to narrow down the controlling factors that influence the life cycle of this organism. During a bloom, *A. catenella* inundates the water while secreting the neurotoxin that causes Paralytic Shellfish Poisoning (PSP). The toxin is then absorbed by organisms such as clams, oysters, and geoduck larvae. The chemical is not toxic to these organisms because it only affects organisms with complete nervous systems. When humans consume the shellfish farm products, they also ingesting the toxin, which can be fatal. Consumers may be unaware of the pending danger in the shellfish they consume. Some may even assume because it is locally grown, shellfish stock is safe to eat under any condition. However, *A. catenella* blooms and high saxitoxin levels can go unnoticed until the effects are experienced first hand. If the blooms can be predicted with certain probability, toxic events can be avoided. Shellfish farmers can take precautions such as moving their harvest temporarily or safeguarding it in isolated tanks with clean water sources. This would save farmers from needing to dispose of contaminated stock and reduce public health risk. The question then becomes one of the predictability of *A. catenella* blooms that can be assessed using patterns in behavior and conditions.

Understanding the life cycle of *A. catenella* is necessary to help sustain the economic stability of the shellfish industry and provide advanced warnings for customers.

The first step in establishing predictability is determining under which conditions these cysts settle in the sediment. This dinoflagellate has a complex life cycle: under warm water conditions, it is actively swimming and dividing in the water column and under cooler conditions it forms a resting cyst in the sediment. *A. catenella* can form both a temporary and permanent cyst (hypnocyst) until the environmental conditions are favorable for it to metamorphose into and active (vegetative) cell (Blackburn and Parker, 2005).

The first step in establishing predictability is determining under which conditions these cysts settle in the sediment. All regions in Puget Sound have had shellfish harvest closures due to Hazardous Algal Blooms (HABs) caused by *A. catenella* with the exceptions of Southern Hood Canal and the Southern Puget Sound (Rensel, 1993). Discovery Bay and Penn Cove in particular each have a history of *A. catenella* blooms. These blooms are thought to have originated from high concentration of cysts in those environments, also known as breeding bays (Nishitani and Chew, 1984). Both areas are of economical interest because of the number of shellfish farms in each region. The results of a previous survey conducted in 2006 provided evidence that both Discovery Bay and Penn Cove have among the highest *A. catenella* cyst counts in Puget Sound (Horner et al., 2007). Large numbers of cysts forcast an impending bloom the following spring. Both of these regions are enclosed areas that may have a unique environment that is preferred for encystment.

I hypothesize that enclosed regions provide a trigger temperature at which *A. catenella* vegetative cell encyst most readily. The environmental factors for *A. catenella* vegetative cell excystment have been measured, but the environment for encystment has not been well studied in Puget Sound. Both changes in temperature and nutrient levels, such as high nitrogen, are suggested to provoke cell excystment (Navarro et al., 2006; Hallegraeff et al., 1997; Figueroa et al., 2005). Increased light abundance and oxygen levels have also been observed to facilitate excystment in *A. catenella* specifically (Andersen et al., 1987). Enclosed regions may have preferred temperatures for encystment. Encystment serves as a precursor to a bloom, whose magnitude may be estimated by the number of cysts present in the sediment. If temperature is a major player in encystment and enclosed areas such as Discovery Bay and Penn Cove do serve as preferred environments for *A. catenella* cysts then one more step can be taken towards the predictability of location, size, and timing of HABs.

The objective of this study is to quantify the presence of *A. catenella* cysts in the sediment in Discovery Bay, Penn Cove, and the Main Basin and to identify a critical temperature that causes encystment (Figure 1, Table 1). Discovery Bay and Penn Cove were chosen based on a previous survey taken in 2006 that showed increased numbers of cysts in the sediment when compared to the Main Basin (Horner et al., 2007). The next step is a laboratory- based investigation, in a laboratory environment, of what temperatures cause the active, or vegetative cells, to encyst. Once the critical temperature is identified, one can use the numbers of active cells present during the onset of this condition, assess the numbers of possible cells that would encyst, and establish a probability of a HAB during the following spring based on those data.

METHODS

Field/ Sampling Methods

The first phase of the research enumerated presence of cysts in the sediment of Discovery Bay, Penn Cove, and the Main Basin to provide a survey of the current distribution of *A. catenella* cysts (Table 1, Figure 1). The platform for the field component of this research was the *R/V Thomas G. Thompson* from 19 March 2007 to 23 March 2007 . Sediment samples were collected using a Van Veen at two stations each in the Main Basin, Penn Cove, and Discovery Bay. One sediment grab was taken per station and 2 sub- samples of the top ~3 cm of sediment were taken for analysis. The sediment samples were stored in plastic storage bags and kept in the dark at 4°C aboard the *R/V Thomas G. Thompson.*

Lab/Analytical Methods

The laboratory component for both the enumeration of cysts and encystment experiments took place at the University of Washington School of Oceanography. Sediment samples collected in the field were quantified using the epifluorescent microscopy and primuline staining protocol established by Yamaguchi et al. (2002) (Figure 2). Dilutions were made when necessary and accounted for in the final calculations.

