Effect of thermal stress on fluorescence and dinoflagellate density in the captive coral, *Anthelia* spp.

Joleen Santos

Department of Biological Sciences

MacEwan University, Edmonton, Alberta, Canada

Abstract

The obligate mutualistic relationship between corals and dinoflagellates is a classic example of symbiosis. Over the last few decades, coral reefs have been devastated by warm temperatures, hence, the necessity to develop a method to predict future mass bleaching events is higher than ever. Fluorescence might be used as an indicator of coral health, but very few studies have attempted to utilize it as a proxy for dinoflagellate density, which was the scope of the present research. The goal of this study was to determine the effects of thermal stress on fluorescence and dinoflagellate density in the captive coral, *Anthelia* spp.. Over a five-week period, tanks filled with *Anthelia* spp. underwent gradual increases in temperature, beginning at 28°C and ending at 33°C. Samples were quantified for fluorescence and dinoflagellate density using fluorescence microscopy and a maceration method. As the temperature gradually increased, fluorescence values subsequently decreased. In contrast, dinoflagellate density first increased until it reached a threshold, followed by a sudden drop in numbers. Symbionts might be increasing their mitotic rate in response to thermal stress to compensate for the shortage of photosynthate supply for their host. Therefore, fluorescence analysis may be a potential predictor of coral bleaching in *Anthelia* spp..

Keywords: coral, heat, dinoflagellate, density, fluorescence

Introduction

Coral reefs are often referred to as the rainforest of the ocean because they are inhabited by a cornucopia of organisms (Sheppard et al. 2009). Although they may appear as large, stony structures, corals are Cnidarians made of small polyps. More specifically, the polyps of soft corals (Order Alcyonacea) are of the autozooid kind, which have eight tentacles with pinnules on both sides. Reefs are of economic significance to the countries near them as they attract tourists.

Besides their importance to humans, they support a vast array of organisms such as fish, turtles, sea stars, etc. and without a healthy reef system, all of these organisms will be without a home.

Dinoflagellates

Zooxanthellae come from the genus *Symbiodinium* (Sheppard et al. 2009). They are 5-10µm in diameter, spherical, and appear brown in colour. Symbionts can be categorized into eight different clades (A-H) which are characterized by different factors such as geographical distribution, temperature preferences, etc.. Clade C dinoflagellates are found primarily in areas with more stable conditions, and usually in deeper water (Rowan and Knowlton 1995). It has been suggested that symbionts from Clade C are heat-sensitive compared to other phylogenetic groups (Chen et al. 2005)

Corals have established an obligate mutualism with zooxanthellae which are embedded within the gastrodermal cells of polyps (Davy et al. 2012). The average density of dinoflagellates is $1.45x10^6$ cells/cm^2, but some species (*Plerogyra*) can be inhabited by up to $15x10^6$ cells/cm^2 (Drew 1972). These dinoflagellates can be transmitted vertically from parent to offspring, or horizontally from free-floating symbionts, ingested zooplankton, and expulsion from other corals (Sheppard et al. 2009). Symbionts undergo photosynthesis while inside their host providing up to 98% of its photosynthates to the coral (Muscatine et al. 1984), while obtaining plant nutrients and protection in return. Unfortunately, this mutualistic relationship has been over-disrupted by recent climate patterns.

Coral Bleaching

Over the last century, average sea temperatures have increased yearly (McClanahan et al. 2007, Abdo et al. 2012). While the effects of global warming are evident in virtually any ecosystem, its effects on coral reefs have been deleterious. An abundance of studies exists

showing that warm water temperatures increase the rate of symbiont expulsion (Jokiel and Coles 1974; Hoegh-Guldberg and Smith 1989; Lesser et al. 1990). Thus, thermal stress has been accepted as the main cause of coral bleaching events, which in essence is the expulsion of dinoflagellates (Sheppard et al. 2009). Briefly, the photoinhibition model explains that thermal stress combined with ultraviolet rays triggers the denaturing of proteins and destruction of the photosynthetic apparatus of dinoflagellates (Lesser et al. 1990). Particularly, areas within photosystem II are susceptible to damage by increase in temperature (Warner et al. 1999). Furthermore, heat induces apoptosis in coral cells, hence bleaching is a response to stress imposed on both organisms (Sheppard et al. 2009).

