A Histological Assessment of the Effects of Elevated Temperature and Nitrogen on the Symbiodinium of Palythoa Toxica (Walsh and Bowers 1971)

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A HISTOLOGICAL ASSESSMENT OF THE EFFECTS OF ELEVATED TEMPERATURE AND NITROGEN ON THE SYMBIODINIUM OF PALLYTHOA TOXICA (WALSH AND BOWERS 1971)

by

Molly Westbrook

A Thesis Submitted in Partial Fulfillment of the Requirements for a Degree with Honors (Marine Science)

The Honors College
University of Maine
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Coral reefs around the world are suffering mass bleaching events caused by a combination of stressors, including rising ocean temperatures, acidity, pollution, increased suspended sediments, and increasing nitrogen levels. Corals harbor a complex microbial ecosystem consisting of bacteria, and algal symbionts known as *Symbiodinium*. This study examines the effects of elevated temperature, a known cause of bleaching, and elevated nitrogen, an increasingly important potential stressor for reefs, on the *Symbiodinium* of a zoanthid coral, *Palythoa toxica*. A total of 65 polyps were subjected to 5 different water treatments of 2 levels of elevated nitrogen, and 2 levels of elevated temperature in populations of 12. Samples were removed every four days and at the experiment’s termination. The samples were fixed for histological examination. *Symbiodinium* size was analyzed using Feret diameter. The results of this experiment confirm the effect of elevated temperature as a bleaching mechanism for *P. toxica* and provide evidence that elevated nitrate is a potential bleaching trigger in prolonged exposure. Furthermore, there is evidence that combined elevated nitrate and temperature compound bleaching mechanisms, with the highest amount of bleaching occurring in the most stressed treatments. When exposed to low levels of nitrate and low levels of temperature elevation, there is a trend of *Symbiodinium* Feret diameter increase. With time, however, there is an eventual collapse of the system, resulting in *Symbiodinium* Feret diameter decrease and an increase of algal mass potentially from cell lysis within bleached *Symbiodinium* colonies remaining between tentacles and within the coelenteron.
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# TABLE OF CONTENTS

Table of Figures v  
Introduction 1  
  Anthropogenic Effects on Coral Reefs 1  
  *Symbiodinium* and the Adaptive Bleaching Hypothesis 2  
  The Interaction Between Nitrogen, Coral, and *Symbiodinium* 5  
  *Palythoa toxica* Overview and Study Objectives 6  
Materials and Methods 8  
  System Design and Sampling 8  
  *Symbiodinium* Cell Size Analysis 10  
  Statistical Analysis 11  
Results 12  
  Experiment One 12  
    Effects of Elevated Nitrogen 14  
    Effects of Elevated Temperature 17  
    Variance Among Treatments 20  
  Experiment Two 21  
    Effects of Combined Elevated Nitrogen and Temperature 22  
    Variance Among Treatments 27  
Discussion 28  
  Nitrogen Effects on *P. toxica* 28  
  Temperature Effects on *P. toxica* 30  
  Combined Stress Effects 31  
  Conclusions 33  
References 35  
Appendices 40  
  Appendix 1: ImageJ Feret Diameter Calculations 41  
  Appendix 2: R Script for Statistical Analysis 45  
Author’s Biography 49
TABLE OF FIGURES

Figure 1. Image of *P. toxica* 6
Figure 2. Image of care system 8
Figure 3. Image of experimental system 9
Table 1. Treatment water quality parameters of experiment 1 and 2 9
Figure 4. Images of histological slides (1) 12
Figure 5. Images of histological slides (2) 12
Figure 6. Images of histological slides (3) 14
Figure 7. Images of histological slides (4) 14
Figure 8. Images of histological slides (5) 15
Figure 9. Box plot of *Symbidoinium* size comparison (1) 16
Figure 10. Images of histological slides (6) 17
Figure 11. Images of histological slides (7) 17
Figure 12. Images of histological slides (8) 18
Figure 13. Box plot of *Symbidoinium* size comparison (2) 19
Figure 14. Box plot of *Symbiodinium* size variance (1) 20
Figure 15. Images of histological slides (9) 21
Figure 16. Images of histological slides (10) 22
Figure 17. Images of histological slides (11) 22
Figure 18. Images of histological slides (12) 23
Figure 19. Images of histological slides (13) 24
Figure 20. Images of histological slides (14) 25
Figure 21. Box plot of *Symbidoinium* size comparison (3) 25
Figure 22. Box plot of *Symbiodinium* size variance (2) 27
INTRODUCTION

Anthropogenic Effects on Coral Reefs

It is abundantly clear that anthropogenic effects are changing the nature of coral reefs (Hughes et al 2003, Bellwood et al 2004), and that as humans continue to impact the global climate, the negative effects of these changes on coral reefs will increase (Hughes et al 2003, Hughes et al 2017). There are a number of different factors that can influence these effects, including stress effects from sedimentation and pollution (ISRS 2004), temperature (Glynn 1990, Winter et al 1998), high levels of solar radiation (Lesser et al 1990, Gleason and Wellington 1993, Brown 1997), ocean acidification (Anthony et al 2008), and nitrogen enrichment (Wiedenmann et al 2012, Vega-Thurber et al 2014).

One of the most well-known coral stress indicators is that of coral bleaching, or the expulsion of symbiotic dinoflagellate algae known as Symbiodinium normally maintained in coral polyp tissues (Brown 1997). This loss of Symbiodinium results in the coral tissue becoming pale or transparent, causing the skeleton to become visible and resulting in a white color. The first observed and published mass bleaching event of a wide range of coral reefs simultaneously occurred in Gulf of Chiriquí, Panama in 1982 during the El Niño temperature anomaly (Glynn 1984). Since this event, there have been numerous local and widespread mass bleaching events worldwide, including three widespread mass bleaching events in 1998, 2002, and from 2016 to 2017, the first back-to-back mass bleaching event (Hughes et al 2017) on the Indo-Pacific Archipelago. Other major bleaching events include the 2005 Caribbean reef bleaching event (Eakin et al 2010), and the 1982 Eastern Pacific bleaching event (Harriot 1985, Glynn 1990). The
result is clear: bleaching events are becoming increasingly common, and understanding these events is essential for reef protection and recovery (Hughes et al. 2003).

