ZOOXANTHELLAE COUNTS IN BLEACHED CORAL

An Undergraduate Research Scholars Thesis

by

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This project did not require approval from the Texas A&M University Research Compliance & Biosafety office.

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ABSTRACT

Zooxanthellae Counts of Bleached Coral

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Coral reefs are an essential part of the world. They provide the world with over half of the produced oxygen and take up over one-third of the carbon dioxide produced by mankind. Part of the success of these reef systems is due to the symbiotic relationship between coral and zooxanthellae (unicellular algae), and the different clades that reside within them. The relationship between these living organisms is essential for the reef systems survival, and for its ability to thrive. Zooxanthellae produce over 90% of the corals food and provide them with their vibrant color. The greatest threat to these delicate creatures is bleaching (loss of Zooxhantellae) due to increased water temperatures. *Xenia elongata* (Pulsing Xenia) is a soft coral that is easy to maintain in the laboratory, but also easy to bleach. In this project, I compared the concentration of zooxanthellae in Xenia, before and after a bleaching event induced by local temperature increase. The average loss of symbionts within these corals after bleaching was over 80%. This

significant loss contributed to the mortality and complete disintegration of the observed colonies. The loss of zooxanthellae, resulted in coral starvation, that subsequently caused coral death. In this paper I also discuss how the increased temperatures may have caused an increased buildup of hydrogen peroxide which disrupted the extracellular matrix protein 67 and its calcium bonds. The loss of protein-calcium bond may then have caused the disintegration and overall death of the coral.

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1. INTRODUCTION

1.1 Coral Reefs

Coral reefs are an essential marine habitat that house a variety of varied species. These reef systems are one of the most biologically diverse and most productive systems in the world. The health of these reef systems is imperative to the success of many different fauna, including humans. Seafood, recreation, aesthetics, and cultural benefits are some of the few things coral reefs provide humans (Moberg and Folke, 1999). Coral reefs have an estimated cover range of 0.1-0.5% of the entire ocean. Yet this ecosystem holds 25-35% of the earths marine species. These reef systems are found in tropical waters along the equator. It is estimated 10% of the consumed fish are caught at coral reefs (Moberg and Folke, 1999). The biodiversity of these habitats is important and dependent on coral health. When these habitats are endangered by environmental stressors the fragile coral will begin to bleach and die. This causes millions of people to lose their food source, since there are more than 100 countries that have coral reefs within their coastlines.

Many different environmental factors contribute to the overall health of a coral reef. Salinity, temperature, dissolved oxygen, and pH are the main points of observation when a coral reef is found ill. However, there are factors such as structural complexity that can influence a coral reefs ecosystem. Structural complexity refers to the physical three-dimensional structure of an ecosystem (Graham, and Nash, 2013). This structure is provided by the complexity of living organsims such as kelp and coral. These organisms are described as foundation species. These complex structures allow for microhabitats that will lead to greater diversity. The structural complexity can have positive or negative affects on population densities. For instance urchins

have a negative relationship with structural complexity, while coral has a positive relationship (Graham, and Nash, 2013).

The continuing degradation of these coral reef systems has caused many issues within the oceans delicate ecosystem. Without the reef many marine species are negatively affected. Biodiversity declines, thereby harming the goods and services the reefs provide the world (Pratchett et al. 2014). The loss of sustained coral causes shifts in the biotic composition of benthic habitats. Which further degrades the coral reef. The abundance of fish associated with coral reefs is an important ecological key which is in decline. As coral loss continues, the ability to sustain large populations of fish declines with it. Several species of fish have even declined in body mass due to the loss of this essential habitat (Pratchett et al. 2014).

The restoration of coral reefs has become a huge effort across the globe, from the Great Barrier Reef in Australia to the reef system in Florida. A huge effort is carried out to salvage coral Many of these corals, for examples, are shipped to different zoos to be maintained and to make sure they are healthy for re-establishment. In some cases they are kept in captivity in hopes to be able to reproduce (Jaap, 2000). Three-dimensional structures are placed in the water to serve as reef structures. These structures are made to optimize recruitment of many different species of fish, coral, and invertabrates. In some cases, old boats are sunk to the bottom and used as artificial reef structures. Although this efforts are very important, bleaching (the loss of the symbiotic zooxhanthellae due to increased temperature) is still the major threat to coral (Jaap, 2000).

Zooxanthellae

Zooxanthellae are dinoflagellate algae with three representative classes: *Symbiodinium*, *Cryptomonas*, and *Chrysidella* (Riddle, 2006). There are at least eight clades of *Symbiodinium*, which have different traits. Different clades are found on coral, depending on the location the

zooxanthellae are harvested from. Zooxanthellae are protected by carotenoid pigments called Xanthophylls. These pigments help protect the algae from harmful visible light. There are even mycosporin-like amino acids (MAAs) that protect them from UV radiation. The coral *Xenia*, used in this project, comes originally from the Indo Pacific. The main clade of zooxanthellae found in this region is described as clade F (Riddle, 2006). This clade is not tolerant of high light intensity, although it has been shown it contains MAAs (Riddle, 2006), However it has been shown that there no production of protective xanthophylls as a response to super saturating irradiance. This means that this clade of zooxanthellae and their host are vulnerable to light intensities.

Zooxanthellae have been found to be able to resist bleaching to some extent (Tchernov et al., 2004). In a paper written by Ray Berkelmans and Madeleine Van Oppen (2006) coral were able to acquire increased thermal tolerance by switching the dominant zooxanthellae from Symbiodinium type C to type D. Type C is often noted in bleaching events, while type D is found to be relatively resistant to bleaching (Riddle, 2006).

The dinoflagellate algae are photosynthetic, and trade food for carbon dioxide produced by the coral. Coral also offers protection. Coral relies on the symbiotic relationship with zooxanthellae to provide roughly 90% of their food. The uptake of food happens in the gastrodermis of the cell (Hu, Zheng, X., Zheng, Y. 2020). Without their symbionts, corals starve as they cannot take enough food from its surroundings to feed itself. That is why zooxanthellae are an essential part of the coral survival.

This study focuses on the visual quantification of zooxanthellae of two genera: *Symbiodinium* (which is brown), and *Cryptomonas* (which is green) (Douglas, 2003).