Fluorescence

Corals produce an abundance of fluorescent proteins (FP's) of mainly three different colours: green, cyan, and red (Field et al. 2006). These FP's absorb high-energy photons of light, then re-emit them at a lower energy (Roth and Deheyn 2013). Historically, corals only used to produce green FP's, but a mutation at some point gave rise to the other two colours, implying a selective advantage (Field et al. 2006). Although it has been established that corals produce FP's of different kinds, their function remains undetermined (Roth and Deheyn 2013). Furthermore, the diversity in colour does not contribute to photoprotection (Fields et al. 2006), which is one of the most prominent hypotheses for coral fluorescence (Salih et al. 2001). Despite understanding very little about their role, change in fluorescence is one of the quickest responses to environmental stress (Roth and Deheyn 2013). Hence, fluorescence has been used as an indicator of coral health.

Effects of thermal stress on coral fluorescence is scarcely studied, and the results obtained have been inconsistent with one another (Rodriguez-Lanetty et al. 2009, Roth and

Deheyn 2013). Rodriguez-Lanetty et al. and Roth and Deheyn showed that FP expression (molecular level) decreased with increasing temperature (2009, 2013). However, Roth and Deheyn's study demonstrated that despite a decrease in FP expression, observable fluorescence remained consistent throughout the study (2013). Therefore, it is inconclusive if fluorescence can be used as an indicator of coral health in this context.

Furthermore, long-term average temperature is more significant in causing coral bleaching and mortality than short-term rapid fluctuations (Jokiel and Coles 1990). A criticism of thermal stress experiments is that researchers essentially subject corals to heat shock treatments (Rodriguez-Lanety et al. 2009, Roth and Deheyn 2013), which is not reflective of real climate patterns. Although such studies have been very informative, there is room for improvement such that the results are more scientifically significant.

Lastly, there is little research on both fluorescence and dinoflagellate density of corals under thermal stress (Roth and Deheyn 2013). The objective of this study is to determine if fluorescence can be used as an indicator of dinoflagellate density in corals and hence, as a proxy for coral health. I hypothesize that fluorescence and dinoflagellate density reflect the health of corals and predict that as temperature gradually increases, fluorescence and dinoflagellate density will both decrease.

Materials and Method

Coral Species and Tank Setup

The species of coral used in this study was *Anthelia* spp., provided by Dr. Ross Shaw. This species is inhabited by Clade C zooxanthellae (Goulet et al. 2008), has a preferred temperature range of 23°C to 28°C, and contain red fluorescent proteins. Corals were established among twelve 28.8L tanks layered with rubble for the organisms to attach to. Once they were

anchored on, *Anthelia* spp. were fragmented into groups of 3-5 polyps using a razor blade. Each tank was filled with freshwater (using RiOs 100 reverse osmosis system (EMD Millipore, Billerica, Massachusetts, USA)) in which the specific gravity was adjusted to 1.025. Above each tank was a light system (with two 24W bulbs: one Aquablue+ and one Actinic+) set at a six-hour photoperiod. Inside the tanks was a circulation pump (Koralai Nano 420) and a heater (Fluval E100 100W) fixed at 27°C prior to the start of experimentation.

Tank Maintenance

Throughout the study, tanks were topped with freshwater to maintain specific gravity. Fifty-percent of the water for each tank was replaced about every five days, or whenever the water became unclear. Algal growth on the side of the tanks were wiped down to prevent them from overgrowing.

Heat Stress Treatment

Experimentation occurred over a five-week period. Each week the temperature was increased gradually, starting from 28°C on the first week, and ending at 33°C. A 29°C treatment was excluded due to a lack of time and a shortage of tanks. On each day of temperature change, heaters were adjusted by 0.5°C every twelve hours, except for the transition from 28°C to 30°C, in which temperature was increased every six hours by 0.5°C. Every 72 hours, samples were obtained for fluorescence analysis and counting dinoflagellates. Two sets of control tanks were maintained at 28°C throughout the experiment, and samples from these tanks were analyzed only on the 144^{th} hour.