The second phase of the research was to test the range of temperatures vegetative cells from Puget Sound prefer to encyst. 50 mL aliquots of *A. catenella* vegetative cells were separated into four 125 mL Erlenmeyer flasks with F/2 medium. The vegetative cells had been cultured for four weeks prior in a 13°C cold room with a 12/12 day/night cycle. Cultures were redistributed into new flasks with F/2 every two weeks. The flasks were replenished with F/2 medium and two were housed in an 8°C cold room with a 14/10 day/night cycle and two in a 10°C cold room with a 14/10 day/night cycle. Initial population counts were taken for a control sample in the 13°C cold room, and both samples in the 8°C and 10°C room, respectively. Following final counts, growth rates were calculated using the Malthusian parameter (Blackburn and Parker, 2005):

$$r = [\ln (N_t / N_0)] / \Delta t$$

The variable r represents the growth rate. N_t is the population size at the end of the time interval and N_0 is the population size at the beginning of the time interval.

Samples from each flask were collected every other day during a period of 21 days for each cold room. To ensure homogeneity of samples, the flasks were swirled gently to aggregate the cells without compromising cellular structure. ~2mL of sample was fixed with one drop of 10% formalin. A 0.1mL aliquot is taken with a glass micropipette for counting on a Palmer-Maloney slide with a 25x objective and 10x ocular lens. Abundances of vegetative cells, hypnocysts, and vegetative cells were recorded from 1- 2 slides per sample, whichever allowed for counts that totaled over 300 cells. This allowed for statistically significant data collection with a precision of + or -10% and a 95% confidence interval (Venrick, 1978). Chain formation among cells was also recorded according to the percentages of the total population that formed chains of 2 to 5 cells in length per time interval.

One consideration in the cell enumeration was identifying these cells in a temporary cyst stage and distinguishing them from the cells in the hypnocyst stage (Blackburn and Parker, 2005). The two stages can be recognized by the difference in shape. The hypnocyst is more oval in appearance and has two clearly identifiable cells walls. During cyst counting, the difference between the two cysts was noted as these represent various stages in the life cycle and reactions to stress. Numbers of cysts present in Discovery Bay and Penn Cove were compared with the distribution of cysts observed in the Main Basin.

<u>RESULTS</u>

Sediment cyst count results:

Cyst counts were highest in Penn Cove with variation on locations within Penn Cove. DM1 averaged 52.5 cysts/mL whereas DM2 averaged 7.5 cysts/mL. The north Main basin held the second highest cyst counts with 17.5 cysts/mL at DM5. The next highest counts were found in Discovery Bay 2 (DM4) with an average count of 20 cysts/mL. Discovery Bay station DM3 yielded an average of 10 cysts/mL followed. The Main Basin at West Point had the lowest counts at an average of 5 cysts/mL but a raw data count as low as 0 cysts/mL (Figure 3).

A second species found at all of these locations was *Scrippsiella trochoidea* (Figure 4). The highest abundance of this cell, also found in a resting cyst state in the sediment, was found in Penn Cove at station DM1 with an average of 47.5 cysts/mL. This follows a similar trend as the abundance of *A. catenella* cyst abundance (Figure 5). The next highest abundance of *S. trochoidea* is in Discovery Bay at DM4. Abundance of this cell decreases from DM2, DM3, DM5, and DM6. The null count of *S. trochoidea* at DM6 follows the same pattern as *A. catenella* at DM6.

Encystment results:

Chain Formation

Patterns emerged as the cells have reacted to stress. Active cells multiplied in both the 8° C room and 10° C room. The growth rate (r) for these populations of cells is 0.14 day $^{-1}$. Active, motile cells were not in chain form upon initial counts in the 8°C room but formed chains after 6 days in the cold room. Chains ranged in size from 2- to 4- celled chains, with chains of multiples of 2 as the most common occurrence at all temperatures. The percentage of cells that formed chains of two cells in the 8°C room peaked at 1.9% after 21 days. The numbers of cells forming 2-celled chains fluctuated between .05% and 0.8% between day 1 and day 13 with no discernable trend. The motile cells formed 3-celled chains on days 4, 11, and 21 ranging from 0.19% to 0.09% of the total population with no discernable pattern.

Cells in the 10° C were in chain formation during initial counts. The percentage of cells that formed chains of two cells in the 10° C room peaked at 3.6 % after 9 days in the cold room. The percentage of two-celled chains decreased to 0.18% on day 14 and ranged between 0.18% and 0.93% from day 14 to day 18. By day 2, 4- celled chains reached a maximum abundance at 0.08% of the total population. The motile cells formed 3- celled chains intermittently on days 2, 4, 9, and 14 ranging from 0.02 % to 0.003% of the total population.

Cyst Formation

In the 8°C room, 2.3% of the active formed temporary cysts after 6 days of exposure. The maximum percentage of temporary encystment occurred on day 8 at 7.8%. Between day 8 and day 11, cyst abundance decreased from 7.8% to 0.39% of the total population. During this same time interval, motile cells abundance increased from 99.4 % to 99.6%. Both motile cell abundance and cysts abundance peak on day 15 and on day 21 with a decrease in both on day 18 (Figure 6).

Motile cell and cyst formation have similar increasing and decreasing trends over the same time scale when plotted logarithmically. Betweens days 6 and 8, 13 and 15, and 18 and 21, motile cells and cysts show the same increasing and decreasing trends. Between day 8 and day 13, the abundance relationships are inverses of one another (Figure 7).