1.2 Bleaching

The term bleaching is not only meant for coral. Many different organisms that use a symbiotic relationship with zooxanthellae can undergo bleaching events. However, recent focus has been on massive bleaching events in coral. Bleaching is defined as the loss of color, from the partial or total loss of the *Symbiodinium* population or degradation of algal pigments (Douglas, 2003). In simple terms: the loss of color in corals indicates that the symbioses between dinoflagellate algae (zooxanthellae) and coral have been disrupted, and corals are losing their symbionts. Coral bleaching occurs when the thermal tolerance of corals and their symbionts is exceeded (Baker et al. 2008). When the temperatures of the water rise above a certain threshold, the coral ability to retain the zooxanthellae decreases. The environment becomes unstable for the zooxanthellae and are forced to leave the corals. The higher temperatures cause weakening in the endoderm which in turn causes cell death. This leads to the expulsion of the algal symbionts into the immediate surroundings. Energy reserves within the coral are not enough to sustain its life for long periods of time after bleaching (Rodrigoes and Grottoli, 2008). This causes starvation in the coral, which is the cause of death. Temperature-induced bleaching essentially affects the carbon dioxide fixation mechanism. The primary site of heat damage is in carboxylation within the Calvin cycle (Jones, Larkum, and Schreiber, 1998). Since the first description of this issue in 1984, bleaching events have been reported regularly in the Caribbean Sea, Indian, and Pacific Oceans (Brown, 1997). More recently these events have been sighted in waters around Mexico, Belize, Papua New Guinea, and Hawaii. Understanding how fast coral bleaches and how many zooxanthellae remain available in the coral after bleaching is important to assess their capability to return to a healthy state after bleaching events.

1.3 Bleaching on a Chemical Level

A common misconception is that temperature causes a decrease in pH. This is not true; the decrease of pH is due to an influx of carbon dioxide. Both cause bleaching, however, reduced pH is known for destroying the calcium carbonate structures in hard corals as well as in other organisms such as bivalves. The bleaching observed in this experiment is due to thermal increases in the water. Both hard and soft coral have Ca2+ ions within their structures. Hard corals form aragonite needle like crystals, while soft corals from calcite crystals (Rahman, Oomori, and Wörheide 2011). The difference in formation has to do with the magnesium ions within the water. Hard corals perform crystallization in vivo while soft corals do not, they perform crystallization in vitro (Mass T. Et al, 2014). The protection of the crystallization, in vivo, process allows for the formation of aragonite crystals to be formed. Soft corals mix proteins (matrix proteins 12 and 13) with the Ca2+ ions structure which gives them the name soft coral. The most potent protein bound calcium ions was the extracellular matrix protein 67 (Rahman, Oomori, and Wörheide 2011). When thermal conditions increase a breakdown of the protein and the calcium ions causes a disintegration of the cell wall. This breakdown of the cell leads to disruption of the calcium exclusion system then to apoptotic or necrotic cell death. This causes an event known as blebbing zooxanthellae (Sandeman, 2006). This is not a direct process; hydrogen peroxide (H2O2) is involved. Many dinoflagellates are known to produce H2O2. “Red tide” or Cochlodinium polykrikoides are dinoflagellates that produce a superoxide and a hydrogen peroxide. Zooxanthellae are another that can secrete H2O2. This is normal H2O2 (Sandeman, 2006). This is because a higher temperature causes a higher/faster metabolism. Peroxide is a performs radical reactions which causes the destruction of many proteins. The

destruction of the extracellular matrix protein 67 is why the soft coral disintegrates (Rahman, Oomori, and Wörheide 2011).

1.4 Xenia *Elongata* (Pulsing Xenia)

This study focuses on one species of coral known as silver pulsing xenia (*Xenia elongata*). This species of coral is fast growing and easy to care for. *Xenia* tend to have varying hardiness. Some do not grow well while others (silver pulsing *Xenia*) can be invasive. This species can reproduce quickly by growing in colonies and spreading into mats across the available surface. *Xenia* is found in the Indo Pacific Ocean and has a dominating clade F type of zooxanthellae. The species was first described by Dana, J. D. in 1846.

Kingdom: Animalia

Phylum: Cnidaria

Class: Anthozoa

Order: Alcyonacea

Family: Xeniidae

Genus: Xenia

Species: Xenia *Elongata* (Cordeiro, McFadden, van Ofwegen, and Williams, 2021).

1.3 Study

Bleaching is a common occurrence in today's world. Understanding how it is happening, and if it can be stopped, is important to the survival of marine ecosystems. For this to be done some questions must be asked. How many zooxanthellae are left in bleached coral? How quickly does the zooxanthellae leave the coral, and how many (if any) zooxanthellae remain in bleached coral? Quantifying zooxanthellae in bleached coral will bring an understanding on how quickly

bleaching occurs. Then when bleaching has occurred it will show how long it takes to return to its healthy state if it can return at all. This study aims to understand a portion of these questions by looking into the concentrations of zooxanthellae before and after a thermal induced bleaching event and investigating whether corals return to a healthy state depending on the concentration of zooxanthellae that remained after the bleaching event. In summary, this study aims to investigate what percentage of zooxanthellae is lost in the bleaching event and link it to the coral survivability.

Throughout this study some complications caused the original research plan to slightly change. One unanticipated challenge was due to the speed at which the bleaching events occurred. It was believed bleaching would occur over a period of two days, but in this study, it happened within a couple of hours. This made sampling of the bleached coral challenging. Another complication was due to type of coral used in this study. The initial plan aimed to the use two species of coral (Xenia and ADD name). However, the protocol for extracting zooxanthellae did not work on the second species (*Pachyclavularia violacea* or green star polyp). Furthermore, bleaching did not occur to this coral species, possibly due to the zooxanthellae clades that reside them. Bibliographic research indicated that green star polyps had predominantly Clade D zooxanthellae which are known to resist thermal bleaching. These issues caused the study to be forced into one large, but replicated, experiment that focused on *Xenia* colonies only.

2. METHODS

2.1 Selection of species and tank

Each species of coral requires different water parameters and environment to survive. The selection of *Xenia elongata* for this study was due to multiple factors. *Xenia* is a hardy species which colonizes new environment quickly. It requires adequate water flow and light. *Xenia* is also a fast-growing coral, making it ideal for laboratory experiment. This coral is also inexpensive, unlike most other corals which can be very pricey. *Xenia elongata* does not detach from its location once the colonies are established and does not move in the tank when stressed. This is important for the experimental design, where colonies are identified by the position they are holding in the tank.

