Recovery Ability of Thermally Stressed Captive Coral *Anthelia* spp., as measured by Dinoflagellate Density

Dana Dunbar and Ross Shaw

Department of Biological Sciences

MacEwan University, Edmonton, AB, Canada

dunbard8@mymacewan.ca

Abstract

Warming ocean temperatures are leading to an increase in coral bleaching events. These rising temperatures are fatal to coral species as they disrupt the symbiotic relationship between corals and dinoflagellates. Among other factors, thermal stress results in dinoflagellate damage and the loss of these symbionts. The recovery ability of corals exposed to this stress is a small area of research within the larger body of coral conservation. This study aims to add to that field by examining how soft corals, specifically *Anthelia* spp., react to thermal stresses. Over a nine-week period, 4 different experimental tanks will be raised from 28º to 32ºC before returning to 28ºC to observe recovery potential. Dinoflagellate density was examined twice per week using a maceration method on tissue samples, viewed under a compound microscope. These densities were used as an indication of coral health and successful recovery. Expanding the knowledge of the recovery ability of soft corals is imperative to continuing the existence of these species.

Keywords: dinoflagellate, coral recovery, coral bleaching, thermal stress, density

Introduction

Coral reefs are well-known for their high productivity, diversity, and provision of habitat for many marine organisms (Coker et al. 2014). A quarter of all marine species live on or are associated with coral reefs (Coker et al. 2014). In addition to providing a home and shelter for these species, reefs have a significant influence on the bordering countries by providing food and jobs through the tourism industry centered on reefs (Sheppard et al. 2009). In Florida alone, a revenue of over $4 billion is generated in association with coral reefs (Riegl et al. 2009).

Although they often appear like one large, stony structure, corals are made of multiple repeating units called polyps. These can be separated and grown individually to create coral

colonies (Sheppard et al. 2009). Corals exist in both hard and soft forms. Both have rings of tentacles which surround the mouth, leading to the body cavity (Sheppard et al. 2009).

Corals are found in a wide variety of conditions and have adapted to differences in microalgae presence, carbon dioxide, water flow rate, and light levels, to name a few. (Donovan et al. 2021; Edge et al. 2013; Woesik et al. 2012). These sessile organisms have adapted to environment fluctuations; however, recent anthropogenic changes have caused increased stress on corals, beyond which they can tolerate (Edge et al. 2013). These conditions elicit a stress response in corals (Edge et al. 2013). These responses however are no longer able to compensate for the once modest condition fluctuations (Edge et al. 2013). Worldwide, there has been a decline in the quality and diversity of reefs, with 20% already being lost (Edge et al. 2013; Riegl et al. 2009).

Dinoflagellates

A large component both in coral function and health depends on the mutualistic relationship corals have with dinoflagellates (Sheppared et al. 2009). Dinoflagellates, or zooxanthellae, are single cellular, phototrophic organisms which live inside the gastrodermis tissue layer of corals (Rowan and Powers 1991). They belong to the genus *Symbiodinium* and are yellow brown in colour (Santos and Shaw 2018). They provide corals with energy rich foods and in return receive inorganic carbon (Sheppard et al. 2009).

Dinoflagellates are organized into eight different clades (A-H) based on their gene sequences (Rowan and Powers 1991; Sheppard et al. 2009). The different clades have different resistance abilities and are thus found in the best suited conditions (Winters et al. 2009). For example, clade C is found in deeper water whereas clade A is found in shallow waters (Winters

et al. 2009). A study by Winters et al. (2009) found Clade A to have a higher resistance ability when the heat stress is over a short period of time.

The level of symbiosis can determine the trophic mode of soft corals (Slattery et al. 2019). Dinoflagellates are acquired through either vertical transmission through asexual reproduction, or through horizontal transmission from taking up free floating dinoflagellates in the surrounding water (Sheppard et al. 2009; Slatterly et al. 2019). When stress is elicited is corals, they can lose their zooxanthellae symbionts (Slatterly et al. 2019). This is significant as dinoflagellates have an obligate mutualistic relationship with the corals they inhabit (Santos and Shaw 2018). This loss of the algae removes the coral's ability to gain food from the photosynthetic products of the dinoflagellates and ultimately results in coral death (Riegl et al. 2009).