Following 2 days in the cold room, 0.11% of the active cells in the 10° C room formed temporary cysts The maximum abundance of temporary cysts were present on day 11 at 1.7% and the minimum number of cysts were present on day 6 at 0.05% of the population (Figure 8). Plotted logarithmically, motile cells and cysts follow similar increasing trends from days 4 to 6 and days 11 to 13. Between days 6 and 8, the abundance relationships are inverses of one another with an increase in motile cells and a decrease in temporary cells (Figure 9).

Permanent cysts did not form in the 8° C room or the 10° C room at any point during the cold room experiment. Temporary cysts come in various stages of development. For the purposes of this experiment, any stage of development of a temporary cyst was enumerated as a temporary cyst.

DISCUSSION

Cyst abundance patterns:

The distribution of *A. catenella* cysts in Puget Sound did not match the ~80 cells/mL pattern found by Horner et al., (2007) during their 2005 survey. The highest values were found in Penn Cove, not Discovery Bay as predicted. Several factors can account for a shift in distribution including bioturbation, predation, and environmental effects.

Cysts are found in the top ~3-5 cm of the sediment layer. The stations sampled were all nearshore and bay areas where benthic foraging is high. Several species of benthic organisms other than bivalves have been documented to feed in the uppermost layer of sediment in Puget Sound and bioconcentrate PSP toxins. Among these species is the moonsnail, *Polinices lewisii* (Matter, 1994). *P. lewisii* feeds in the sediment directly but also may feed on bivalves that have bioaccumulated saxitoxin. During foraging, *P. lewisii* would decrease the abundance of *A. catenella* cysts if the cysts themselves were inadvertently taken up with other food items. Although there is no current direct evidence that gastropods concentrate saxitoxin, there is direct evidence that tetrodotoxin (TTX) and phycotoxin are concentrated in crustaceans and gastropods (Shumway, 1995). Currently, there is no research focusing on the population abundance of *P. lewisii* in the stations sampled and the PSP toxin concentrations found in the tissue.

Bioturbation, the turning or movement of sediment due to biological processes, can also shift the concentration of cysts and other organisms in the sediment. Bivalves and polychaete worms are examples of an organism that routinely displaces sediment during movement and foraging, therefore influencing microorganism distribution in the sediment (Kanaya et al., 2005)**.** These populations would have to be substantial to have an influence on the distribution of *A. catenella* cysts over an entire region. Bioturbation occurs but is less likely to change regional distribution of cysts.

DM5 had the second highest cyst counts out of the stations sampled. The Main Basin is more flushed than Discovery Bay and Penn Cove. Any organism in the top layer of the sediment would not have an opportunity to settle in and would have a shorter residence time when compared to enclosed regions. The sediment is categorized as sandy-gravel in comparison to the sediment found in Penn Cove and Discovery Bay where the sediment is sandy-mud (Roberts, 1979); this would allow for the cysts to be transported more readily because they are not compacted into a mud bed.

DM1 counts were ~40x those of DM2. Both of these stations are located in Penn Cove but DM2 is closer to the shore than DM1. DM1 and DM2 have a depth of 24 m and 26.8 m respectively. The difference is unlikely to be explained by depth difference and therefore circulation difference, assuming the circulation pattern to be a function of the depth. If the depth is consistent between any two locations, the circulation may be assumed to be comparable.

Encystment:

Organisms can react to stress in different ways. When *A. catenella* feels physical and/or chemical stresses, it reacts accordingly. Within 6 days, the cells in the 8°C room started to form temporary cysts. Temporary encystment is a reaction to an environmental change that the cell anticipates will change in a relatively short period of time (Blackburn and Parker, 2005). The same cells (in 8°C) also began to form chains after exposed to 8°C after 4 days. These results show a progression of behaviors in reaction to stress and perhaps a hierarchy of the reactions. The peak of the chain forming was from day 18 to day 21. The time for these cells to form chain in reaction to stress is longer than the time needed to form temporary cysts. Forming a temporary cyst, a faster reaction, is a more efficient way to shield the cell from stress. Cells exposed to 10°C formed temporary cysts at a lower percentage (.05% - 0.4%) after day 5. When compared to the 8°C cells, the time frame for formation of the temporary cysts is consistent. It can also be argued, however, that the abundance of cell chains occurs not due to physical stress, but rather as a function of cell division. When *A. catenella* divides, chains result. Most chains for this cell are observed in multiples of two with the original cell present and the new cell attached. These chains that are the result of population and cell growth, can grow to up to 64 cells in length (Personal communication, Rita Horner).

From the data, it is difficult to say whether a consistent relationship exists between the abundance of motile cells and temporary cysts. From cells in both cold rooms, there are clear trends in the increase of motile cells correspond to a decrease in temporary cysts (Figure 7, Figure 9). As motile cells increase, the number of cysts would decreases if previously existing cysts germinated into motile cells once again. A contrary trend is seen in the graphs for both cold rooms. Days 6 and 8, 13 and 15, and 18 and 21, show the same increasing and decreasing trends in motile cells and temporary cysts in the 8° C room (Figure 9). These occurrences are more common than the inverse relationships indicating that an increase in cysts does not correspond to a decrease in motile cells. One way to account for the differences in the trend is the complexity of population growth in a culture. Cells can be in any stage of motile or cysts development and change these forms within days. The motile cells could be forming cysts then germinating in motile cells within days. The cysts can either remain cysts for extended periods or go into motile stage and back into cyst state. The complexity of the population life cycle dynamics makes it difficult to distinguish a predictable behavior in the cell development and timing of the various stages in the life cycle.