Fluorescence Analysis

Fluorescence analysis was carried out through fluorescence microscopy, similar to the study done by Jones (2017). Prior to any analysis, an acclimation tank was set up near the

stereomicroscope (Olympus SZ61 Zoom Stereo Microscope at 2.5X magnification and on full brightness, with a blue UV filter and X-Cite Series 120Q EXFO with excitation 630nm). The heater and light settings were adjusted to match the conditions of the treatment tank that the fragments were obtained from. *Anthelia* spp. were transferred and underwent acclimation for at least thirty minutes before analyzing fluorescence. In a small dish, a single fragment was viewed under the microscope, and a camera (Olympus XM10 Monochrome fluorescence CCD camera) mounted onto the scope gave a live stream to the program Olympus cellSens Standard (exposure time = 0.98ms, gain = 12.5dB, resolution = 1376x1038). The focus was adjusted to individual tentacles and their images were taken. Four randomly selected photographs were uploaded to ImageJ (National Institutes of Health, Bethesda, Maryland, USA) to quantitatively measure fluorescence of five areas along a tentacle. Average background reading was calculated by selecting five of the darkest areas of the image. Corrected fluorescence was determined using the formula: Corrected Fluorescence = Integrated Density - (Area x Mean Background Reading). A clear ruler was used to set the scale for all images (407.921 pixels/mm). The analysis consisted of two replicates (n = 1 from two tanks) for the heat treatment, and one (n = 1 from one tank) for the control treatment.

Dinoflagellate Density

Fragments were obtained and analyzed one at a time. Using a dissecting kit, tentacles were cut off, blotted on a Kimwipe, and weighed on a Mettler Toledo scale normalized to a weigh boat. Tentacles were added until a total mass of $1x10^{-5} \pm 2x10^{-7}$ kg was reached, followed by maceration with curved forceps to remove the dinoflagellates. Small drops of seawater were added with a micropipette, and the mixture was added into a small tube. The weigh boat was rinsed out into the tube until the 1ml line was met, which was followed by vortexing. A 1:10

dilution was performed into a larger tube followed by vortexing, and a 10µl sample was dropped onto a gridded microscope slide. Dinoflagellates were counted under a Zeiss Primo Star compound microscope. On the 72nd hour, four counts were done: two fragments from two separate treatment tanks. On the 144th hour, six counts were done: two fragments from two separate treatment tanks, and one from two separate control tanks. The average number of dinoflagellates was calculated after.

Statistics

All statistical analyses were carried out using the software JMP 14 (SAS Institute Inc.). Two one-way ANOVA tests were done to analyze the effects of heat stress on each fluorescence and dinoflagellate density. For all significant p values ($p<0.05$), a post-hoc Tukey Kramer analysis determined which values among heat treatments were significantly different.

Results

Effect of Thermal Stress on Fluorescence

As temperature gradually increased, fluorescence in *Anthelia* spp. steadily decreased (Fig. 1B). Corrected fluorescence at 32°C (hour 144), and 33°C (hour 72 and 144) was significantly lower than all of the values obtained from less intense heat treatments (Fig. 1B, one-way ANOVA test, $F = 8.6796$, $p < 0.0001$). Fluorescence in *Anthelia* spp. from the control tanks increased slightly, but the trend was not statistically significant (Fig. 1B, one-way ANOVA test, $F = 1.1087$, $p = 0.387$).

Effect of Thermal Stress on Dinoflagellate Density

As temperature gradually increased, dinoflagellate density in *Anthelia* spp. remained stable up until 31°C where it began to increase (Fig. 1A). The amount of symbionts continued to increase until 33°C, which was followed by a rapid drop in cell counts. Dinoflagellate density

after 144 hours at 33°C dropped three-fold compared to just 72 hours prior (Fig. 1A, one-way ANOVA test, $F = 2.8872$, $p = 0.0139$). *Anthelia* spp. from the control tanks exhibited a decrease in dinoflagellate density, and cell counts on the fifth week were lower by half the counts at week two (Fig. 1A, one-way ANOVA test, $F = 7.4375$, $p = 0.0407$).