Fluorescence

Another important component of coral biology is their ability to fluoresce. Coral fluorescence fulfills a distinct biological function and can be used as an indicator of coral health (Eyal et al. 2015; Hender and Shaw 2020). Eyal et al (2015) reported that fluorescence enhances the functioning ability of zooxanthellae. Both the dinoflagellates and host coral contribute to the fluorescence (Hender and Shaw 2020). Many corals produce pocilloporin, a fluorescent protein in the green spectrum, and dinoflagellates contain chlorophyll-a that fluoresces red (Hender and Shaw 2020). A healthy symbiotic relationship will display fluorescence.

Coral Bleaching

The loss of dinoflagellates, either partially or completely, is referred to as coral bleaching (Sikorskaya et al. 2020). Since corals live close to their thermal tolerance, it is relatively easy for a change in temperature to induce bleaching (Sheppard et al. 2009). This stress damages the

photosynthetic apparatuses and thylakoid membranes of dinoflagellates, subsequently causing expulsion from the organism (Sikorskaya et al. 2020). Losing the symbionts is detrimental and a major energetic cost, which leads to reduced colony fitness and often coral death (Slatterly et al. 2019). However, recovery can be achieved if corals are able to switch to heterotrophy for short periods of time and increase the rate of mitotic division in dinoflagellates (Slatterly et al. 2019; Santos and Shaw 2018). This is referred to as the thermal compensation point (Hender and Shaw 2020).

The greatest threat to corals is this climate driven bleaching (Graham et al. 2007). For reefs to be conserved, mediating coral bleaching needs to become a priority (Graham et a. 2007). However, an often-overlooked aspect of reef conservation is this area of coral recovery (Slatterly et al. 2019). Most of the research in this field is centered on the threats experienced by corals, and at what point they can no longer compensate for changes in their environment. Most studies are focused on dying reefs, the causes of coral bleaching and the numbers of corals that are dying. My goal in this research is to help broaden the knowledge surrounding coral recovery. I focused on determining the recovery potential of soft corals based on their dinoflagellate densities after being exposed to various and gradually increasing levels of heat stress. I hypothesize that if *Anthelia* spp. can successfully recover after the removal of thermal stress, they will have similar dinoflagellate density levels to the control tanks. I further predict that recovery will not be exhibited in corals that are exposed to 32ºC. However, recovery will be seen in the corals that are raised into the range of 29-31ºC.

Materials and Methods

The methods followed in this study were adapted from Joleen Santos (2018) and Rebecca Hender (2020). This study will take place at MacEwan University.

Coral Species and Tank Setup

I studied *Anthelia* spp. coral, inhabited by Clade C zooxanthellae, which was provided by Dr. Ross Shaw (Santos and Shaw 2018). This coral fluoresces red and has an optimal temperature range of 23ºC to 28ºC (Hender and Shaw 2020). Six tanks were filled with freshwater (using RiOs 100 reverse osmosis system (EMD Millipose, Billerica, Massachusetts, USA)) and the specific gravity was adjusted to 1.025. Each tank was equipped with a heater (Fluval E100 100W) and circulation pump (Koralai Nano 420). A light system was set up above each tank containing two 24W bulbs: one Aqublue+ and one Actinic which together mimic the natural light experienced by reefs (Hender and Shaw 2020). These lights were set on a six-hour photoperiod and opaque black corrugated plastic was placed between the tanks to prevent any bleeding of excess light. The tanks are not a confounding variable, nor do they add any extraneous variables, as determined by a pilot study ran by Rebecca Hender (2020).