Motile cell versus cyst abundance were plotted using absolute values and again logarithmically. There are three peaks for both motile abundance and cyst abundance in the 8° C room when plotted with absolute counts. These occur on day 6, 15, and 21 (Figure 6). Although the graph can represent marked peaks in these values, there is the issue of sampling from a homogenous mixture in which the cysts may settle to the bottom. The presence of these cysts and motile cells could have been observed on these days, however, were present in the sample before counting. These peaks are then a representation of cells taken in that sample that may or may not have been in the sample prior to that particular count. Similar peaks occur in the 10 ° C room on day 13. The motile cell count and the cysts both increase with similar slopes indicating that the relative abundance of each has not changed rather the absolute count has (Figure 8). Again, the population growth dynamics factor in when attempting to discern predictability.

The experiments set up in this study did not test a range of temperatures. Further research is needed cover a range of 1° C to 2° C differences in an attempt to enduce encystment. Culture populations can react to the environment over short- term or long-term time intervals. Since life cycles of *A. catenella* can be seasonal, new experiments should be run on those time frames for a more complete assessment of the overall population reaction to environmental change.

CONCLUSION

Although temperature plays a role in determining encystment, the cells appear to adapt to temperature changes over time. The duration of encystment varies with the temperature. Neither 8° C nor 10° C is a trigger temperature for permanent encystment under the conditions used in this study.

Reactions to environmental stress can take permanent or temporary forms. The results of my experiment show that stages of physical change, chain forming and temporary encystment, occur when the cells are exposed to 8° C and 10° C. The onset of these physical changes is observable in initial stages, but there is no collective population change.

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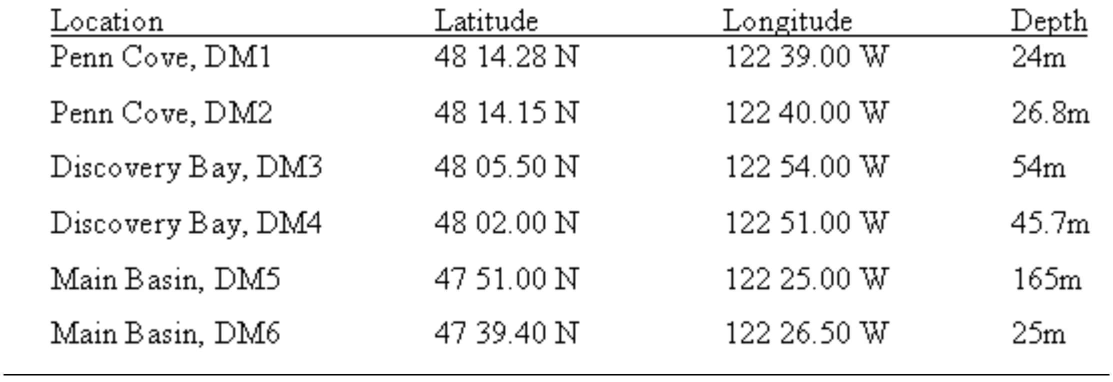
Table 1. Latitudes, longitudes, and sampling depth of each station.



Location	Latitude	Longitude	Depth
Penn Cove, DM1	48 14.28 N	122 39.00 W	24m
Penn Cove, DM2	48 14.15 N	122 40.00 W	26.8m
Discovery Bay, DM3	48 05.50 N	122 54.00 W	54m
Discovery Bay, DM4	48 02.00 N	122 51.00 W	45.7m
Main Basin, DM5	47 51.00 N	122 25.00 W	165m
Main Basin, DM6	47 39.40 N	122 26.50 W	25m

FIGURE LEGENDS

Figure 1. Sampling stations for *A. catenella* are in Penn Cove, Discovery Bay, and the Main Basin. There are two stations per site and one Van Veen was taken at each station.

Figure 2. *A. catenella* cysts were enumerated using an eipfluorescent microscope and identifiable by the ovaline shape and double cell wall. Cyst size ranged from 25 µm to 40 µm.

Figure 3. *S. trochoidea* cysts were found with *A. catenella* at all stations sampled with the exception of DM6 in the Main Basin near West Point. *S. trochoidea* is smaller than *A. catenella* and has a less symmetrical ovaline shape that narrows at one end of the cell. S. trochoidea cyst sizes ranged from 20 µm to 30 µm.

Figure 4. *A. catenella* cyst abundance is plotted for all six stations in cells/mL. DM1 = Penn Cove 1, DM2 = Penn Cove 2, DM3 = Discovery Bay 1, DM4= Discovery Bay 2, DM5= Main Basin near Triple Junction, DM6 = Main Basin at West Point.

Figure 5. *S. trochoidea* is a dinoflagellate also found in cyst stage in Discovery Bay, Penn Cove, and the Main Basin. *A. catenella* and *S. trochoidea* share the same niche in the sediment. *S. trochoidea* was not found at DM6.

Figure 6. Motile cells plotted with temporary cysts with a secondary axis in the 8° C cold room.