Dinoflagellate Morphology

Cells occurring as doublets were observed in both control and heat treatments however, only those from the 33°C tanks were recorded (Fig. 2A). The mitotic index was not formally analyzed. Samples from the 33°C treatment exhibited the greatest variation in size among dinoflagellates (Fig. 2B). Cells from the control tanks were relatively consistent in size (Fig. 2C), whereas zooxanthellae in the 33°C samples consisted of cells that were both normal and smaller in size (Fig. 2B). Furthermore, the smaller sized dinoflagellates appeared to be darker in pigmentation.

Discussion

As predicted, fluorescence in *Anthelia* spp. decreased as a result of thermal stress, hence supporting that it can be used as an indicator of coral health. On the other hand, although statistical analysis determined a difference in means in dinoflagellate density, a steady decrease was not observed. It was unexpected that cell counts increased along with temperature (Fig. 1A) as the health of corals declined. If both zooxanthellae and their hosts depend on a mutualistic relationship, dinoflagellate density and coral health should move in the same direction together. These results are in agreement with Bhagooli and Hidaka who found that after seven days of exposure to 30°C, *Galaxea fasicularis* lost control of zooxanthellae division (2002). An interpretation of these results is that in response to thermal stress, dinoflagellates increased their mitotic rate to compensate for the shortage, or anticipated shortage, in supply of photosynthates

for the host. In order to increase their mitotic rate, the symbionts require use of their own products, transferring less to their host. Therefore, despite the increase in numbers of dinoflagellates, the health of *Anthelia* spp. may have dropped as a result of insufficient energy source. This notion is supported by Woolridge who proposed that prior to a bleaching event, the primary effect of thermal stress on zooxanthellae is the dedication of photosynthates to new dinoflagellate cell growth rather than allocation to the host (2013). The initial strain in their mutualism will eventually lead to the bleaching event. One can detect this by observing a higher rate of cells appearing as doublets. At 33°C, dinoflagellates existing as doublets were observed (Fig. 2A, B, C). However, the rate at which they occurred compared to the controls was not analyzed, so their presence in the heat treatments alone is not enough to support that mitotic rate increased. Instead, what could support this notion is the difference in cell sizes between the control and 33°C treatment (Fig. 2E, F, G). The emergence of smaller cells in the heat-stressed *Anthelia* spp. may suggest a higher rate of cell division. To verify this, future studies should look to analyze mitotic index as well as photosynthetic productivity of symbionts. Additionally, Chen et al. observed that density of Clade C zooxanthellae increases from March to May, then drops rapidly around June (2005). In other words, as temperature gradually increased, dinoflagellate density increased until it reached a threshold, followed by expulsion when the water temperature was at its warmest. Hence, it is possible that what has been captured in the present study's results is the event leading up to coral bleaching. If this is the case, then the decrease in fluorescence suggests that it can be used as an indicator of dinoflagellate density, and hence as a proxy for coral health, supporting the hypothesis.

It was unexpected that dinoflagellate density in *Anthelia* spp. maintained at 28°C would decrease over five weeks (Fig. 1A). There are two main things to consider in order to make sense

of this observation. First, to establish the corals, they first had to undergo tissue damage and therefore required recovery before starting the experiment. Second, additional fragments were brought in during the middle of the study to analyze preliminary data. It was found that the average corrected fluorescence was 24 and dinoflagellate density was 400, and in Fig. 1, both control curves are approaching these two values. It is possible that the *Anthelia* spp. had not fully recovered yet in the start of the experiment, and the patterns seen in the control tanks could reflect an increase in health over the five weeks under ideal conditions. The decrease in dinoflagellate density can be explained by the host not needing as many cells anymore, since each symbiont is photosynthesizing sufficiently. If this is true, then perhaps when corals are unhealthy, dinoflagellates immediately regulate their own density, but when the corals are healthy, the host is the primary regulator. This is a different project on its own, and could be analyzed further in depth.