Xenia colonies of *X. elongata* were purchased from Vivid Aquariums, a commercial coral supplier. Colonies were shipped overnight to Texas A&M at Galveston and were set up in tanks at the Sea Life Facility, upon arrival. Colonies were kept under the following conditions: 30-33 ppt salinity, 23-26 °C, 8.1-8.4 pH, 420-440 ppm Ca, 8-9.5 Alk dKH, 1260-1350 Mg, and <10 ppm Nitrates. The aforementioned water quality parameters are standard for soft polyp coral species, such as *X. elongate*.

Three tanks were used in this experiment (figure 2.2). The main tank held the coral and was 101.6 cm by 40.64 cm by 20.32 cm. The second tank was used as a sump tank and was 50.8 cm by 31.12 cm by 31.75 cm. The final tank was used as a top off tank and was 40.0 cm by 25.4 cm by 37.47 cm. The shallow depth of the holding tank allowed for ease of access to the coral and pinpoint feeding. Two inflows from the pump return line generate water flow over the coral. A Kessil series Tuna Blue light hanging 35.56 cm over the top of the tank provided the required

light spectrum to promote coral growth. The temperature probe was in the top tank to always keep a track of what the temperature was throughout the experiment. A UV sterilizer was attached to the top tank. The coral rested in a grid in the top tank (figure 2.1). The second tank, or sump tank, held 2 heaters both being 50-watt Hydor heaters. The sump tank included mechanical and biological filtration as well as an output from the third tank. The third tank held excess water that was pumped in using an apex auto top off. Since this experiment investigated the thermal tolerance of the coral, elevated temperatures were necessary, and evaporation was unavoidable. The auto top off helped keep the tank at the desired water level. The pH, temperature, and conductivity were continuously monitored throughout the experiment using the Neptune Apex monitoring system and online Apex fusion platform. All probes were in the top section of the tank to monitor the water around the coral. The Apex Fusion allows to control and monitor the temperature of the tank from a computer or phone. The Apex Fusion App allows to remotely raise the temperature, turn on and off heaters, and has an alarm that can communicate when the temperature is off range.

The sump tank heaters were the main heaters used in this experiment however, when the temperature was raised past 32 °C the top heater was turned on to push passed this temperature.

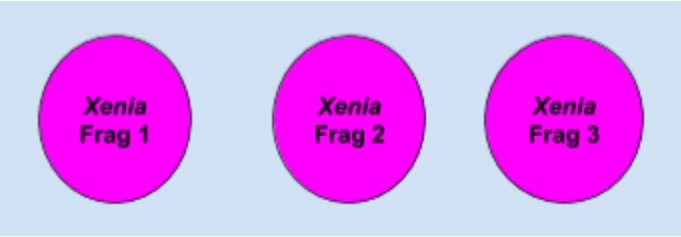

Figure 2.1: *Arrangement of the three coral in a rack within the tank.*

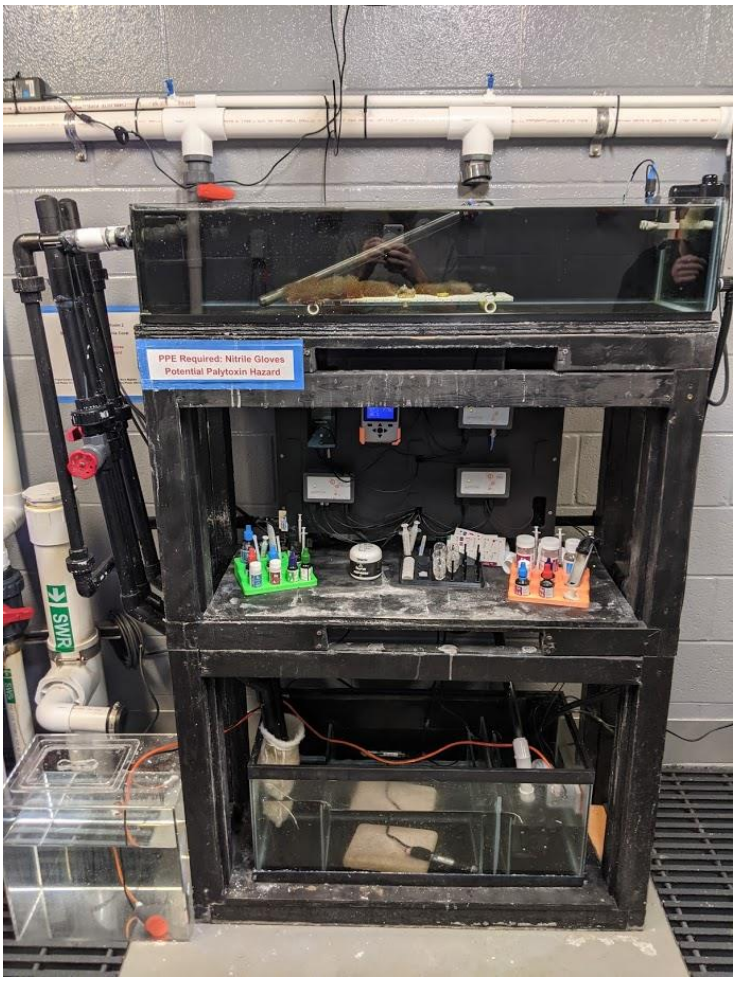

Figure 2.2: *Coral tank set up. Top tank held the coral, probes, 300-watt heater, and UV sterilizer. Bottom middle tank is the sub tank. This tank held two 50-watt heaters, a sock, bio substrate and auto top off output. Left bottom tank was the top off tank that holds the auto top off intake.*

2.2 Experimental design

2.2.1 Each experiment consisted of three parts:

Sampling of the non-bleached coral. From each polyp, one neck was cut at 1.5 cm using sharp scissors and Kelly forceps. The neck is 1.5 cm long when fully open and shrinks once cut. This was done between the body of the coral (anthostele) and the neck zone (figure 2.3). The entire neck and head of the coral (anthocodia) was kept and used for extraction. The corals

natural defense is to shrivel up, so this step must be done quickly. The forceps help hold the polyp still and provide a strong grip to dab the sample. Samples were dried with a paper towel then put into a conical vial filled with 500 µL of 4 M NaOH. The vial was properly labeled to indicate date and polyp/coral colony from which it was obtained. After sampling the coral showed signs of stress, so the next step of the trial was not started until the coral was seen back fully open and pulsing. The second part of the trial was started a week after the completion of this first step.