Figure 7. Motile cells plotted with temporary cysts without a secondary axis in the 8° C cold room. Note this figure is on a logarithmic scale.

Figure 8. Motile cells plotted with temporary cysts with a secondary axis in the 10° C cold room.

Figure 9. Motile cells plotted with temporary cysts without a secondary axis in the 10° C cold room. Note this figure is on a logarithmic scale.

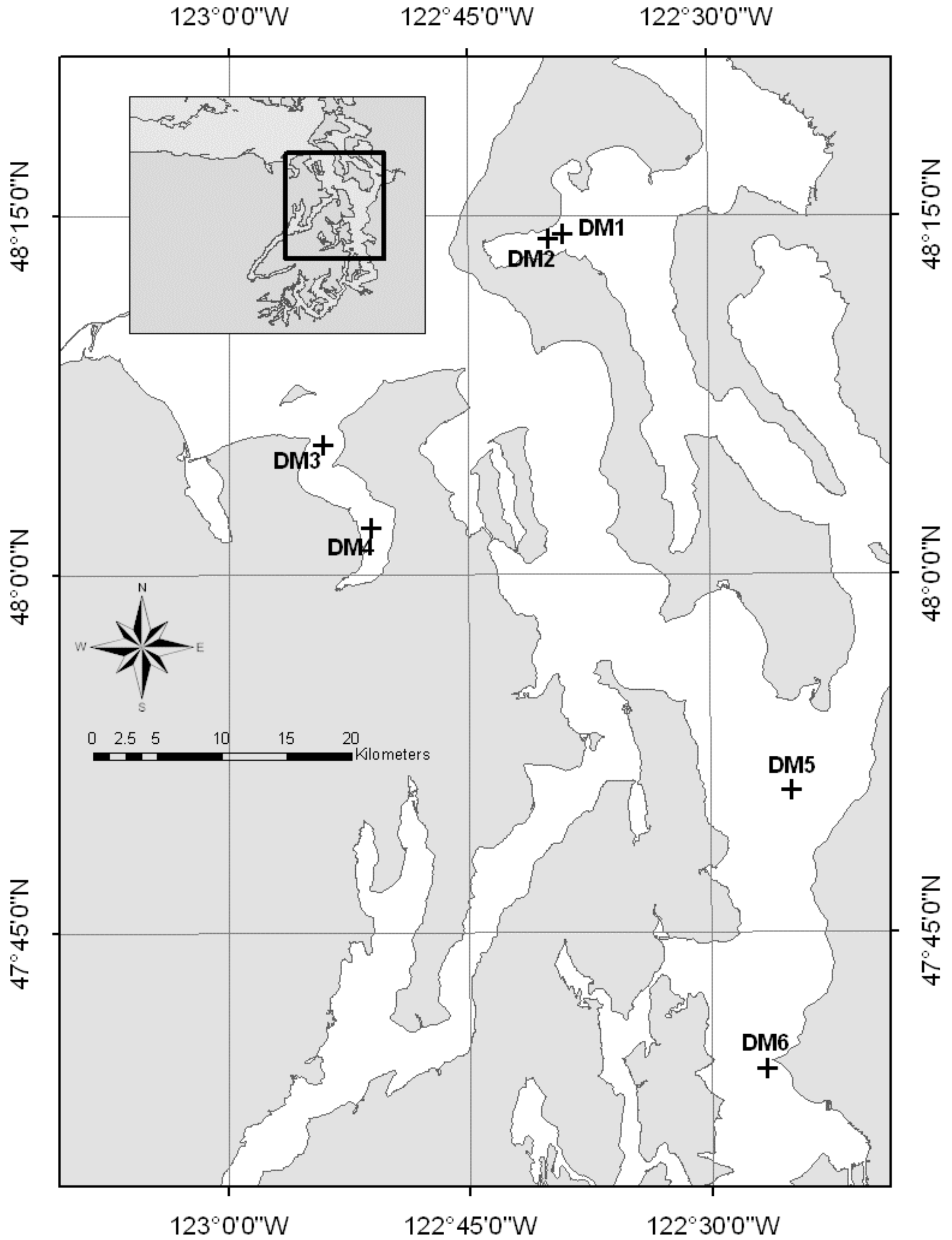
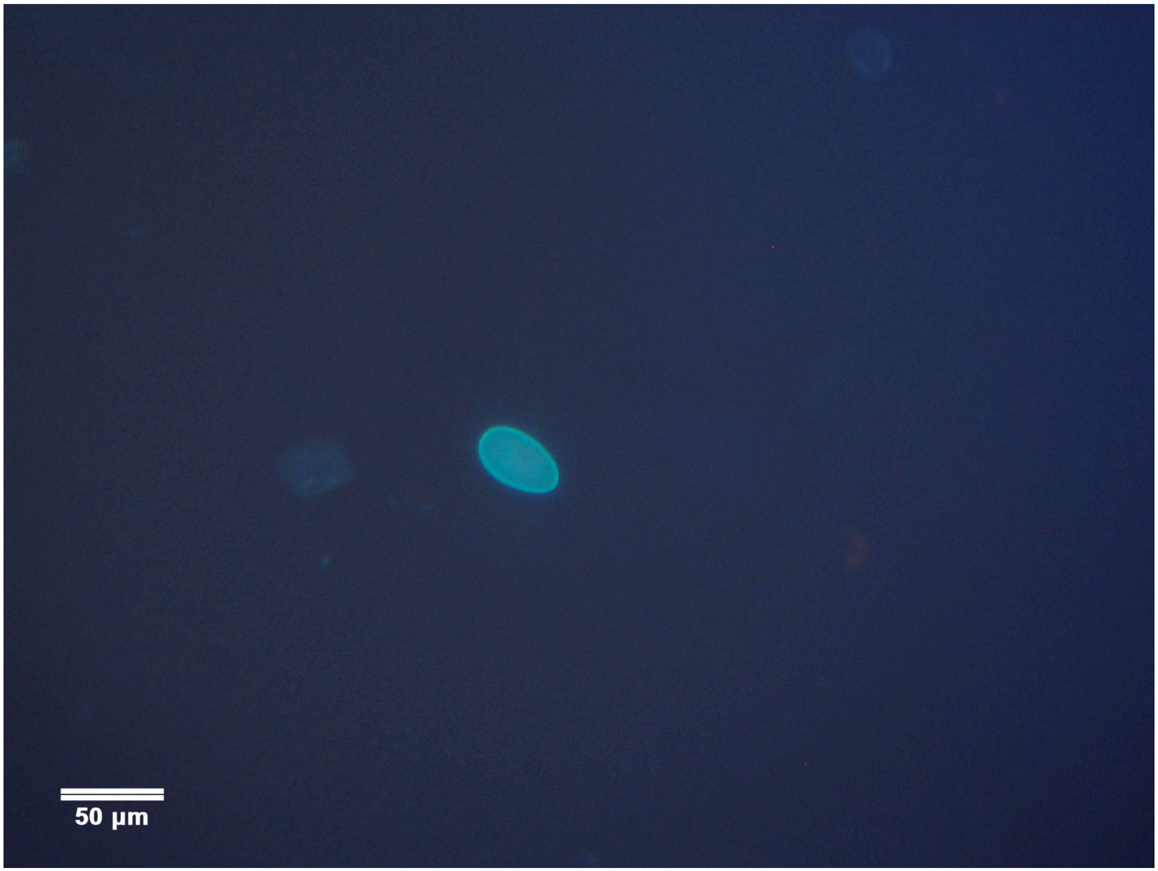