The bleaching mechanism lies in the symbiotic relationship between *Symbiodinium* and the coral animal, and understanding this relationship is essential to the coral bleaching mechanism. With a more complete understanding of this mechanism, human conservation policy can be accurately shaped to protect reefs from anthropogenic-caused stress.

**Symbiodinium and the Adaptive Bleaching Hypothesis**

Symbiosis, or “the living together of unlike organisms” (Douglas 1994) has been a long-observed relationship in biology. First used to describe the relationships among lichens in the late 1800’s (Trappe 2005), there are several required factors for a relationship to be classified as symbiosis. First, at least one organism must play a significant role in the life cycle of the second, and second, the organisms are in sustained and close contact throughout their lifetimes (Lewis 1973).

*Symbiodinium* are a genus of dinoflagellates found maintaining symbiotic relationships with several marine invertebrate phyla including Cnidaria, Mollusca, Platyhelminthes and Porifera (Stat et al. 2006). The most well-known relationship of these groups is that between *Symbiodinium* and the Cnidarian *Anthozoa*.

The relationship between coral animals and *Symbiodinium*, known colloquially as zooxanthellae, is an essential relationship within the coral animal life cycle. *Symbiodinium* provide essential nutrients to the coral animal through photosynthesis for cell nutrition, maintenance and reproduction (Muscatine, Porter, 1977), fixing up to four times as much carbon as coral lacking *Symbiodinium* in their tissues (Muscatine et al.
1984). Maintaining symbiotic relationships with algae allow coral to function as primary producers as well as primary and secondary consumers (Muscatine, Porter 1977), resulting in the role of coral in reef ecosystems as a keystone species. The carbon fixed by *Symbiodinium* plays an especially important role in the complex calcium carbonate skeleton certain coral secrete that comprises the physical shape of the reef itself (Anthony et al 2008). These calcifying coral not only function as animals within the reef ecosystem, coral compose the shape of the reef itself literally through their skeletal structures (Tambutté et al 2011). Furthermore, *Symbiodinium* play an important role in juvenile coral growth, fixing carbon as the coral polyp settles on the reef (Cantin et al 2008). *Symbiodinium* have only recently become the focus of understanding the adaptive mechanisms of coral to stress.

*Symbiodinium* were first grouped into 22 different taxonomic groups of indeterminate level using restriction fragment length polymorphisms from the small ribosomal subunit RNA (Rowan and Powers 1991). Later these groups became the modern clade taxonomic classification of these algae, composing nine clades (A-I), and a number of subclades, with primary associations of clade C with coral. *Symbiodinium* are acquired in coral tissue through two ways: horizontally from the environment, or vertically from parental populations (Byler et al 2013). Originally thought to be specific to the host, *Symbiodinium* transmission is flexible both between species of coral and within species, with species maintaining multiple subclades of a single *Symbiodinium* clade within their tissues, and some maintaining even multiple clades (Little et al 2004). It has been shown that different *Symbiodinium* clades adjust the metabolism and stress coping mechanisms for different aspects of the holobiont system. For example, hosts
harboring clade C *Symbiodinium* have an increased growth rate (Little et al 2004), whereas populations maintaining clade D have a higher tolerance of thermal stress (Rowan 2004, Baker et al 2004).

Herein lies the key to coral bleaching and coral adaptation to stress conditions. Individual clades of *Symbiodinium* have relatively narrow ranges of stress tolerance due to their thylakoid lipid-membrane composition (Tchernov 2004), and thus it is very difficult for populations of *Symbiodinium* within a single clade to survive alone with the holobiont system in changing environments. However, there are two mechanisms that allow corals to shift the dominant clades of *Symbiodinium* maintained within their tissues: switching, or completely expelling the symbionts to replace with others taken up horizontally, and shuffling, or rearranging existing populations within their tissues for a different symbiont clade to influence the coral’s adaption to the environment (Reich et al 2017). Bleaching was first hypothesized in 1993 and later refined as an adaptive mechanism through which coral could shuffle *Symbiodinium* within their tissues to increase tolerance to local conditions known as the Adaptive Bleaching Hypothesis (Buddemeier and Foutin, 1993; Buddemeier et al 2004). Further research into this mechanism lead to the hope that corals would be able to adapt for the short term to human-influenced changes, such as increasing ocean temperatures up to a point (Berkelmans, Oppen 2004), though it is increasingly clear that the complicated nature of *Symbiodinium* taxonomy and nuanced understanding of thermal and stress tolerance requires reclassification from the current taxonomic clade system (Sampayo et al 2008). Whether or not scientists can use this relationship to help protect and potentially recover reefs in future anthropogenic climate fluctuations is unknown.
The Interaction Among Nitrogen, Coral, and Symbiodinium

The interaction between coral reefs and nitrogen has been a subject of study since the 1970’s, when the observation was first made of the coral reef nutrient paradox. Reefs are located in areas of relatively low nutrient levels and yet are able to maintain high levels of productivity (Webb et al 1975), thus the mechanisms through which coral are able to efficiently use nitrogen are of major concern. Nitrogen is the primary limiting nutrient for marine systems within coral- reef systems (Thomas 1969), and it has been shown that coral’s response to the nitrogen cycle and internal nitrogen cycle are closely tied with their susceptibility to bleaching and disease (Weidenmann et al 2013, Vega-Thurber et al 2014, Rädecker et al 2015).

In brief, the nitrogen cycle begins in the atmosphere as N₂, nitrogen is fixed by archaeobacteria and eubacteria located in sediment in an anaerobic process called denitrification to NO₃⁻ or nitrate, which is then taken up by organisms, usually plants, and converted to ammonium (NH₄⁺). Microbial processes convert NH₄⁺ to NH₃, then NO₂⁻, and finally to NO₃⁻, and the cycle can repeat. These converted forms of nitrogen, especially NO₃⁻ and NH₄⁺, can be taken up by organisms and transformed back to NH₄⁺ or urea, which then sink down into the sediment and are converted again. NH₄⁺ can also be nitrified back into N₂, where it is returned in the atmosphere (Falkowski 1997).

However, human impact on the nitrogen cycle through artificial fertilizers in modern agriculture and burning fossil fuels have added a second influx of NO₃⁻ into the system, where it is washed into the ocean, most notably manifesting as algae blooms and areas of anoxic or dead zones along the coast (Vitousek et al 1997, Canfield et al 2010). There is clear evidence that these anoxic blooms are increasing with time, resulting in
decreasing diversity along coastal dead zones including among seagrass beds and coral reefs, lower fish and shellfish resources for human fisheries and aquaculture, increasing dominance of nuisance algae and increasing occurrences of toxic dinoflagellate blooms or “red tides” known to cause shellfish poisoning among humans (Vitousek et al 1997).