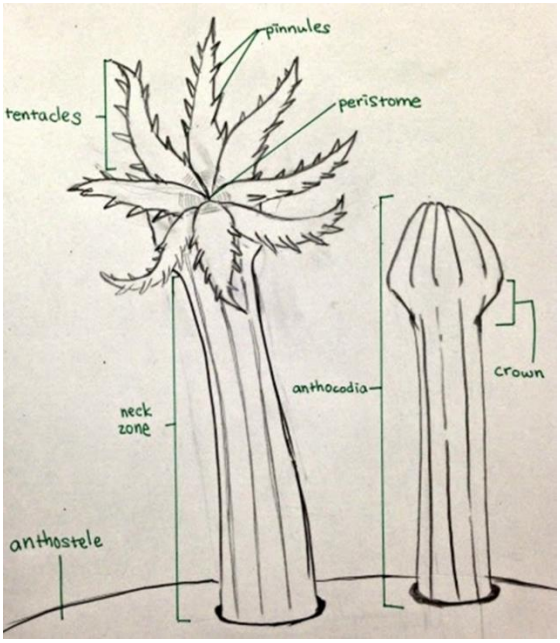

Figure 2.3*: Drawing of Xenia anatomy to describe where to cut a sample. Picture provided byhttps://www.gbri.org.au.*

Increase of temperature. In the second part of the experiment the temperature of the tank was risen 0.5 °C every 4-6 hours (Zamoum, Thamilla, and Paola Furla, 2012). The coding for the temperature change had to be between a ±1.5 °C change. Any coding below this causes the switch to malfunction and not work properly. Each time the temperature was adjusted, and to avoid false alarm, the alarm settings were changed accordingly. Sample coding for the alarm is

provided in figure 2.4. The coral was observed between 8 am and 4 pm (2-hour increments) to look for signs of bleaching. As a proxy of coral health, the percent of open polyps on the coral, and their color was recorded. Observation continued until either the coral showed signs of bleaching, or 35 °C was reached. If the temperature cap of 35 °C was reached, then the specimen was held at 35 °C, and the coral observed until bleaching. The coral started to bleach at 31.5-32 °C. At the first signs of bleaching, samples of each coral polyp were taken. This proved to be challenging due to the fact the coral shrivels up as a sign of bleaching. The target length of each fragment was 1.5 cm. Samples where carefully dabbed dry and put into 500 µL of 4 M NaOH, then properly labeled. After bleaching. After the bleaching, polyps were abundantly fed to investigate whether feeding was able to increase coral survival after the bleaching event. Feedings were increased from twice a week to once a day using reef roids.

$$Set\ OFF \qquad (2.2.2)$$

$$If\ Tmpx3 > 27.5\ Then\ ON$$

$$If\ Tmpx3 < 26.0\ Then\ ON$$

$$Set\ OFF$$

$$If\ Tmpx3 > 28.0\ Then\ ON$$

$$If\ Tmpx3 < 26.5\ Then\ ON$$

Figure 2.4*: Coding for temperature increase during the bleaching experiment. The settings set on and off the heaters at certain temperatures. Each increase in temperature was done at 0.5 °C increments. The coding could only handle an on and off ±1.5 °C change.*

Return to normal (pre-bleaching) temperature. The final part of the experiment was the decrease of the temperature back to a stable range (pre-bleaching temperature). This followed the same protocol for increasing the temperature, just in the opposite direction. Observations of the

coral continued to be recorded every two hours till the temperature was returned to its original range, and the coral demonstrated non-bleaching activity, such as pulsing, being fully opened, and when the color returned.

2.3 Zooxanthellae Extraction

To extract the zooxanthellae from the coral polyp fragments, a centrifuge and incubator were used. The incubator was set at 37 °C. The samples were then put into the incubator for 4 hours (Zamoum, Thamilla, and Paola Furla, 2012). Every fifteen minutes throughout incubation the samples were momentarily removed and vortexed. The *Xenia* coral disintegrates at these elevated temperatures. Some larger pieces of tissue will be floating around. A centrifuge was used for 3 minutes at 3000 rpm to force the large sections of tissue to stick to the bottom of the vail. This left-over mass of tissue holds little to no zooxanthellae. This tissue can be incubated longer to disintegrate into the solution or can be removed and rinsed into the vial with 1 mL of DI water to wash off remaining zooxanthellae.

Counting the zooxanthellae required a hemocytometer and a compound microscope. To stir up the contents of the vial. The extracted sample was vortexed before zooxanthellae counting in order to resuspend the content. Using a micropipette 10 µL of the sample were extracted and put it on the cross section of a hemocytometer. A slide cover was then used to carefully cover the preparation, trying not to get bubbles under the slide cover. The excess sample will flood into the canals of the hemocytometer. Using a tally counter zooxanthella were counted in each of the 9 squares within the hemocytometer and numbers were recorded. Each square contains 0.1 µL of

fluid (0.9 µL total). The number of zooxanthellae in the sample was thus calculated and R-studios was used to statistically analyze the data.

2.4 Weight of Coral Samples

Weighing the coral samples provides a rough estimate of the mass to zooxanthellae concentrations. This was done by taking a sample (1.5 cm long anthecodia) and letting it shrivel up in the test tube with water from the tank. Once completely shriveled, the sample was removed and set on paper wipes for 15 seconds to dry. Samples were weighed on a fine balance scale to get the closest weight possible. Weights were averaged.

3. RESULTS

3.1 Weight of Coral Samples

Three sample weights were taken on 1.5 cm cut anthecodias. The average weight of the three weighed samples were 7 mgs (±1 mg). This is model mass used as the basis for the project as extracted polyps could not be weighed individually.

3.2 Feeding results

The increased feeding yielded no difference in the survivability of the coral. All coral died except for one small polyp on fragment 1.

3.3 Water Parameters and Coral health

The parameters of the tank remained the same throughout the trial. No changes were observed in the pH or salinity. Table 3.1 shows the water parameters during the trial. Parameters were recorded every day at 8 am. Trial started on the 16th of October 2020 after temperature recording. Table 3.2-3.4 shows the recordings of coral health during the trial. The color and polyp status were recorded. White indicates bleaching as well as having low polyp percent status. If there is a white color with 0% polyp status, then the fragment was dead.