Fragments of *Anthelia* spp. were taken from a communal tank and placed into the six tanks. The total fragment number is n=66 and these were divided between the tanks according to the number of samples that will be taken from each tank throughout the weeks. Each fragment contains between 2 and 10 polyps and was given a two-week acclimation period in the tanks.

Tank Maintenance

Throughout the study, water was topped up to maintain specific gravity, and fifty percent of the water was replaced every five days, or whenever it became murky. The tanks were also continually wiped down to be kept clean from excess algae growth or cyanobacteria.

Heat Stress Treatment

This study took place over a nine-week period, beginning following the two-weeks of acclimation. Two of the six tanks functioned as controls and were kept at 28ºC for the duration of the study. The remaining 4 tanks were experimental and underwent different phases during the study. The first was the baseline phase in which all the tanks began at 28ºC. Following this was the thermal maximum phase where the different tanks were brought up to 29, 30, 31 and 32ºC. Once a tank reached its maximal temperature, it entered the Recovery Phase where it was gradually brought back down to 28ºC. All the temperatures were adjusted ± 1º on the first day of each week, depending on their specific schedule as outlined in Figure 1.

Dinoflagellate Density

Twice each week on the 3rd and 6th day, an *Anthelia* spp. fragment from each experimental tank was sampled for dinoflagellate density. Once a fragment was selected for analysis, tentacles were cut off, blotted on a Kimwipe, and added to a weigh boat until a mass of $1x10^{-2} \pm 2x10^{-4}$ g was reached on a Mettler Toledo scale. The sample was then macerated using forceps for dinoflagellate removal. Using a micropipette, 10µl of seawater were added, and maceration continued. Using a pipette, 1mL of seawater was then added and used to wash the sample, weight boat and tweezers into a 10mL tube. The sample was then vortexed and titrated 10 times using a 1mL pipette to aid in even cell distribution throughout the sample. Following this, two 10µl samples were observed on a hemocytometer and the number of cells within each nine square side were counted using a tally counter. The average number of cells in the entire 1mL sample was then calculated.

$$cells\ per\ mL\ = Average\ number\ of\ cells\ per\ 1\ mm\ square\ \times\ 10^4$$

$$Total\ cells\ = (cells\ per\ mL)(Sample\ volume\ in\ mLs)$$

Two different fragments were observed each week and the dinoflagellate densities recorded. The number of doublet cells on every slide was also counted to calculate mitotic index.

$$Mitotic\ Index\ = \frac{Number\ of\ cells\ undergoing\ mitosis}{Total\ number\ of\ cells} \times 100$$

Each fragment in this study will only be sampled one time to avoid pseudo replication.
The control tanks will be sampled following identical methodology, however, will only be sampled once on the 6th day of the week.

Fluorescence Imaging

In contrast to my original proposal, fluorescent imaging did not take place due to faulty microscopes and software that was unable to be fixed before sampling began. Removing this from my study does not alter its purpose as although I would have been taking images weekly of the corals, I was not going to analyze them myself due to time constraints.

Statistical Analysis

All analyses were completed using JMP 16 (SAS Institute Inc.). A Wilcoxon / Kruskal-Wallis test was used to analyze the difference between tank treatments on dinoflagellate density. Nonparametric Wilcoxon methods were used for post hoc analysis where $P<0.05$ to determine which treatments were significantly different from each other.

Results

As temperature increased, a decrease in dinoflagellate density was seen in the experimental tanks, and the degree of recovery varied between treatments (Fig. 2). Analysis revealed that there was a statistically significant difference among the dinoflagellate density means for the different tanks ($Chi^2 = 24.7086$, $P = 0.0002$). Post hoc analyses suggest that tanks 5 and 1 ($Z = -2.74$, $P = 0.0061$), tanks 6 and 4 ($Z = -2.54$, $P = 0.0110$), tanks 6 and 2 ($Z = -2.90$,

$P = 0.0037$), 6 and 3 ($Z = -2.98$, $P = 0.0030$), and 6 and 1 ($Z = -3.78$, $P = 0.0002$) were significantly different.