Tropical coral reefs are most often located in coastal or near-coast ecosystems and are thus subject to these anthropogenic influxes of nitrogen into the system. Because coral maintain symbiotic algae within their tissues, it is likely that this influx of nitrogen into the ocean is causing changes to these communities. Very little research has been done on the interactions between coral and the nitrogen cycle, though there is increasing evidence that nitrogen plays an important role in the dynamic of the coral holobiont and the bacterial and algal members of its symbiotic community (Siboni et al 2008, Pogoureutz et al 2017)

*Palythoa toxica* and Study Objectives

*Palythoa toxica* (Walsh and Bowers 1971) is a zoanthid hexacoral that produces the infamous palytoxin, one of the most deadly toxins within the natural world. This

Figure 1. A colony of *Palythoa toxica* (Walsh and Bowers 1971) from the University of Maine Aquaculture Research Collection used in this experiment. Photo credit to Allyson Redcay.
species is not a reef-building scleractinian coral. Instead these animals have relatively large anemone-like polyps, but maintain similar symbiotic algal associations as scleractinian coral. The species was first isolated in 1971 from Hawaii from a tropical coral referred to as limu-make-o-Hana, and was the first organism discovered to produce palytoxin (Walsh and Bowers, 1971). Since its discovery, palytoxin and its effects on humans have become the focus of research on this group (Moore and Scheuer, 1971; Deeds and Schwartz 2010). Currently there is very little research on histological changes during prolonged stress periods, though ecological studies of *Symbiodinium* in zoanthid coral have been conducted (Kemp and Cook 2006; Burnett 2002) and indicate zoanthid coral exhibit similar bleaching mechanisms as scleractinians (Kemp and Cook, 2006). Furthermore, *P. toxica* is relatively easy to obtain and culture, and was used for this experiment due to its accessibility and lack of research focusing on this particular symbiotic relationship.

The goal of this study is understanding the relationship between *P. toxica* and its associated *Symbiodinium* when exposed to elevated levels of nitrogen and temperature both separately and in combination using histological analysis. *Symbiodinium* were not genetically analyzed to determine clade. The hypothesis of the study was based on increased growth of dinoflagellates when exposed to elevated nutrient levels (Parkhill and Cembella 1999), and tests for a direct relationship between the level of nitrogen within the coral tissues and the size of the *Symbiodinium* cells. Temperature was added as a second factor to understand stress effects on coral in combination with nitrate stress, and to retest the effects of temperature on coral bleaching, especially for the relatively understudied zoanthid clade.
MATERIALS AND METHODS

System Design and Sampling

*Palythoa toxica* samples were acquired from a local aquarist in Orono, ME, and moved to the University of Maine Aquaculture Research Center where they were maintained in a 710 liter raceway (290 L raceway, 420 L bottom sump) under a Tek light T5. The water was maintained around 26˚C, 32 ppt salinity, a pH of approximately 8, and a water exchange rate of 35 L/sec.

Figure 2. The system in which colonies of *P. toxica* were maintained before the experiment.

The samples comprised three genetically identical colonies of various size. 125 polyps were cloned from these samples using an Ecotech Marine Coral Propagation Kit, glued onto cement coral plugs using Ecotech coral glue, and maintained in the raceway for two weeks prior to the experiment. The design of the experiment comprised one control and four experimental treatments with one 4-L tank per treatment. 60 polyps were used for each experiment, in groups of 12 polyps per treatment.
Two experiments were run over the course of this project, both lasting 14 days, of two levels of elevated nitrate and two levels of elevated temperature (Table 1). Nitrate levels were elevated by adding powdered NaNO$_3$. After completion of experiment one tanks were drained and cleaned using a water and bleach rinse before being randomly rearranged for experiment two. Experiment two consisted of the same experimental setup. Nitrate levels were readjusted for the second experiment, and the same temperature levels were used.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
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<tbody>
<tr>
<td>1</td>
<td>26°C, 0 ppm</td>
<td>26°C, 0 ppm</td>
</tr>
<tr>
<td>2</td>
<td>26°C, 7 ppm</td>
<td>30°C, 10 ppm</td>
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<td>3</td>
<td>26°C, 15 ppm</td>
<td>30°C, 20 ppm</td>
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<tr>
<td>4</td>
<td>30°C, 0 ppm</td>
<td>35°C, 10 ppm</td>
</tr>
<tr>
<td>5</td>
<td>35°C, 0 ppm</td>
<td>35°C, 20 ppm</td>
</tr>
</tbody>
</table>

Table 1. Treatment temperature and nitrate levels for experiment 1 and experiment 2.
All tanks contained a fish tank heater of 50 or 100 watts to maintain temperature, and were maintained beneath two 50 watt lights, on a light schedule of 12 hours on, 12 hours off (Figure 3).

For both experiments two polyps were sampled from the control treatment on day one, and then from every treatment every four days. On day 14 all remaining polyps were sampled from each treatment. Histology fixation was conducted using a standardized procedure as follows: samples were placed in 10% neutral buffered formalin, placed in a standard tissue processor for 16.5 h, then embedded in paraffin. Five-µm thick sections were cut and stained with hematoxylin and eosin. Sampling protocol was designed using IACUC Power Study protocol (University of Maine). Nitrogen-enrichment levels were based on two Australian reefs with higher levels of nitrate: Yongola Reef in the mid-Great Barrier (Vridaka) as the low-enriched level, and Rottnest Island, in Western Australia as the high-enrichment level (Thomson et al 2011). Nitrogen levels were tested every day for the first four days of each experiment using a LaMotte water quality testing kit and then every other day for the duration of the experiment. When nitrogen levels were lower than the required level, approximately 0.01 grams of NaNO₃ was added to the tanks (increasing NO₃⁻ by approximately 2 ppm).