The main finding in this study is significant because it implies that fluorescence is an appropriate predictor of coral bleaching in *Anthelia* spp. As reefs continue to be devastated by warm water temperatures, the requirement for a noninvasive method of checking coral health is higher than ever, and fluorescence analysis is a promising direction. This project is especially important because it was designed to reflect realistic climate patterns, whereas the few existing studies have utilized heat-shock-like experiments (Roth and Dehyn 2013). To build on this current research, increasing the sample size of the controls would be beneficial. Future studies should seek to analyze different species of coral, as well as testing different environmental stressors besides temperature.

Acknowledgements

This project could not have been completed without Jill Anderson who supplied the needed materials quickly, as well as all of the laboratory technicians in the biological department who ensure that labs are well-stocked and resourced. Thank you to Tara Androschuk for instructions on fluorescence analysis, and Averil Jones who determined the temperature threshold for *Anthelia* spp.. Lastly, I extend my gratitude to Dr. Ross Shaw for his guidance throughout the semester, and whose excitement and passion for the subject served as great encouragement for this research.

Literature Cited

Abdo, D.A., Bellchambers, L.M., and Evans, S.N. (2012) Turning up the heat: increasing temperature and coral bleaching at the high latitude coral reefs of the Houtman Abrolhos Islands. *PloS ONE* **7**, 1-11

Bhagooli, R., and Hidaka, M. (2002). Physiological fascicularis responses of the coral *Galaxea* and its algal symbiont to elevated temperatures. *Journal of the Japanese Coral Reef Society* **4**, 33-42

Chen, C.A., Wang, J-T., Fange, L-S., and Yang, Y-W. (2005) Fluctuating algal symbiont communities in Acropora palifera (Scleractinia: Acroporidae) from Taiwan. *Marine Ecology Progress Series* **295**, 113-121

Davy, S. K., Alleman, D., and Weis, V.M. (2012). Cell biology of cnidarian-dinoflagellate symbiosis. *Microbiology and Molecular Biology Reviews* **76**, 229-261

Drew, E.A. (1972). The biology and physiology of alga-invertebrates symbioses. II. The density of symbiotic algal cells in a number of hermatypic hard corals and alcyonarians from various depths. *Journal of Experimental Marine Biology and Ecology* **9**, 71-75

Field, S.F., Bulina, M. Y., Kelmanson, I.V., Bielawski, J.P., and Matz, M.V. (2006). Adaptive evolution of multicolored fluorescent proteins in reef-building corals. *Journal of Molecular Evolution* **62**, 332-339

Goulet, T.L., Simmons, C., and Goulet, D. (2008). Worldwide biogeography of *Symbiodinium* in tropical octocorals. *Marine Ecology Progress Series* **355**, 45-58

Hoegh-Guldberg, O., and Smith, G.J. (1989). The effect of sudden changes in temperature, light and salinity on the population density and export of zooxanthellae from the reef corals and *Stylophora* pistillata Esper and *Seriatopora* hystrix Dana. *Journal of Experimental Marine Biology and Ecology* **129**, 279-303

Jokiel, P.L., and Coles, S.L. (1974). Effects of heated effluent on hermatypic corals at Kahe Point, Oahu. *Pacific Science* **28**, 1-18

Jokiel, P.L., and Coles, S.L. (1990). Response of Hawaiian and other Indo-Pacific reef corals to elevated temperature. *Coral Reefs* **8**, 155-162

Lesser, M.P., Stochaj, W.R., Tapley, D.W., and Shick, J.M. (1990). Bleaching in coral reef anthozoans: effects of irradiance, ultraviolet radiation, and temperature on the activities of protective enzymes against active oxygen. *Coral Reefs* **8**, 225-232

McClanahan, T.R., Ateweberhan, M., Muhando, C.A., Maina, J., and Mohammed, M.S. (2007). Climate change and spatio-temporal variation in seawater temperature effects on coral bleaching and mortality in East Africa. *Ecological Monographs* **77**, 503-525