Figure 1. Deni Malouf



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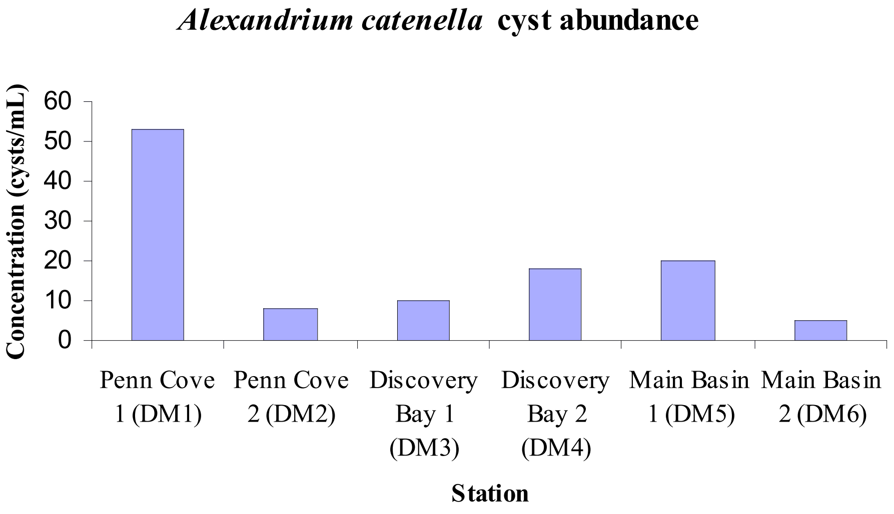

Figure 3.