*Symbiodinium* Cell Size Analysis

Feret diameter was used as a quantitative measurement of the change of size in *Symbiodinium* (See Appendix 1). Slides were photographed using an Olympus CX31 microscope with Infinity Capture software. Photographs were analyzed using ImageJ version 1.51s and Java1.8.0_151. Analysis was calibrated for 40x images using a Motis calibration for 40x, 100x and 400x magnification. The perimeters of 100 randomly
selected *Symbiodinium* were traced using the freehand trace function and measured for each polyp for a total of 200 Feret diameters for each treatment barring the samples from Trial 1 Treatment 1 Day 1 (3 polyps), Treatment 3 Day 12 (3 polyps), Treatment 4 Day 8 (1 polyp), Treatment 5 Day 8 (3 polyps), and Trial 2 Treatment 1 Day 1 (1 polyp), as well as samples from the 14th day of the experiments (3-4 polyps). Due to stress, the polyps from the 12th day of Trial 2 for treatments 4 and 5 were too degraded for histology, and thus there are no data for those samples. *Symbiodinium* were treated as the individual (N=1) for this process instead of polyps within the treatment.

**Statistical Analysis**

All statistical analysis was conducted in RStudio version 3.3.3 on a basis of 95% confidence. Data were organized by Experiment (1 or 2), treatment (control, high/low nitrate, high/low temperature), time of sampling (Day 4, 8, 12, or 14) and polyp. Each *Symbiodinium* Feret measurement was assigned a number, and average Feret diameter was calculated by averaging the Feret maximum and minimum. One-way analysis of variance (ANOVA) was used to compare mean Feret of treatments (See Appendix 2). Outliers were removed from each dataset and assumptions of the ANOVA were met by each set (Levene’s test for symmetry, p>0.05, Shapiro-Wilk test for normality, p>0.05). Two ANOVA analyses were conducted. The first ANOVA used treatments as the explanatory variable, and the second used day of sampling as the explanatory variable. A Tukey HSD post-hoc was used for both ANOVA results for individual mean comparison.
RESULTS

Experiment One

Figure 4. Control Day 4, from left to right, top to bottom: 400x, 100x and 40x images of Polyp II.

Figure 5. Images of control polyps throughout different sampling times of the experiment. Green arrows indicate healthy *Symbiodinium* cells.

To establish a concept of a ‘healthy’ polyp as a basis of comparison for the experiment, samples of the control polyp were taken at every sampling step of the
experiment. There are several factors that characterize a healthy polyp for this experiment, as can be demonstrated by Figures 4 and 5. Healthy polyps contain no faded tissue; in other words, polyp tissue is tinted darker shades than bleached counterparts. Healthy *Symbiodinium* are in general maintained in one of two places: either along the tentacles near the top of the polyp (Figure 4, 400x, Figure 5, Day 12, 400x) or maintained in gastrodermis in the mesenteries of the polyp (Figure 5, Day 14 400x Polyps 1 and 3). Small numbers of *Symbiodinium* could also be found maintained in vesicles within the polyp epidermis (Figure 5, Day 14 Polyp 3). Finally, there is no sign of *Symbiodinium* lysis; in other words, all cells of *Symbiodinium* are whole and contain plastids (Figure 4, 400x, Figure 5, all 400x images show relatively healthy *Symbiodinium*). Not every control polyp displayed these characteristics; bleaching occurred in the control treatment of the second experiment on the 14th day.
Effects of Elevated Nitrogen

Figure 6. Images of polyps taken from Treatments 2 and 3 (elevated Nitrogen treatments) at 40x throughout the experiment. Polyps in treatment 3 days 4, 8 and 14 exhibit moderate bleaching.

Figure 7. Images of polyps taken from Treatments 2 and 3 (elevated Nitrogen treatments) at 400x throughout the experiment.

Within elevated nitrogen treatments, there was a major difference between these treatments and the control treatments. Full bleaching of coral tissue did not occur; however, there was a trend of gastrointestinal pockets of Symbiodinium among the
tentacles and mesenteries being expelled into the coelenteron of the polyp (Figure 7).

Near the end of the experiments, some of the *Symbiodinium* had lysed within Treatment 3 (Figure 7; Treatment 3, Day 14).

![Image](image1.png)

Figure 8. A comparison of *Symbiodinium* on the last day of the experiment for the control (treatment 1) and elevated nitrogen treatments (2 and 3) at 400x.

There appears to be a trend with time between different levels of elevated nitrate. Small levels of nitrate may stimulate growth of *Symbiodinium* (Figure 8) to an unhealthy degree. Higher levels of nitrate cause coral tissue to bleach partially, but retain those *Symbiodinium* within their system.
Elevated nitrate affected *Symbiodinium* Feret diameter, however, how nitrate affected the size of the algae cells varies between time and treatment (Figure 9). For the first half of the experiment, there was a clear trend of increasing size with increased nitrate levels within the water. As the experiment continued however, the average Feret diameter of treatment 3 decreased from almost 10 µm in diameter on Day 4 at the highest to as low as 9 µm on Day 12. Within nitrate treatments, *Symbiodinium* Feret diameter remains between 8 and 10 µm. For both treatment 2 and 3, diameter increased in the first half of the experiment and appears to decrease in the second half.
Effects of Elevated Temperature

Figure 10. Images of polyps taken from Treatments 4 and 5 (elevated temperature treatments) at 40x throughout the experiment. Treatment 4 on day 14, and treatment 5 on day 4 exhibit symptoms associated with bleaching.

Figure 11. Images of polyps taken from Treatments 4 and 5 (elevated temperature treatments) at 400x throughout the experiment.

Elevated temperature in general caused minor bleaching (Figure 10, Treatment 4 Day 14, Treatment 5 Day 4 and Day 14). In general, similar to nitrogen treatments, there was a trend of *Symbiodinium* being expelled from the polyp tissues to coagulate in spaces
among tentacles and within the coelenteron of the coral (Figure 11, Treatment 4 Day 14, Treatment 5 Day 4). Some breakdown of gastrointestinal tissue occurred in particularly unhealthy polyps (Figure 10, Treatment 4 Day 14, Figure 9, Treatment 4), however the major trend remained change in *Symbiodinium* location, moving out of gastrointestinal lining into spaces inside the polyp (Figure 10). Bleaching remained minimal, with only one polyp in treatment 4 and one polyp in treatment 5 displaying signs of moderate bleaching.