An increase in mitotic index was also observed (Fig. 3). The tanks that were raised to 30, 31 and 32ºC all saw a rise in the index. The largest increase in mitotic index was when the temperatures first reached 30ºC, which occurred during week 3. A decrease was then seen in tank 5 when it was brought back down to 30º during week 5, and for tank 6 in week 7 when it returned to 30ºC.

As a result of the temperature changes, dinoflagellate appearance changed (see Fig. 4). Doublet cells were seen in every sample that was raised above 30ºC (Fig. 4A). As the control tanks did not move from 28ºC, these cells remained similar in pigmentation throughout the nine weeks (Fig. 4B). Samples from tanks 5 and 6 appeared grainy after 30ºC was reached and these cells covered a wide range of colour and size (Fig. 4C).

Discussion

As predicted, tank 6 (32ºC thermal maximum) was unable to recover as it did not have similar overall dinoflagellate densities to the control tanks. Throughout the study, however, this tank decreased steadily at a rate which was unexpected when compared with similar research (Hender and Shaw 2020). An explanation for this degradation of the coral fragments could be found in week 4. When monitoring the conditions of tanks that day, the specific gravity of tank 6 was well outside the ocean seawater range of 1.024–1.026. While the reason for this occurrence remained unknown, the salinity shock may have further affected the appearance and recovery ability of these samples. Similarly, Gegner et al. (2017) found that changes in salinity could significantly alter the degree of bleaching when they studied the sea anemone Aiptasia. The largest change in the *Anthelia* spp. of tank 6 was in appearance: they shrunk to a fraction of their

original size. As a result, it was difficult to collect samples which may have also affected the observed densities.

An unexpected result was that tank 5 (31ºC thermal maximum) differed statistically from tank 1 (first control). This was not expected as corals which reach 31º are expected to recover successfully (Hender and Shaw 2020). And importantly, tank 5 does not show statistical difference from tank 2, the other control. The difference between tanks 1 and 2 may be explained by their setup. During setup, coral fragments were distributed into the different tanks. The second control was the last tank set up and the stock of *Anthelia* spp. from the communal tank ran out. To make up the missing numbers, fragments were selected from the other tanks and the cement plugs to which the corals were attached were broken into two using a chisel, and the base tissues were then separated. Therefore, all the corals in tank 2 underwent a slight stress at the beginning of the experiment which may explain both why the counts are lower in this control at the start of the experiment but then follow similar trends to control 1 (see Fig. 2). This may explain why tank 5 does not show a difference to both controls.

Interestingly, tanks 5 and 6 are not different from each other. Since these were raised to similar thermal maximums, they underwent a similar stress pattern for the longest time in the study and their symbionts therefore had the most similar response before the divergence in temperature began.

This similarity can also be seen when examining the mitotic index (Fig. 3). All the observed mitotic index responses were expected. Corals, specifically *Anthelia* spp., have a thermal compensation point around 30ºC (Hender and Shaw 2020). At 30ºC the dinoflagellates respond to the thermal stress by increasing their rate of division. After this point, however, they are unable to compensate and coral recovery is not seen at 32ºC, both in my study and others

(Hender and Shaw 2020). The 30ºC mark for my study was reached at week 3, and it is at this point the mitotic index can be seen to increase (Fig. 3). Similar trends were also seen in a study by Levin et al. (2016). Above 30ºC, both of their coral populations inhabited by C1 *Symbiodinium* had increased regulation of meiosis and division genes by 4-fold (Levin et al. 2016).