Muscatine, L., Falkowski, P.G., Porter, J.W., and Dubinsky, Z. (1984). Fate of photosynthetic fixed carbon in light- and shade- adapted colonies of the symbiotic coral. *Stylophora pistillata*. *Proceedings of the Royal Society B: Biological Sciences* **222**, 181-202

Rodriguez-Lanetty, M., Harii, S., and Hoegh-Guldberg, O. (2009) Early molecular responses of coral larvae to hypothermal stress. *Molecular Ecology* **18**, 5101-5114

Roth, M. S., and Deheyn, D. D. (2013). Effects of cold stress and heat stress on coral fluorescence in reef-building corals. *Scientific Reports* **3**, 1-8

Rowan, R., and Knowlton, N. (1995). Intraspecific diversity and ecological zonation in coral algal symbiosis. *Proceedings of the National Academy of Sciences* **92**, 2850-2853

Salih, A., Hoegh-Guldberg, O., and Cox, G. (1998). Photoprotection of symbiotic dinoflagellates by fluorescent pigments in reef corals. *Proceedings of the Australian Reef Society 75th*

Anniversary Conference, Heron Island October 1997 **1988**, 217-230

Sheppard, C.R.C., Davy, S.K., and Pilling, G.M. (2009) *The Biology of Coral Reefs,* 1st Ed., Oxford University Press, Oxford, NY

Woolridge, S. (2013). Breakdown of the coral-algae symbiosis: Towards formalising a linkage between warm-water bleaching thresholds and the growth rate of the intracellular zooxanthellae. *Biogeosciences* **10**, 1647-1658

Warner, M.E., Fitt, W.K., and Schmidt, G.W. (1999). Damage to photosystem II in symbiotic dinoflagellates: A determinant of coral bleaching. *Proceedings of the National Academy of Sciences* **96**, 8007-8012