Figure 12. A comparison of *Symbiodinium* on the last day of the experiment for the control and elevated temperature treatments at 40x.
The pattern of *Symbiodinium* diameter size change in elevated temperature treatments was more random than elevated nitrate treatments but displayed a similar pattern. In general, treatments 4 and 5 had significantly different means than the control (Figure 13). In the case of the beginning of the experiment, both treatment 4 and 5 had larger Feret diameters on average, with treatment 4 being the largest of the three. However, by the end of the experiment, the average Feret diameter of treatments 4 and 5 had decreased. By Day 12, there was no significant difference between treatment 4 and the control. By Day 14, there was no significant difference between treatment 5 and the control, and treatment 4 had decreased significantly to below the average Feret diameter of the control.
Variance Among Treatments

Figure 14. Box plots of average Feret diameter within each individual treatment throughout the experiment. Matching colors represent insignificantly different samples (ANOVA, P>0.05). In the case of multiply colored samples, a significant difference occurred for at least one treatment comparison. For treatment 2, Day 12, Feret diameter was significantly different from Day 14 but equivalent to Days 4 and 8. In Treatment 4, Day 14 was not significantly different from either Day 4 or Day 12, but both Day 4 and Day 12 varied from each other. Finally in Treatment 5, Day 8 and 14 were significantly different than Day 12, but not Day 4.

There was significant variance among individual tank Feret diameters as time passed during the experiment. Among treatments 1, 2 and 3, treatment Feret diameter remained consistent for several days at a time but still varied at different points of the experiment. Treatments 4 and 5 varied to a higher extent, with Treatment 4 containing the most variation among Feret diameters within the experiment.
Experiment Two

Figure 15. Images of the control polyps throughout experiment 2 and 40x and 400x.

Control polyps of experiment 2 matched the healthy characteristics from experiment 1 with little change. Healthy *Symbiodinium* were maintained between tentacles, among mesentarial tissues and within vesicles along the epidermis of the polyp. Bleaching occurred within the control tank on Day 14.
Effects of Combined Elevated Nitrogen and Temperature

Figure 16. Images of polyps from treatments 3 (low temperature, high nitrate) and 4 (high temperature, low nitrate) at 40x.

Figure 17. Images of polyps from treatments 3 (low temperature, high nitrate) and 4 (high temperature, low nitrate) at 400x.

Within varying combined levels of stress, there was a clear trend that higher temperatures in combination with nitrate stress of any level resulted in increased bleaching (Figure 16, Figure 17). High elevated nitrate levels and lower elevated
temperature levels did not cause bleaching to occur in treatment 3 (whereas moderate and some severe bleaching occurred in highest nitrate levels in experiment 1). The most severe cases of bleaching occurred on Day 12 within treatments 4 and 5 as samples were too damaged to be histologically fixed. Within treatment 3, however, high temperature in combination with stress resulted not only in more frequent bleaching, but more severe bleaching when it did occur, though there was no bleaching on the final day of experiment 2 in either treatment 3 or 4. Bleaching generally resulted in *Symbiodinium* still being maintained within the coral coelenteron. Fading of coral tissues was more obvious within experiment 2 (Figure 17 Treatment 4 Days 4 and 8).

Figure 18. Images of polyps from treatments 2 (low temperature, low nitrate) and 5 (high temperature, high nitrate) at 40x.
Treatment 2 contained no bleached samples, generally maintaining relatively healthy tissues and *Symbiodinium* compared to control treatments. Treatment 5 contained the most occurrences of bleaching, with bleaching occurring severely in every sample taken (Figure 19, Figure 20, Treatment 5), and the samples from Day 12 were too damaged to even histologically fix. Movement of *Symbiodinium* bleaching is most obvious within these treatments. Tissues are completely pale, *Symbiodinium* are found exclusively in either the coelenteron or between tentacles, and contain several instances of what may be lysed cell material among surviving *Symbiodinium* (Figure 19, Treatment 5, Day 14, Figure 19).
Figure 20. A comparison of *Symbiodinium* between all four treatments of experiment 2 at 400x on the final day of the experiment.

Figure 21. Box plots comparing average Feret diameter within each individual treatment throughout the experiment. Matching colors represent insignificantly different samples (ANOVA, P>0.05). In the case of Treatment 4, Day 4, Feret was significantly different from treatment 5, but not treatment 1. Within Day 8, treatments 2, 3 and 5 were significantly different than treatment 1, but not treatment 4. Within Day 14, treatments 2, 3 and 4 were significantly different than treatment 5, but not from treatment 1.
Within experiment 2 *Symbiodinium* Feret diameter varied generally differently than the control treatment (with the exception of Day 14) with no other discernible pattern. It seems with multiple stressors on the polyp, the Feret diameter generally increases in size, with the major exception of samples from Day 14.
Variance Among Treatments

In general, Feret diameter varied strongly within each treatment more so than in experiment 1. In the case of all experimental treatment, there is strong variation throughout the experiment with nearly every average value varying significantly from the previous sample with the most variation occurring in treatments 2 and 3.
DISCUSSION

Nitrogen Effects on *P. toxica*

There is a general trend of size increase among elevated nitrate treatments in the Feret diameters of *Symbiodinium*; however, this trend is inconsistent throughout the experiment. Furthermore, there is a trend of variation within each system. There is significant variation within the control treatment with a general trend of Feret size increase; however these conditions were kept constant. This is potentially a sign that when *P. toxica* is maintained at constant condition, healthy *Symbiodinium* will grow, causing the increase in size (Figure 14, Figure 22). Both treatments displayed a similar trend: near the beginning of the experiment, size was elevated directly with size, though by the end of the experiment both treatments dropped to either an insignificantly different size to the control, or significantly lower. This could mean that with elevated nitrogen there is a promotion of growth due to these excess nutrients, then a collapse of the system, resulting in the cell lysis displayed in both nitrate treatments and an overall decrease in size.

Size trends within each treatment varied significantly as well. The control treatment varied significantly, increasing with time during the experiment. Originally it was thought that if conditions remained constant and healthy, *Symbiodinium* size would not be affected. However, this could support the idea that a healthy coral displays *Symbiodinium* growth. Meanwhile, Treatment 2 remained consistent, with a slight insignificant decrease. Treatment 3 varied significantly, decreasing and then increasing.
Nitrate uptake in *Symbiodinium* has been studied in the past, and there is a consensus of *Symbiodinium* nitrogen uptake in forms of ammonium and nitrate, with a preference for ammonium (D’Elia et. al. 1983). Furthermore, sudden increases in nitrogen cause an increase in uptake of nitrogen in *Symbiodinium* and storage as uric crystals (Kopp et al 2013). This could be an explanation of the sudden increase and subsequent decrease in size of the *Symbiodinium*, however, within this experiment the timeline of *Symbiodinium* increase and decrease within treatments 2 and 3 occurs more slowly than formation of uric crystals, and these crystals were not examined within the cell. Corals generally display a nutrient limitation on their symbionts, disrupted by organic carbon (Wang and Douglas 1998), and the effects of nutrient stimulation on corals is potentially helpful in the short term, promoting growth in the symbionts, but eventually leading to a collapse in growth, especially with compounded stressors. There is also evidence that the coral microbiome plays a large role in the nitrogen cycle within corals (Pogourez et al 2017), so there is good reason to believe that there is more happening within this pattern of *Symbiodinium* growth and collapse than what this study examined. Recent studies have begun to emphasize the importance of nitrogen as a bleaching trigger (Wiedenmann et al 2012).