Date	Temp	Salinity	pH
17-Oct	29.8	29	7.77
18-Oct	29.8	30	7.77
19-Oct	30.1	33	7.76
20-Oct	29.6	30	7.79
21-Oct	30.8	31	7.9
22-Oct	32.4	30	7.78
23-Oct	28.2	30	7.92
24-Oct	28.9	31	7.75
25-Oct	27.6	30	7.76

***Table 3.1**: Water parameters during trial 10/17/20-10/25/20*

			8:00 AM		10:00 AM		12:00 PM		2:00 PM		4:00 PM	
	Date	8 am Temp.	Color	Polyp status	Color	Polyp status	Color	Polyp status	Color	Polyp status	Color	Polyp status
Frag 1	17-Oct	29.8	Healthy	All open	Healthy	All open	Healthy	All open	Healthy	All open	Healthy	All open
	18-Oct	29.8	Healthy	50% open	Healthy	All open	Healthy	All open	Healthy	All open	Healthy	All open
	19-Oct	30.1	Whitish	10% open	Healthy	50% open	Healthy	All open	Healthy	All open	Healthy	All open
	20-Oct	29.6	Healthy	90% open	Healthy	90% open	Healthy	90% open	Healthy	90% open	Healthy	90% open
	21-Oct	30.8	whitish	10% open	Healthy	50% open	Healthy	30% open	Healthy	20% open	white	90% open
	22-Oct	32.4	white	0%	Healthy	50% open	white	20% open	white	10% open	white	10% open
	23-Oct	28.2	white	0%	white	50% open	white	30% open	white	10% open	white	All open
	24-Oct	28.9	white	0%	white	50% open	white	50% open	white	50% open	white	60% open
	25-Oct	27.6	white	0%	white	50% open	white	50% open	white	50% open	white	60% open

***Table 3.2**: Fragment 1 health and polyp status during bleaching trial in 2-hour intervals. Collection of bleached samples on the 21st around 3:00 pm. Coral mortality observed after.*

			8:00 AM		10:00 AM		12:00 PM		2:00 PM		4:00 PM	
	Date	8 am Temp.	Color	Polyp status	Color	Polyp status	Color	Polyp status	Color	Polyp status	Color	Polyp status
Frag 2	17-Oct	29.8	Healthy	All open	Healthy	All open	Healthy	All open	Healthy	All open	Healthy	All open
	18-Oct	29.8	Healthy	All open	Healthy	All open	Healthy	All open	Healthy	All open	Healthy	All open
	19-Oct	30.1	Healthy	All open	Healthy	All open	Healthy	All open	Healthy	All open	Healthy	All open
	20-Oct	29.6	Healthy	All open	Healthy	All open	Healthy	All open	Healthy	All open	Healthy	All open
	21-Oct	30.8	Healthy	All open	Healthy	All open	Healthy	All open	Healthy	0% open	white	0% open
	22-Oct	32.4	white	0%	white	0% open	white	0% open	white	0% open	white	0% open
	23-Oct	28.2	white	0%	white	0% open	white	0% open	white	0% open	white	0% open
	24-Oct	28.9	white	0%	white	0% open	white	0% open	white	0% open	white	0% open
	25-Oct	27.6	white	0%	white	0% open	white	0% open	white	0% open	white	0% open

***Table 3.3**: Fragment 2 health and polyp status during bleaching trial in 2-hour intervals. Collection of bleached samples on the 21st around 3:00 pm. Coral mortality observed after.*

			8:00 AM		10:00 AM		12:00 PM		2:00 PM		4:00 PM	
	Date	8 am Temp.	Color	Polyp status	Color	Polyp status	Color	Polyp status	Color	Polyp status	Color	Polyp status
Frag 3	17-Oct	29.8	Healthy	All open	Healthy	All open	Healthy	All open	Healthy	All open	Healthy	All open
	18-Oct	29.8	Healthy	All open	Healthy	All open	Healthy	All open	Healthy	All open	Healthy	All open
	19-Oct	30.1	Healthy	All open	Healthy	All open	healthy	all open	Healthy	All open	Healthy	All open
	20-Oct	29.6	Healthy	All open	Healthy	All open	Healthy	All open	Healthy	All open	Healthy	All open
	21-Oct	30.8	Healthy	All open	Healthy	All open	Healthy	All open	white	shriveled	white	0% open
	22-Oct	32.4	white	0%	Healthy	0% open	white	0% open	white	0% open	white	0% open
	23-Oct	28.2	white	0%	white	0% open	white	0% open	white	0% open	white	0% open
	24-Oct	28.9	white	0%	white	0% open	white	0% open	white	0% open	white	0% open
	25-Oct	27.6	white	0%	white	0% open	white	0% open	white	0% open	white	0% open

***Table 3.4:** Fragment 3 health and polyp status during bleaching trial in 2-hour intervals. Collection of bleached samples on the 21st around 3:00 pm. Coral mortality observed after.*

The trials began on the October 16th and continued until 2 pm on October 21st. As shown in tables 3.2-3.4 each fragment was healthy and open until 2 pm on the 21st. It was hard to determine whether Fragment 1 was bleaching or not. Each morning, the polyps appeared mostly

closed and the fragment had a white tent to it. To ensure a bleaching event was occurring, sampling was put off till one of the larger fragments showed some signs. A smaller colony was attached to the same plug as fragment one ended up surviving the bleaching trials with 5 tiny anthocodia. These were white in color and around 0.5 cm in length. Sampling of this fragment was impossible due to size of anthocodia. Recording of the small colony continued. The other fragments all ended up dissolving in the water within hours. Since all other water parameters remained the same it can be concluded this was purely thermally induced bleaching and thermal mortality.

3.4 Hemocytometer counts

The following figures (figures 3.6-3.8) show the zooxanthella counts from the hemocytometer. The extraction of zooxanthellae for both non-bleached and bleached samples happened at the same time. The total volume under the cover slide was 0.9 µL of sample (see methods).