The results of this study have relevance to the broader body of research surrounding coral conservation in multiple ways. First, the recovery of corals is a relatively overlooked topic within this field (Slattery et al. 2019). This study also utilized gradual temperature changes which is beneficial as many thermal stress studies are done through heat shock treatments which are often not representative of real-world changes (Roth and Deheyn 2013). Additionally, the health of corals does not decrease linearly while temperature increases which creates important periods of respite (Sheppard et al. 2009). It is imperative to further understand coral health and recovery so these periods may be used to extend the life of these species (Sheppard et al. 2009; Stat et al. 2008). Lastly, a new area of research is developing with the specific focus to extend the life and thermal tolerance of coral species. Depending on the clade present, dinoflagellates can be the limiting factor in thermal tolerance for corals (Stat et al. 2008). Different clades have wider ranges of tolerance and understanding these differences this may be critical for the survival of corals (Stat et al. 2008). As the knowledge surrounding zooxanthellae clades grows, the hope would be to take dinoflagellates with a higher tolerance, such as clade D, and propagate those into less tolerant corals, thus extending their survivability (Sheppard et al. 2009) For example, the symbionts of *S. polydactyla* have shown potential for a larger range of resistance to thermal stress and could benefit other species (Slatterly et al. 2019). Therefore, this study adds value in furthering the knowledge around clade C and what its capabilities are.

An improvement to this study could be found in exploring recovery vs. full recovery. After a tank was brought back down to 28ºC, it was removed from the study. This was partially due to a limited number of coral fragments. Therefore, completing this study again but maintaining these tanks at 28ºC for the entire 9 weeks and having the coral fragments be continually sampled would give an indication of complete recovery. Can these corals get back to the dinoflagellate density levels at which they began the study? To study this, the number of fragments would have to be increased from 66 fragments to 90. A number of these fragments could also come from removing the second control tank from the design as it seemed extraneous. Removing this would make the total number of tanks 5, and the necessary fragments 81.

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Figures

Week	Control 1 (Tank 1)	Control 2 (Tank 2)	Tank 3	Tank 4	Tank 5	Tank 6
1	28ºC	28ºC	28ºC	28ºC	28ºC	28ºC
2	28ºC	28ºC	29ºC	29ºC	29ºC	29ºC
3	28ºC	28ºC	28ºC	30ºC	30ºC	30ºC
4	28ºC	28ºC	X	29ºC	31ºC	31ºC
5	28ºC	28ºC	X	28ºC	30ºC	32ºC
6	28ºC	28ºC	X	X	29ºC	31ºC
7	28ºC	28ºC	X	X	28ºC	30ºC
8	28ºC	28ºC	X	X	X	29ºC
9	28ºC	28ºC	X	X	X	28ºC

Figure 1. Experimental conditions of tanks throughout the study. Each experimental tank underwent three phases. Phase one was the Baseline (orange), followed by the Thermal Maximum (red) and then Recovery Phase (green). The Recovery Phase was the focus of this study. The controls remained at 28ºC for the duration of the study. (This figure is adapted from Hender 2020).