Bleaching occurred for the samples enriched in nitrate with a total of eleven cases of bleaching out of the total of twenty one samples, about 50%. However, the bleaching that did occur was moderate in general, with most *Symbiodinium* colonies remaining in the coelentron of the coral. Only by the very end of the experiment in the highest nitrate treatment did severe bleaching occur in Day 12 and Day 14. Therefore, according to the
results of this experiment, elevated nitrate triggers the bleaching mechanism of corals to some extent.

**Temperature Effects on *P. toxica***

The effect of temperature on the Feret diameter of *Symbiodinium* has a similar trend to the nitrogen enriched treatments. At the beginning of the experiment, there is a trend of increasing *Symbiodinium* size in the elevated temperature treatments. By Day 8, treatment 4 (held at 30°C) had the highest Feret diameter, before crashing in size and falling to a significantly smaller diameter than the control treatment. Treatment 5 had a similar trend of increasing in size and decreasing in size, though these changes were less extreme. By the end of the experiment Treatment 5 had fallen back to an insignificantly different size than the control.

These could be following a similar pattern of the nitrogen enrichment experiment. It has been shown in *Pocillopora* coral that slight temperature elevation can increase the size of *Symbiodinium* for short periods of time (Edmunds et al 2005), however this effect ceased at 27°C. There was significant variation in the temperature of the two treatment tanks during the experiment, so this may have contributed to the slight increase in size and eventual decrease. Furthermore, this effect was only noted for short-term exposure (24 hours), whereas this experiment incubated coral polyps for 14 days.

Within treatments, similar to the nitrogen enriched treatments, there was a trend of variance through time. *Symbiodinium* size in both treatments increased significantly compared to the control (for treatment 4 this occurs on Day 8; for treatment 5 this occurred on Day 12), before a collapse, resulting in sizes significantly lower than the control.
There is very little literature on the dynamics of how *Symbiodinium* change size during temperature elevation, as this method for measuring *Symbiodinium* size change was a novel method developed from coral reproduction methods (Waller, Tyler 2005). However, it has been shown that *Symbiodinium* photosynthesis decreases in response to elevated temperature (Goulet et al 2005), though this response varies depending on the coral acclimation to temperature (Kemp 2006). This experiment on these zoanthids are the first case of a more extended length study on elevated temperature on *Symbiodinium* size, and it is clear that there is a linkage between length of time in treatment and *Symbiodinium* size.

There is an indication that temperature is a bleaching trigger in *P. toxica*, with the highest temperature treatment resulting in six cases of bleaching, the most bleaching occurring at the end of the experiment, linking time to bleaching as well. Prolonged exposure to elevated temperature is the most consistent trigger of coral bleaching and has been studied numerous times, mostly in scleractinian corals (Glynn and D’Elia 1990, Brown 1997, Hughes et al 2017) but in zoanthids as well (Kemp et al, 2006). This experiment serves further confirmation of the bleaching response in *P. toxica*.

**Combined Stress Effects**

Nitrate and temperature enrichment in combination mostly continue the pattern in *Symbiodinium* size displayed in the first experiment. In the most stressed conditions in treatment 5, there is no significant difference from the control. Treatment 4 displayed the pattern of *Symbiodinium* increase in size and decrease in size, which again supports the idea that low amounts of nitrogen stimulate *Symbiodinium* growth. However, for the most
part there is not much significant difference in *Symbiodinium* sizes between treatments within experiment 2.

It is clear that combined stressors within an environment cause further damage to systems than singular stressors alone (Paine et al 1998, Hughes et al 1999). There has been a movement in coral research to understand multiple stressors on coral in combination, especially in combination with temperature (Coles and Jokiel, 1978, Porter et al 1999, Wiedenmann et al 2012). It has been shown that elevated nitrogen increases coral’s susceptibility to bleaching (Wiedenmann et al 2012). There has been some debate in the past as to whether or not elevated nitrogen is unhealthy for coral reefs, with studies that support very different ideas about nitrogen and coral. One study supports the idea that low amounts of nitrogen stimulate coral growth (Atkinson et al 1995). Other studies show that elevated nitrogen does not have a negative effect on coral growth (Szmant 2002, Bongiorni et al 2003). More recently it was shown that elevated nitrogen increases bleaching severity and disease susceptibility (Vega-Thurber et al 2014).

According to the results of this study, however, there is a clear detrimental effect of combined nitrate and temperature stress on coral. The instances of bleaching within the second experiment outnumbered the instances of bleaching in experiment one, with fourteen instances of bleaching across all treatments, as opposed to the eleven cases of bleaching in experiment one. Furthermore, the vast majority of bleaching in experiment one was moderate, with only a slight fading in zoanthid tissue color. *Symbiodinium* cells were maintained within the coelenteron of the polyp rather than being expelled from the polyp completely. Within the second experiment however, nearly every case of bleaching was severe, with high degradation of coral tissues (to the point where samples were
unable to be histologically fixed). Finally, *Symbiodinium* were for the most part lysed, and zoanthid tissue was nearly clear (Figure 16, 18), especially in high temperature treatments. According to the results of this experiment, then, there is evidence that elevated nitrate in combination with temperature is a bleaching trigger within *Palythoa toxica*.

**Conclusions**

Overall, the results of this experiment confirm the effect of elevated temperature as a bleaching trigger for *P. toxica* and provide evidence that elevated nitrate is a potential bleaching trigger in prolonged exposure. Furthermore, there is evidence that combined elevated nitrate and temperature compound bleaching mechanisms, with the highest amount of bleaching occurring in the most stressed treatments.