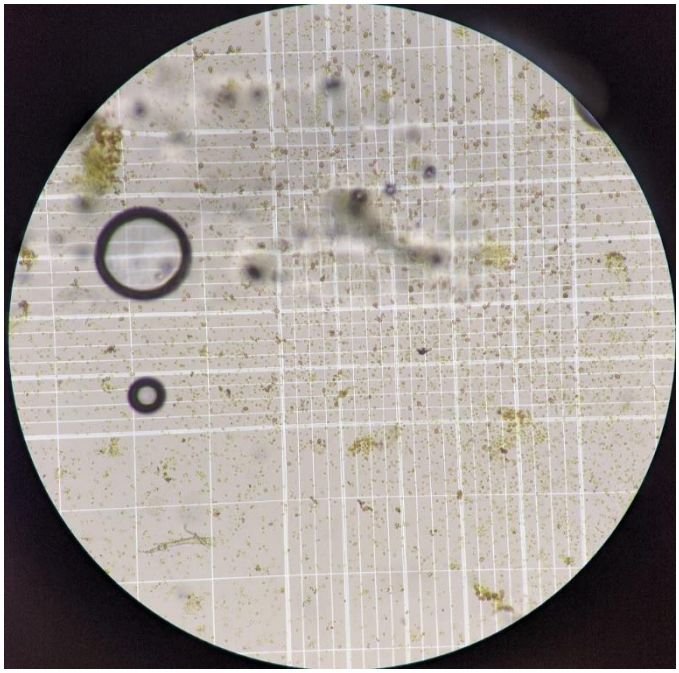

Figure 3.5: *Picture of the hemocytometer under the microscope. The square with the smallest inner squares is the center of the nine square hemocytometer.*

A				B		
401	402	437		79	73	65
486	400	419		74	71	64
417	204	399		64	53	78

Figure 3.6: *Zooxanthellae counts for fragment 1. (A) is non-bleached, (B) is bleached.*

A				B		
437	519	530		73	95	62
448	511	454		91	87	82
515	533	139		61	79	71

Figure 3.7: *Zooxanthellae counts for fragment 2. (A) is non-bleached, (B) is bleached.*

A				B		
516	453	450		75	94	73
446	452	530		96	80	98
443	482	526		115	103	98

Figure 3.8: *Zooxanthellae counts for fragment 2. (A) is non-bleached, (B) is bleached.*

The values in each of the squares was used to calculate the average concentration for each non-bleached and bleached sample for each of the three fragments. Figure 3.9 shows the average zooxanthellae concentrations per 0.1 µL all three fragments, before (blue) and after bleaching (orange). Figure 3.10 shows the average zooxanthellae concentrations in the entire 500 µL sample (±10µL).

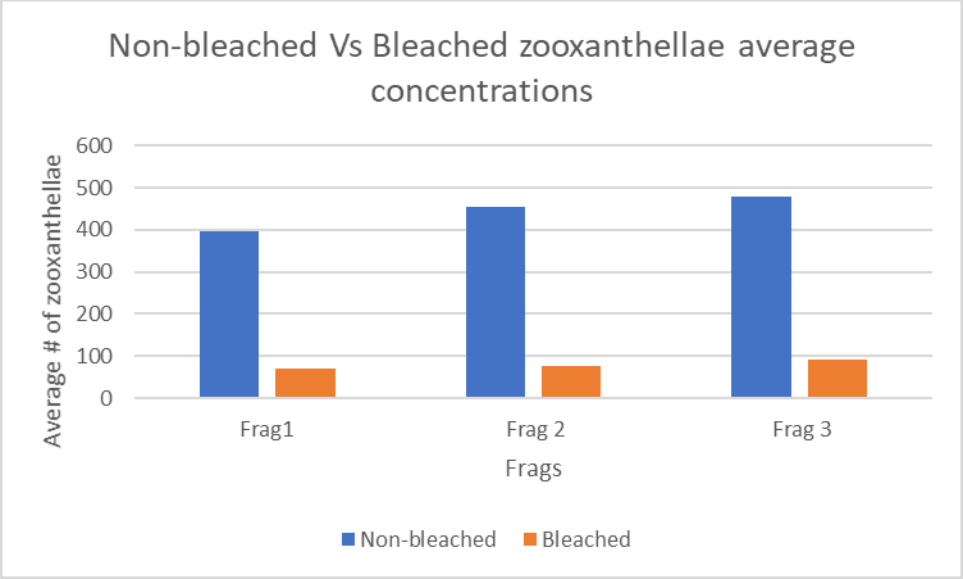

Figure 3.9: *Average zooxanthellae concentration per 0.1 µL of both non-bleached and bleached samples.*

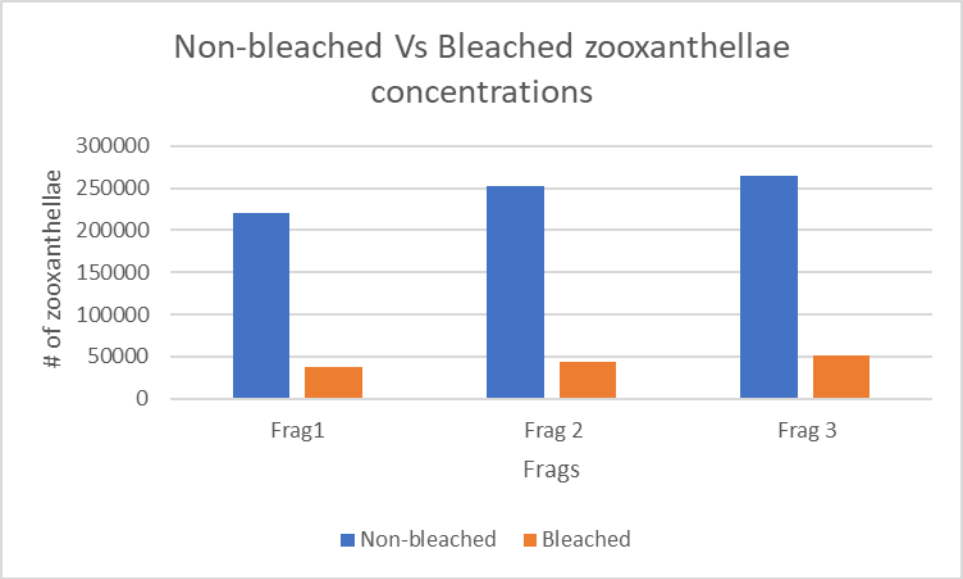

Figure 3.10: *Average zooxanthellae concentration in the 500 µL of both non-bleached and bleached samples.*