Figures

A

B

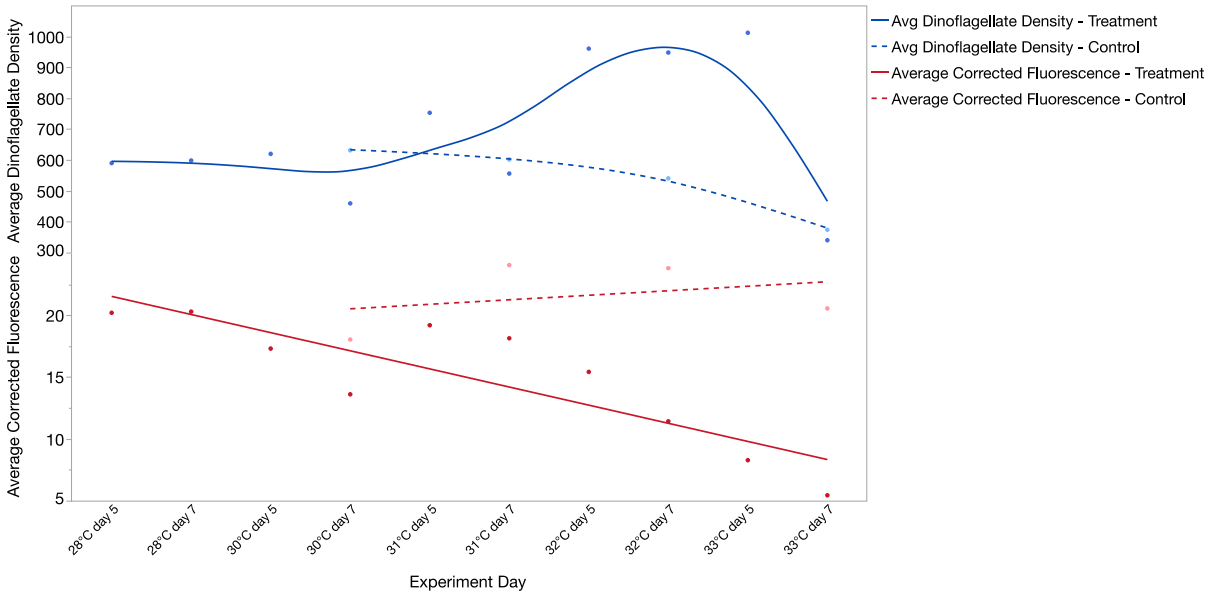

Figure 1. Effects of thermal stress on (A) dinoflagellate density and (B) corrected fluorescence in *Anthelia* spp.. Fragments were subjected to gradual increase in temperature. The heat treatments were 28°C, 30°C, 31°C, 32°C, and 33°C. Analysis occurred twice a week every 72 hours to measure fluorescence of a single tentacle and count the number of dinoflagellates in $1x10^{-5} \pm 2x10^{-7}$ kg worth of coral tissue.

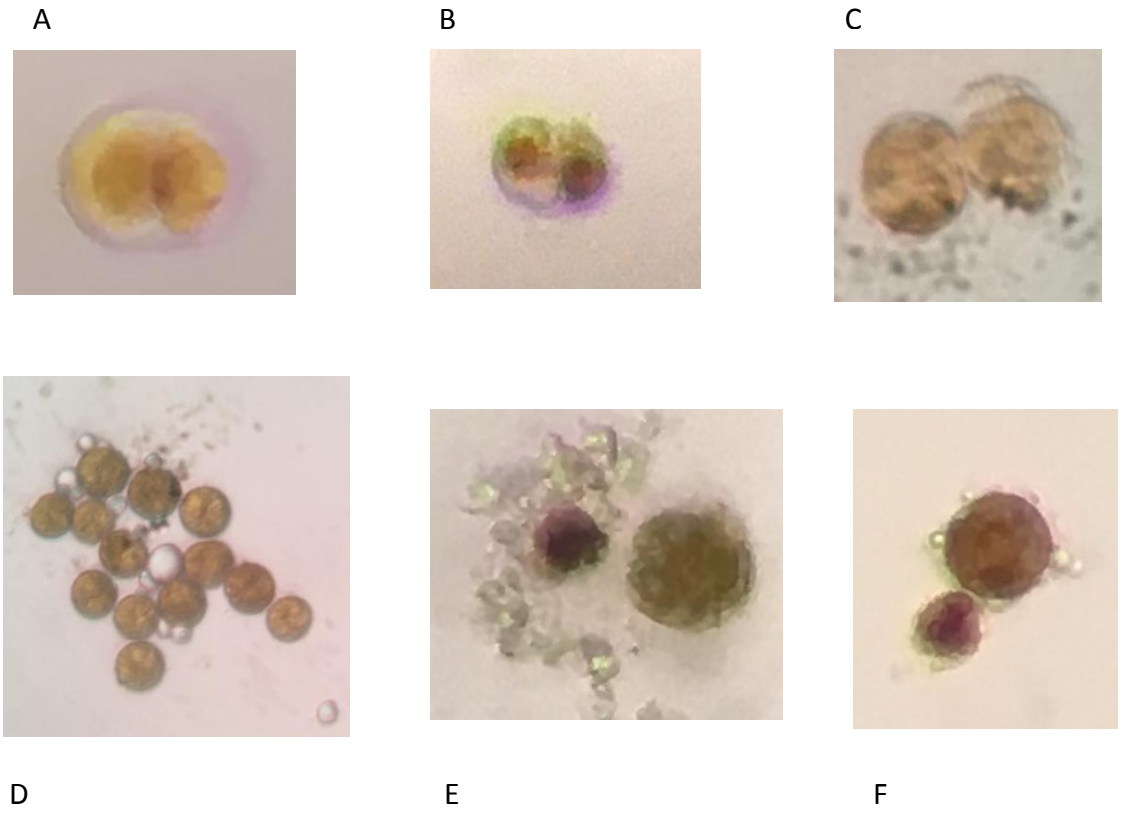

Figure 2. Dinoflagellate Morphology

At 33°C, cells appearing as doublets were observed (A, B, C) under the microscope at 40X and 100X. Dinoflagellates from the control sample were relatively consistent in size (D), whereas cells from the 33°C treatment showed a lack of uniformity in size (E, F). Additionally, the smaller cells were darker in pigmentation.