A pattern of *Symbiodinium* size changes emerges within this experiment as well. When exposed to low levels of nitrate and moderate temperature elevation, there is a trend of *Symbiodinium* Feret diameter increase. With time, however, there is an eventual collapse of growth, resulting in *Symbiodinium* diameter decrease and an increase of algal mass potentially from cell lysis within bleached *Symbiodinium* remaining between tentacles and within the coelenteron.

However, these conclusions come with major caveats. There is no replication within this experiment between coral polyps with only two genetically identical polyps sampled from the same water treatment. In future experiments, it will be necessary to replicate treatments, polyps and genetic individuals to provide for good replication. Furthermore, there was a significant amount of variation within treatments, so further water quality maintenance methods must be developed, as coral prove very delicate in
care (as demonstrated by the bleaching that occurred in the control treatment near the end of experiment 2). That being said, this experiment does provide preliminary evidence of *Symbiodinium* growth dynamics when exposed to prolonged periods of nitrate and temperature stress. It is likely that prolonged stress results in different dynamics of coral adaptation to stressed conditions, and this project is part of that effort to understand sublethal prolonged chronic stress conditions. Future experiments building on the knowledge gained from this project are essential to understanding the true dynamic between nitrogen, temperature and *Symbiodinium*. 
REFERENCES


APPENDICES
APPENDIX 1: IMAGEJ FERET DIAMETER CALCULATIONS

Feret diameter, Feret’s diameter, or the caliper diameter of an object is a measurement used for a non-circular object. It is the measurement between two furthest points on the surface of the object. ImageJ was used to calculate this measurement, and in this case, it was used to measure *Symbiodinium*.

![Figure 1. A Hypothetical *Symbiodinium*](image)

The perimeter of the *Symbiodinium* was used by the program to calculate Feret diameter. The maximum and minimum Feret diameter is calculated by ImageJ by rotating the object and drawing diameters every 2° of rotation. The coordinates of the two furthest points are recorded, and the smallest diameter, the Feret minimum, is located. The angle on which these two lines intersect is the Feret angle (Figure 2). These two Feret measurements were then averaged for each *Symbiodinium*, which were used for all size analysis.
However, ImageJ’s calculation of Feret diameter is not the true Feret measurement, it is a very close approximation. Because the program measures by rotation, there is a slight chance it can miss the true furthest points.

In order to find the true Feret maximum of a small object, in this case a *Symbiodinium*, the perimeter of the object must be drawn. Because of the noncircular nature of *Symbiodinium*, there are places where the cell has concave imperfections, however the only points in the *Symbiodinium* that matter are convex imperfections, as the calculation is for Feret maximum, and the maximum point will not occur at a concave point. A perimeter can be traced where only the convex points of the cell shape touch the perimeter, and all of these measurements can be compared until the Feret maximum is calculated (Figure 4). This object is called the convex hull.
However, this method is tedious for near-circular objects. In just this simple example, the number of pairs a program would have to remember would be 7 choose 2, or 21. In an object with several hundred convex points, for example, 150, a program would have to measure 150 choose 2, or 11175.

An applicable theorem states that “the diameter of a convex figure is the greatest distance between parallel lines of support” [Theorem 4.18, Preparata and Shamos, Computational Geometry, 1985] reduces the number of pairs a program would have to measure. A pair of points in an object that follows this theorem is called antipodal vertices (Figure 3).
The number of antipodal pairs the program would now have to measure is reduced to four pairs, a much easier number of distances to measure and store. A similar method is used to find the Feret minimum with concave points. Hypothetically, if there was a small number of *Symbiodinium*, this method could be used to measure the actual Feret diameter of each *Symbiodinium* cell.

That being said, there are a lot of data in this project, and that data was measured under ImageJ’s assumptions of Feret diameter approximation, and is assumed to be “close enough”.

References:


## ANOVA SET I

### Trial 1

- fetching data
- transfer all cvs from folder to desktop
- note: Feret diameter was misspelled as ‘ferret’ in data column organization
- thus ‘ferret’ was used during this program script in place of ‘feret’

```r
ggplot(T1D4, aes(x = factor(Tank), y = Ferret)) + geom_boxplot()
ggplot(T1D8, aes(x = factor(Tank), y = Ferret)) + geom_boxplot()
ggplot(T1D12, aes(x = factor(Tank), y = Ferret)) + geom_boxplot()
ggplot(T1D14, aes(x = factor(Tank), y = Ferret)) + geom_boxplot()
```

### Trial 2

- fetching

```r
ggplot(T2D4, aes(x = factor(Tank), y = Ferret)) + geom_boxplot()
ggplot(T2D8, aes(x = factor(Tank), y = Ferret)) + geom_boxplot()
ggplot(T2D12, aes(x = factor(Tank), y = Ferret)) + geom_boxplot()
ggplot(T2D14, aes(x = factor(Tank), y = Ferret)) + geom_boxplot()
```

- removed a bunch of outliers in excel to normalize
- test for symmetry

```r
levene.test(T1D4$Ferret, T1D4$Tank)
levene.test(T1D8$Ferret, T1D8$Tank)
```
levene.test(T1D12$Ferret, T1D12$Tank)
levene.test(T1D14$Ferret, T1D14$Tank)
levene.test(T2D4$Ferret, T2D4$Tank)
levene.test(T2D8$Ferret, T2D8$Tank)
levene.test(T2D12$Ferret, T2D12$Tank)
levene.test(T2D14$Ferret, T2D14$Tank)