Calculations for the total concentration within the sample is as follows:

$$\frac{Total\ Pippetted\ Volume}{Zooxanthellae\ Concentration\ in\ Volume} = \frac{Volume\ Counted}{Average\ Zooxanthellae\ in\ Sample}$$

$$\frac{10\ \mu L}{X} = \frac{0.9\ \mu L}{396.1}$$

$$0.9x = 3961$$

$$x = 4401.2$$

$$\frac{500\ \mu L}{z} = \frac{10\ \mu L}{4401.2}$$

$$10z = 2200617.3$$

$$z = 220061.73\ Zooxanthellae\ in\ 500\ \mu L$$

Figure 3.11: *Sample calculations on how to get total concentration of zooxanthellae in sample. 396.1 is the average from the 9 square counts of one sample.0.9 is the volume under the cover slide. X is total zooxanthellae in 10 µL. The same formula was used to get total zooxanthellae in 500 µL sample.*

The number of zooxanthellae left in the samples after the bleaching event, and lost during the bleaching event, was analyzed using single factor ANOVA in R studio (see figure 3.12).

Response: Zooxanthellae					
	Df	Sum Sq	Mean Sq	F Value	P-value
Fragment	2	25370	12685	3.2998	0.04542
Treatment	1	1776704	1776704	462.1813	<2e-16
Fragment: Treatment	2	8769	4385	1.1406	0.32815
Residuals	48	184520	3844		

Figure 3.12: *Single factor ANOVA output for zooxanthellae left compared to zooxanthellae lost in 500 µL. The P-value is significant being below the critical value of 0.05.*

3.5 Percent lost

A comparison between before and after bleaching trials was made to determine the loss of zooxanthellae and showed that 82% of the zooxanthellae were lost during the bleaching event, and 18% were retained. Figure 3.14 shows these percentages in a pie chart for easy comparison.

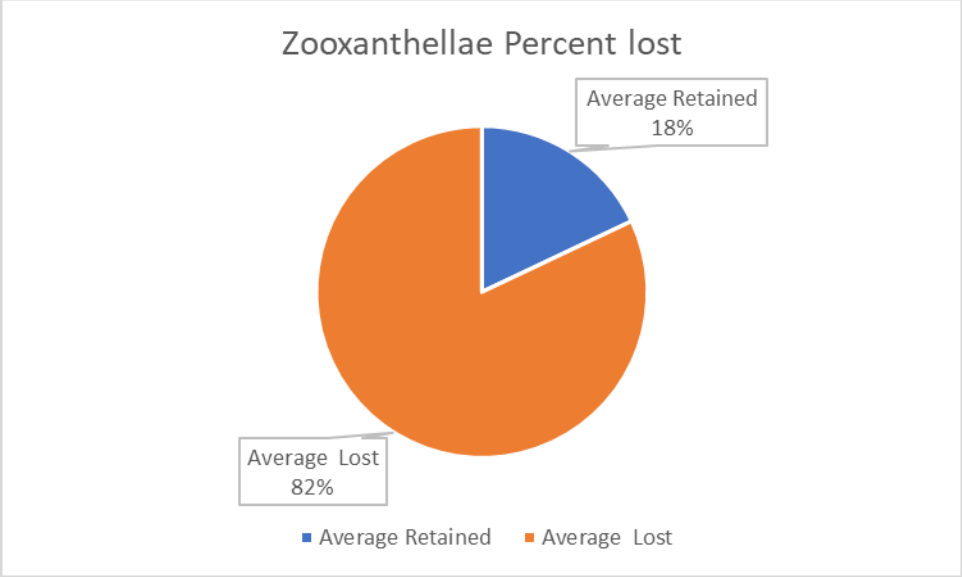

Figure 3.13: *Average amount of zooxanthellae lost after bleaching trials in the fragments. 82% of the zooxanthellae was expelled, while only 18% was retained.*

4. CONCLUSION

4.1 Weight of Coral Samples

The average weight of the coral samples was 7 mg (±1 mg). This step proved difficult. It is possible that during the procedure, the samples lost some zooxanthellae. As the samples dried, they became easy to break apart. The small sample also proved to be difficult to remove from the weight boat. Because of this the 3 samples used for weighing were not used for extraction. Fragment 1 had a total concentration of 220,061.73 zooxanthellae (31,437.39 zooxanthellae per 1 mg). Fragment 2 showed 252,222.22 zooxanthellae (36,031.75 zooxanthellae per mg). Finally, fragment 3 had 265,308.64 zooxanthellae in the whole sample (37,901.23 zooxanthellae per mg). The p-value for the fragment comparison was 0.04542: this is less than the critical value of 0.05 which shows a significant difference. However, this did not effect on the outcome of the treatment when compared to the size of the polyp (or fragment). This can be seen by the ANOVA output giving a 0.32815 p-value for fragment: treatment comparison. Therefore the size of the coral, or fragment, does not affect the outcome of bleaching.

4.2 Trials

Though Xenia are a hardy coral species, bleaching caused an abrupt mortality of fragments 1-3. Xenia started showing signs of bleaching at 31-32.5 °C. Each coral species has a different range of temperature tolerance. The tolerance comes from the species geographical range, their clade of zooxanthellae, and other factors.

4.3 Overall Health and Behavior

The health of the coral was imperative to maintain throughout this experiment. Any changes in the coral's health would alter the concentration of zooxanthellae within the coral.

This Xenia species, much like other species, are prone to getting a black discoloration to their leathery skin. This can affect the number of zooxanthellae within the section infected. Feeding these corals on a regular basis is another essential part to keeping the coral's health at an optimal level. 10% of their food comes from the surrounding water column. Though this is not a lot it still has a significant impact on the coral health. In this experiment we are determining if the coral was bleaching based on its color and its behavior. The color of the coral turns white during bleaching. The behavior of the coral also changes during bleaching. Pulsing Xenia gets its name by the pulsing movements it has with its tentacles. During bleaching the arms will close and shrivel up. The estimated percentage of open polyps within a fragment was used to assess its health status. During the bleaching events, the color changed to a lighter tone of purple, though it was recorded as white to indicate bleaching was occurring. The change in color and a measure 0% open polyps within the fragment was an indication that the coral was dying. Figures 3.3-3.4 show the recordings of the coral health. In figures 3.2-3.4 show how sudden a change in health score happened in the corals.