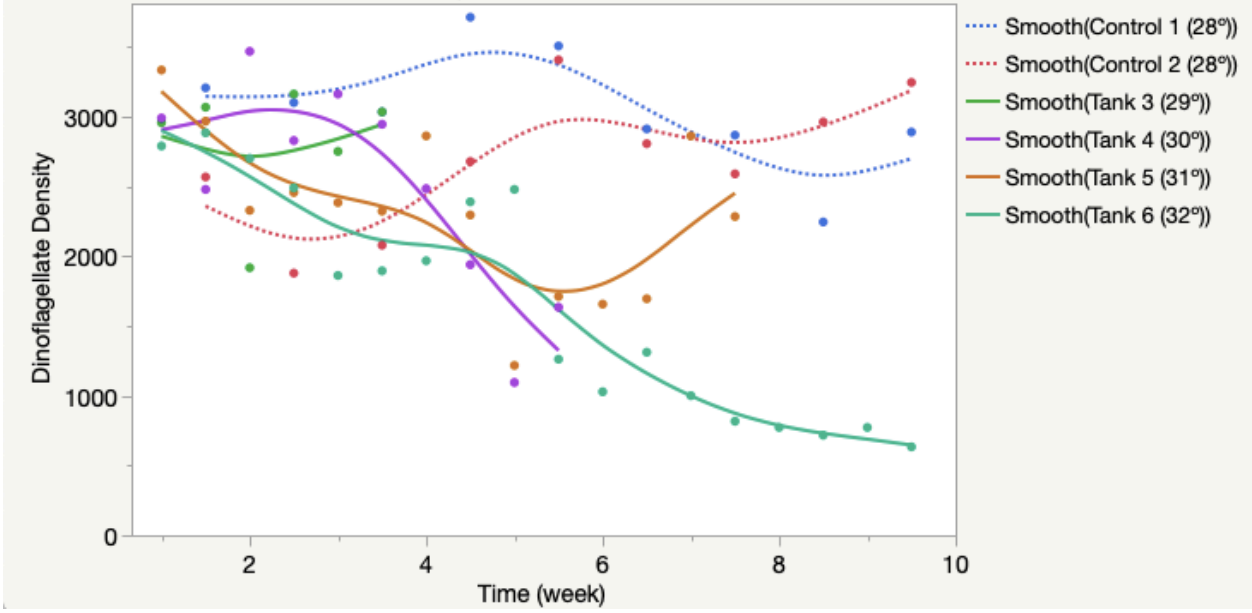


Figure 2. Effects of thermal stress on dinoflagellate density in *Anthelia* spp.. Coral fragments underwent temperature increases from 28ºC to 29ºC, 30ºC, 31ºC and 32ºC, before being lowered again to 28ºC. Control tanks remained at 28ºC. Dinoflagellate density was sampled twice a week on the 3rd and 6th day following a change in the temperature. The dinoflagellates of a $1x10^{-2}\pm 2x10^{-4}$ g sample were found using a hemocytometer.