#test for normality
shapiro.test(T1D4$Ferret [T1D4$Tank=="A"])
shapiro.test(T1D4$Ferret [T1D4$Tank=="B"])
shapiro.test(T1D4$Ferret [T1D4$Tank=="C"])
shapiro.test(T1D4$Ferret [T1D4$Tank=="D"])
shapiro.test(T1D4$Ferret [T1D4$Tank=="E"])
shapiro.test(T1D8$Ferret [T1D8$Tank=="A"])
shapiro.test(T1D8$Ferret [T1D8$Tank=="B"])
shapiro.test(T1D8$Ferret [T1D8$Tank=="C"])
shapiro.test(T1D8$Ferret [T1D8$Tank=="D"])
shapiro.test(T1D8$Ferret [T1D8$Tank=="E"])
shapiro.test(T1D12$Ferret [T1D12$Tank=="A"])
shapiro.test(T1D12$Ferret [T1D12$Tank=="B"])
shapiro.test(T1D12$Ferret [T1D12$Tank=="C"])
shapiro.test(T1D12$Ferret [T1D12$Tank=="D"])
shapiro.test(T1D12$Ferret [T1D12$Tank=="E"])
shapiro.test(T1D14$Ferret [T1D14$Tank=="A"])
shapiro.test(T1D14$Ferret [T1D14$Tank=="B"])
shapiro.test(T1D14$Ferret [T1D14$Tank=="C"])
shapiro.test(T1D14$Ferret [T1D14$Tank=="D"])
shapiro.test(T1D14$Ferret [T1D14$Tank=="E"])
shapiro.test(T2D4$Ferret [T2D4$Tank=="A"])
shapiro.test(T2D4$Ferret [T2D4$Tank=="B"])
shapiro.test(T2D4$Ferret [T2D4$Tank=="C"])
shapiro.test(T2D4$Ferret [T2D4$Tank=="D"])
shapiro.test(T2D4$Ferret [T2D4$Tank=="E"])
shapiro.test(T2D8$Ferret [T2D8$Tank=="A"])
shapiro.test(T2D8$Ferret [T2D8$Tank=="B"])
shapiro.test(T2D8$Ferret [T2D8$Tank=="C"])
shapiro.test(T2D8$Ferret [T2D8$Tank=="D"])
shapiro.test(T2D8$Ferret [T2D8$Tank=="E"])
shapiro.test(T2D12$Ferret [T2D12$Tank=="A"])
shapiro.test(T2D12$Ferret [T2D12$Tank=="B"])
shapiro.test(T2D12$Ferret [T2D12$Tank=="C"])
shapiro.test(T2D14$Ferret [T2D14$Tank=="A"])
shapiro.test(T2D14$Ferret [T2D14$Tank=="B"])
shapiro.test(T2D14$Ferret [T2D14$Tank=="C"])

#boom

#anova time

anova.results <- aov(Ferret ~ Tank, data=T1D4)
summary(anova.results)
TukeyHSD(anova.results)

anova.results <- aov(Ferret ~ Tank, data=T1D8)
summary(anova.results)
TukeyHSD(anova.results)

anova.results <- aov(Ferret ~ Tank, data=T1D12)
summary(anova.results)
TukeyHSD(anova.results)

anova.results <- aov(Ferret ~ Tank, data=T1D14)
summary(anova.results)
TukeyHSD(anova.results)

anova.results <- aov(Ferret ~ Tank, data=T2D4)
summary(anova.results)
TukeyHSD(anova.results)

anova.results <- aov(Ferret ~ Tank, data=T2D8)
summary(anova.results)
TukeyHSD(anova.results)

anova.results <- aov(Ferret ~ Tank, data=T2D12)
summary(anova.results)
TukeyHSD(anova.results)

anova.results <- aov(Ferret ~ Tank, data=T2D14)
summary(anova.results)
TukeyHSD(anova.results)

## ANOVA SET II

# bring in the data

setwd("~/Desktop")
T1 <- read.csv("T1.csv", header = TRUE)
T2 <- read.csv("T2.csv", header = TRUE)
T3 <- read.csv("T3.csv", header = TRUE)
T4 <- read.csv("T4.csv", header = TRUE)
T5 <- read.csv("T5.csv", header = TRUE)

# all the data is already normally distributed and symmetrical so anova time

anova.results <- aov(Ferret ~ Day, data=T1)
summary(anova.results)
TukeyHSD(anova.results)

anova.results <- aov(Ferret ~ Day, data=T2)
summary(anova.results)
TukeyHSD(anova.results)

anova.results <- aov(Ferret ~ Day, data=T3)
summary(anova.results)
TukeyHSD(anova.results)

anova.results <- aov(Ferret ~ Day, data=T4)
summary(anova.results)
TukeyHSD(anova.results)

anova.results <- aov(Ferret ~ Day, data=T5)
summary(anova.results)
TukeyHSD(anova.results)
anova.results <- aov(Ferret ~ Day, data=T5)
summary(anova.results)
TukeyHSD(anova.results)

###now for the final figures

#Experiment 1: Nitrogen Figures

setwd("~/Desktop")
ND4 <- read.csv("ND4.csv", header = TRUE)
ND8 <- read.csv("ND8.csv", header = TRUE)
ND12 <- read.csv("ND12.csv", header = TRUE)
ND14 <- read.csv("ND14.csv", header = TRUE)

library(ggplot2)
ggplot(ND4, aes(x=factor(Treatment), y=Feret)) + theme_classic() + geom_boxplot()
ggplot(ND8, aes(x=factor(Treatment), y=Feret)) + theme_classic() + geom_boxplot()
ggplot(ND12, aes(x=factor(Treatment), y=Feret)) + theme_classic() + geom_boxplot()
ggplot(ND14, aes(x=factor(Treatment), y=Feret)) + theme_classic() + geom_boxplot()

#Experiment 1: Temperature

setwd("~/Desktop")
TD4 <- read.csv("TD4.csv", header = TRUE)
TD8 <- read.csv("TD8.csv", header = TRUE)
TD12 <- read.csv("TD12.csv", header = TRUE)
TD14 <- read.csv("TD14.csv", header = TRUE)

library(ggplot2)
ggplot(TD4, aes(x=factor(Treatment), y=Feret)) + theme_classic() + geom_boxplot()
ggplot(TD8, aes(x=factor(Treatment), y=Feret)) + theme_classic() + geom_boxplot()
ggplot(TD12, aes(x=factor(Treatment), y=Feret)) + theme_classic() + geom_boxplot()
ggplot(TD14, aes(x=factor(Treatment), y=Feret)) + theme_classic() + geom_boxplot()

#end script
AUTHOR’S BIOGRAPHY

Molly Westbrook grew up in Ithaca, NY with a huge passion for the natural sciences. They went on to attend the University of Maine, majoring in Marine Science with concentrations in Aquaculture and Marine Biology, and minoring in Mathematics. Molly’s done several things while at UMaine, including co-founding the UMaine Coral Club and acting as Treasurer for the first year, DJing at WMEB 91.9 fm, performing as a singer-songwriter in the greater Orono area, studying abroad at James Cook University in Australia, working in many labs, analyzing many datasets, and playing many board games. They had to write a lot of bios when they were a stage manager throughout middle school and high school, and ten years later they’re still not great at it.