When looking at figure 3.2, fragment 1, it appears to be unclear if bleaching was occurring because of the constant change in polyp status and or color. Every couple hours polyp status was changed. On the 20th of October there is a shift to a constant healthy and 90% open statues. It is not until the 21st of October that this was suspected to be bleaching. Due to the size of the coral no sample was taken before 2 pm on the 21st. The constant changes during the days prior caused the assumption this was normal. Even before the bleaching trial fragment 1 had some shifts in status. The size of the fragment was roughly half that of fragment 3, the color was a lighter tone as well. This made it difficult to determine if bleaching was occurring. Fragment 1 had a separate colony of Xenia on it that was very small. This colony survived the trials and was

recorded in figure 3.2. The size of this colony was half of fragment 1. It is unclear why this small fragment survived, however, one possible is a transfer of zooxanthellae from another coral species inhabiting the same tank. Green star polyps (Pachyclavularia *violacea*) have a different clade of zooxanthellae which can survive thermal bleaching events (clade D) (Riddle,2006). New polyps can uptake zooxanthellae from the water column surrounding them. Since two distinct species of coral lived in the same environment for months before trials began it is possible for them to have transferred zooxanthellae from one coral species to another. This is a frequent practice for young colonies to partake in. Larger more established corals partake in this as well however, they have an already established colony of zooxanthellae. Having this extra colony on the same fragment ended up causing an issue with the observations. The mortality of the Xenia colonies was not expected to be so quick. Nor was the smaller colony expected to outlive any of the other colonies. Because of this the smaller colony was lumped into the same observation data as fragment 1. The recordings on the 21st show it almost being at 0% polyp status, then returning to 50% and lower. Only to be observed at a higher percentage at 4 pm the same day. If a transfer of clades occurred, then it is possible for the polyp status and color to have been worse than recorded and signs of bleaching possible presented itself earlier.

When looking at figure 3.3, fragment 2, the health of the coral did not change until 2 pm on the 21st. The color of the polyp was of healthy status, or purplish white with silver tent, and the polyp status was 0% at the time of sampling. Any time before sampling shows no signs of bleaching at all. All open polyp status and healthy color presented itself from the start of the trial till 2 pm of the 21st. Again, sampling occurred around the 2 pm mark, then coral mortality was observed a couple hours after sampling. The same occurred to fragment 3 in figure 3.4 with only a slight variation. Fragment 3 start to lose its coloring before it shriveled up. It is important to

note, even though it says white, the coral itself was not white just whiter than the healthy state. The shriveling of the polyps does not mean the polyps closed either. The neck of the polyps was pulled in but still the tentacles were open and pulsing. From this data no matter the size of coral, or age, the mortality of each occurred roughly at the same time. This is not expected, the base idea of the older the coral is the longer it can survive the event is not completely true. There is a reserve of energy each coral has, and it is variant on size and age. The excess is stored in the host at concentrations of up to 10-40% of the total biomass (Rodrigoes and Grottoli, 2008). These reserves decrease in some bleached coral. Some species of coral can recover using these reserves and other tissue biomass (Rodrigoes and Grottoli, 2008). Xenia coral are soft coral which in bleaching events causes most of their tissues to degreed causing them to lose these reserves.

4.4 Bleaching results

The non-bleached sample for fragment 1 had an average of 396.11 zooxanthellae in the 9 squares. Compared to the bleached sample with an average of 69 zooxanthellae in the 9 squares. There was an 82.58% loss in zooxanthellae concentration due to thermal bleaching. There were 38,333 zooxanthellae in the collected anthocodia. 17.42% of zooxanthellae were left in the coral after bleaching.

Fragment 2 showed similar results. The average zooxanthellae in the non-bleached sample were 454, and the average for the bleached sample was 77.89. Again, 82.84% of zooxanthellae was lost from non-bleached to bleached samples. There was a 17.16% retained zooxanthellae within the sample. The non-bleached sample had 252,222 zooxanthellae within the entire anthecodia sample. Whereas the bleached sample had 43,271 zooxanthellae within the entire anthecodia sample.

Figure 3.8 shows the hemocytometer counts for fragment 3. The average zooxanthellae in non-bleached coral 477.56, the bleached sample had an average of 86.78. These are similar results when compared to fragments 1 and 2. The average percent zooxanthellae lost was 80.64% compared to the amount retained with a 19.36%. Calculating the concentration of the entire anthecodia sample yielded 265,308 for the non-bleached sample. Compared to the bleach sample with 51,358.

The data show that the bleaching caused a drastic decrease in zooxanthellae concentration. Figure 3.12 shows the single factor ANOVA output for the concentration of zooxanthellae within the 500 µL sample yielded significant differences between fragment size (fragment 1, fragment 2, fragment 3) and between treatments (non-bleached or bleached). However, it shows no significant difference between fragment size and treatment. This shows that the size of the fragment or colony has no effect on bleaching outcome. On average the coral retained 18% of their zooxanthella and lost 82%.

4.5 Coral mortality

The study shows significant results. The number of zooxanthellae within the non-bleached and bleached coral is an indicator of the coral's overall health. When the temperature was raised to 31-32°C the coral had an average of 82% reduction in zooxanthellae. Out of the 4 polyps analyzed in this study, 3 died and only one survived. The surviving polyp was very small and only made up 1-3% of the total *Xenia* mass within the tank.

In this study we were not able to identify the zooxanthellae clades within the Xenia coral. Every species of coral has their own specific dominating clade which is suited to their environment. Clade D allows for a better chance of survival against bleaching events. Clade F (the clade usually found in Xenia) is less resistant to temperature increase, making Xenia more sensitive to

bleaching and death. It has been shown that it is possible for coral to trade and acquire zooxanthellae from the water column (Riddle, 2006). However, if the coral is not in an early juvenile stage, the introduced clade of zooxanthellae will not be the dominant species within the coral. Future research may investigate which clades (if more than one) are present in Xenia coral, and whether during the bleaching event zooxanthellae belonging to a particular clade make up the 18% that was observed within the coral after the bleaching events. This research showed that bleaching and death occurred very quickly. This indicates that, when temperature raises quickly, increasing feeding for adult corals may not be enough to increase the coral chances of survival. Juvenile, or small coral (carpet breed coral) could potentially benefit from the increased feeding or may be able to uptake new clades of zooxanthellae that allow for better survivability.

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