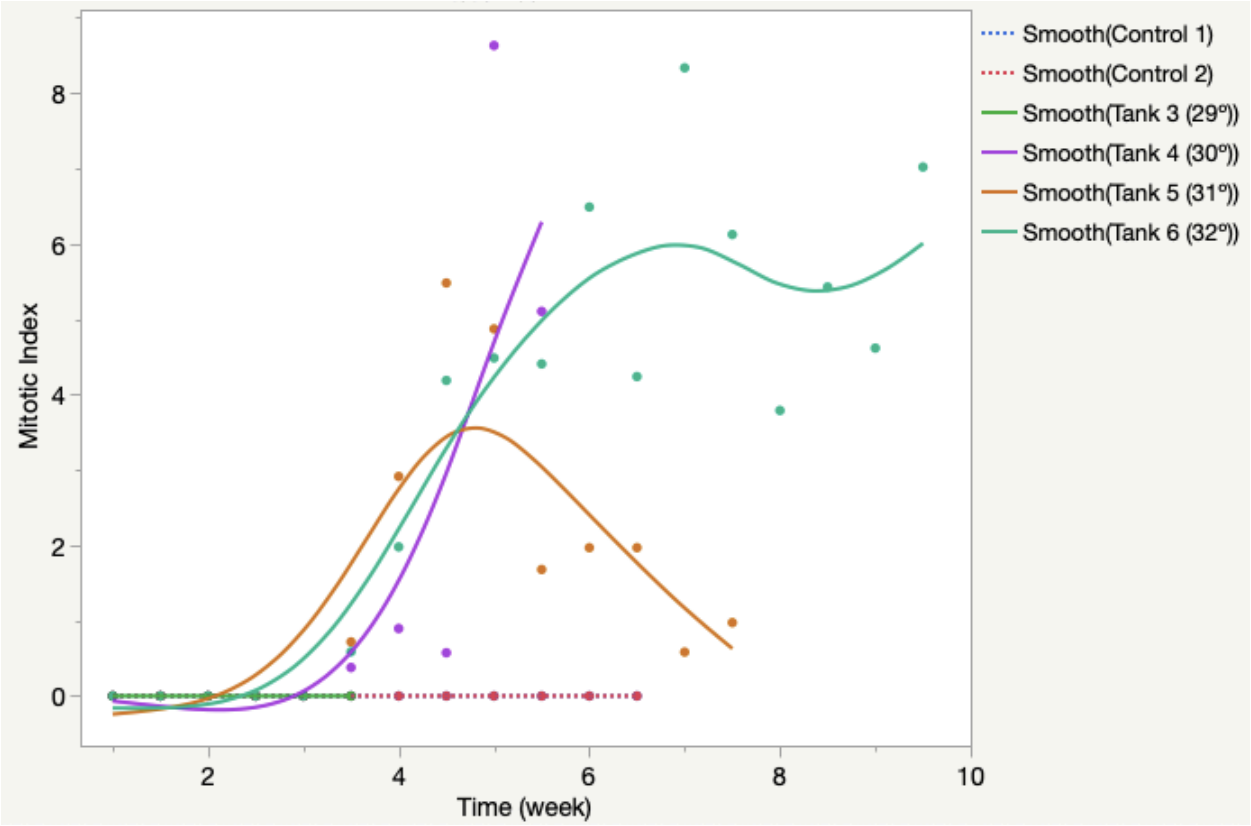
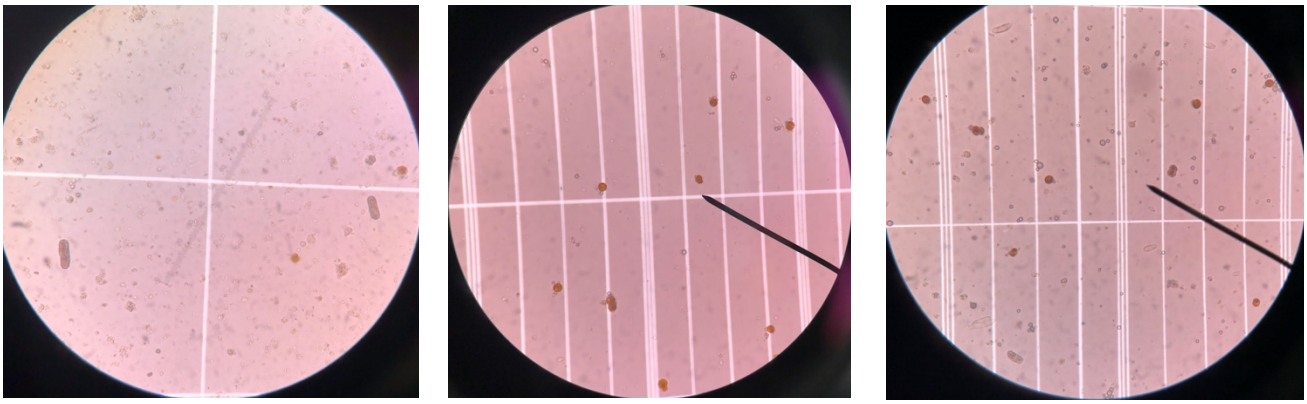


Figure 3. Mitotic index as effected by thermal stress. Coral fragments underwent temperature changes from 28ºC up to 29ºC, 30ºC, 31ºC and 32ºC and back to 28ºC. When dinoflagellate density was sampled, the number of doublet cells was also counted. This was used to calculate mitotic index by dividing these counts by the total number of cells and multiplying by 100.



A B C

Figure 4. Slide and dinoflagellate appearance. Doublet cells were observed in slides beginning at week 3 and continuing until the end (A). Pictured in A are dividing cells from Tank 6 during Week 7, Day 3. Cells from the control tanks remained relatively evenly distributed and similar in pigmentation throughout the study (B). Samples from tanks 5 and 6 appeared grainy and the dinoflagellates were darker in colour once temperatures above 30ºC were reached.