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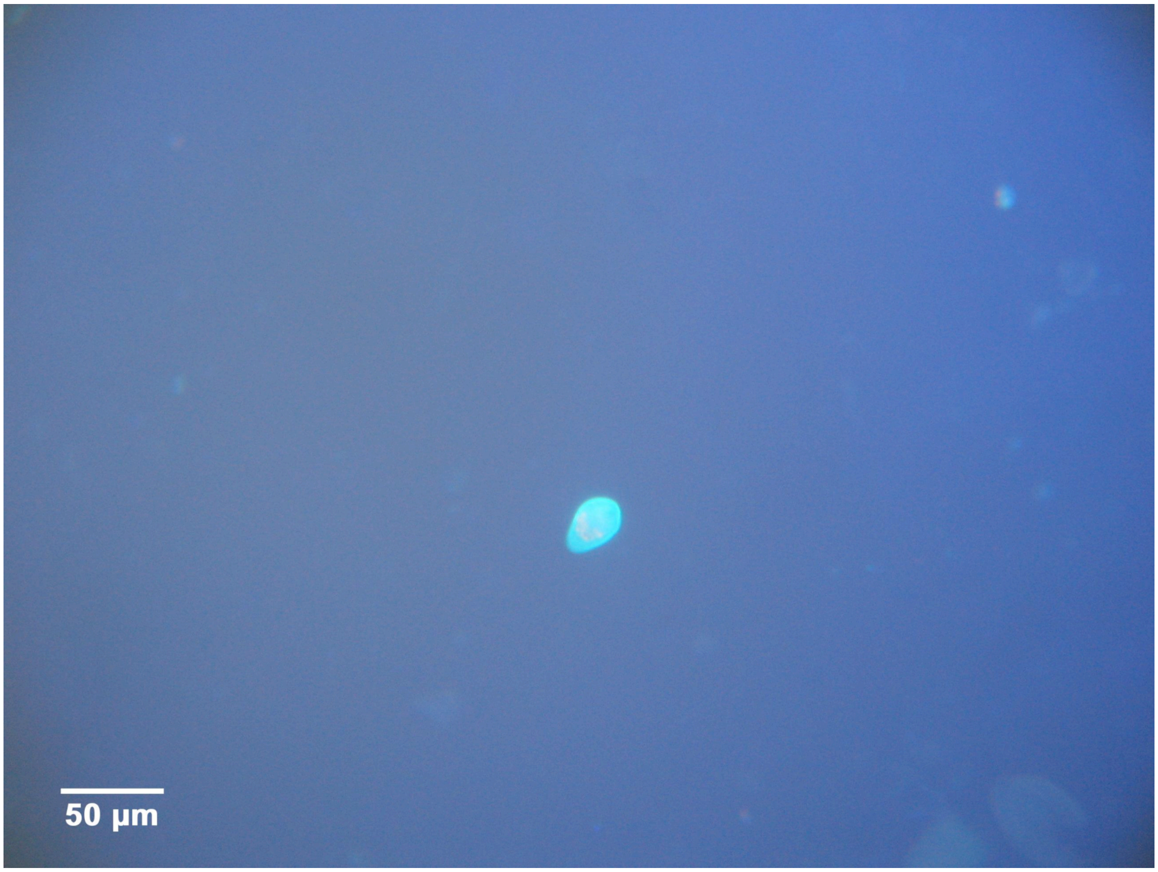

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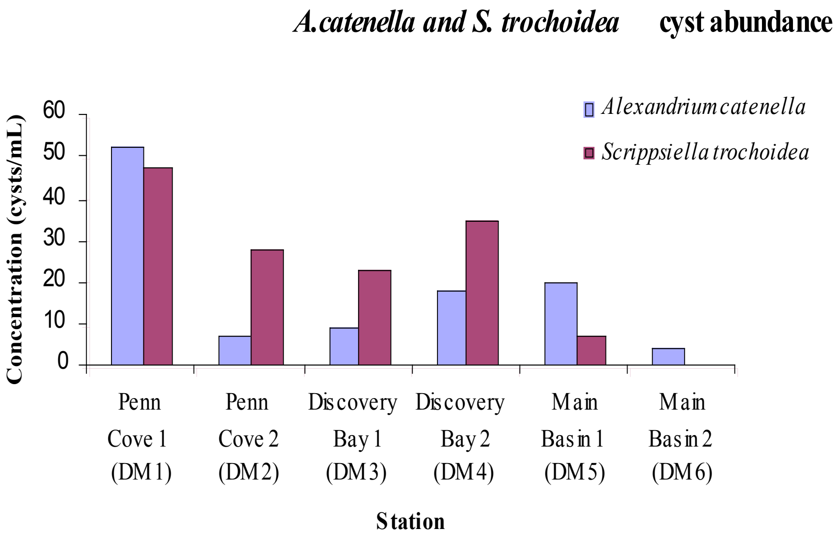

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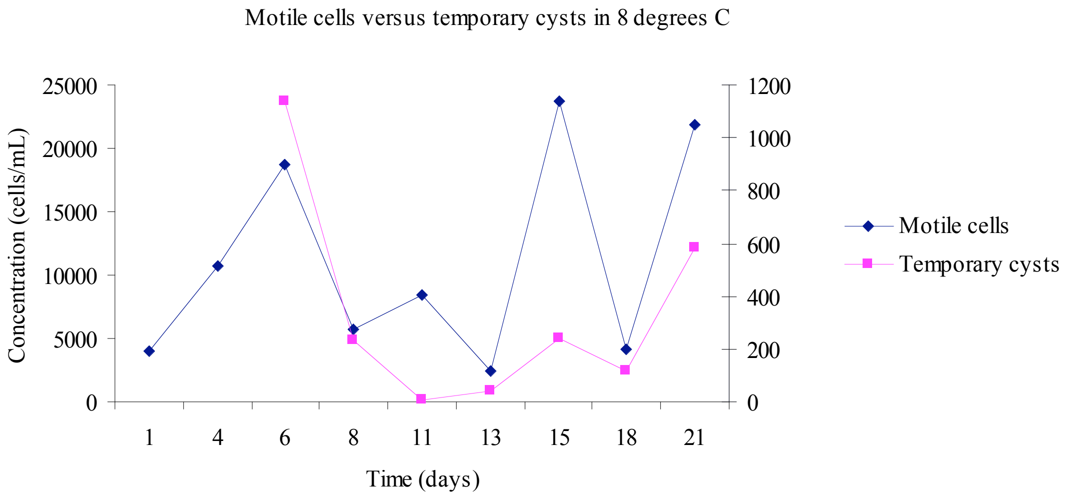

Figure 6.

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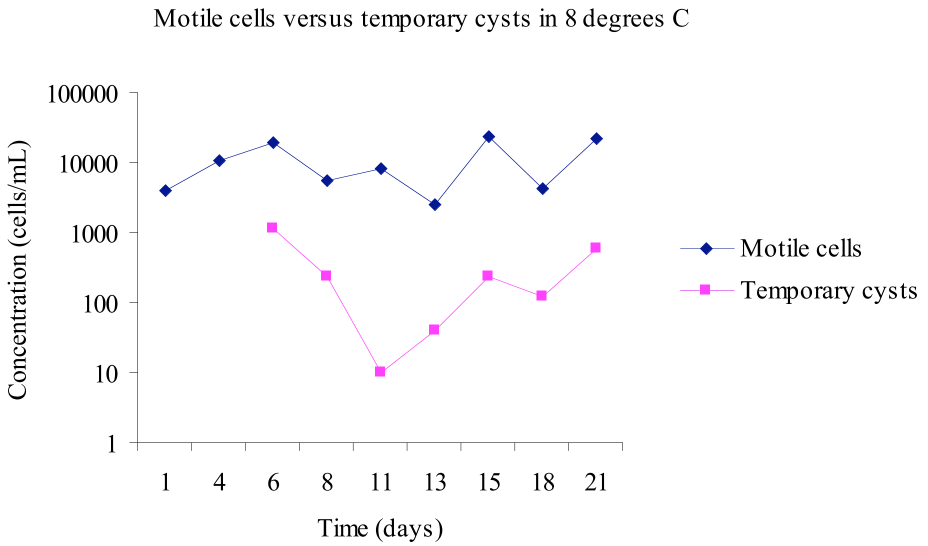

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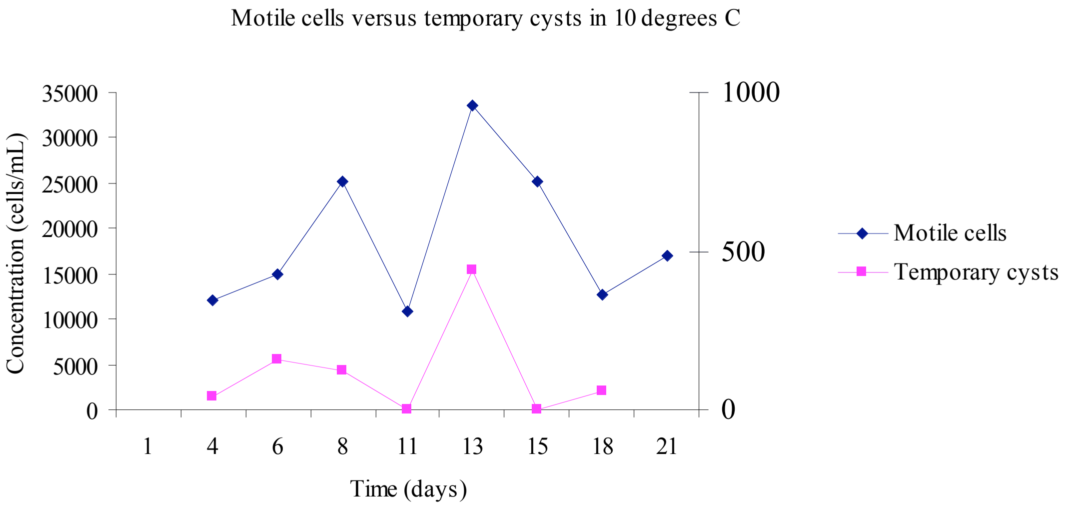

Figure 8.

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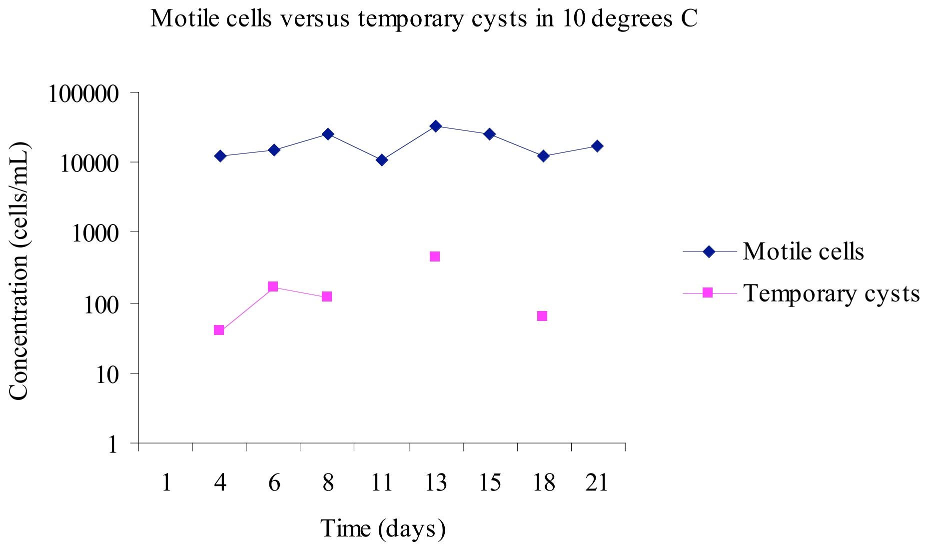

Figure 9.

Deni Malouf