HOMOTREMA RUBRUM (LAMARCK): DISTRIBUTION AND BIOLOGY OF A POTENTIAL REEF BIOINDICATOR AND UNDERWATER ANGLER

by

WILLIAM G. PHALEN

(Under the Direction of SUSAN GOLDSTEIN)

ABSTRACT

Homotrema rubrum is a species of encrusting foraminifera that reinforces the coral-reef framework by calcifying in cracks, crevices and under-populated parts of reefs. *H. rubrum* secretes a hard skeleton that is well preserved in the fossil record and, thus, has the potential to be important in paleoecological analyses. The distribution of this species along transects across Tennessee Reef (Florida Keys, USA) reveals an abundance of encrusting, knobby and hemispherical morphologies on the reef flat. Feeding experiments dispute previous claims of *H. rubrum*'s sole reliance on photoendosymbionts by demonstrating that *H. rubrum* can be an active and efficient carnivore. Photo and Scanning Electron Micrographs reveal that *H. rubrum* utilizes sponge spicules collected from the environment in combination with reticulopodia to trap and consume living prey. Close observation and epifluorescence microscopy suggests *H. rubrum* is capable of calcifying under controlled laboratory conditions.

INDEX WORDS: Foraminifera, Florida Keys, Caribbean, Coral Reefs, Calcein, *Homotrema rubrum*, endosymbiont

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B.S. and B.A., Boston College, 2013

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2015

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May 2015

ACKNOWLEDGMENTS

I would first like to thank my major advisor/dive buddy Dr. Susan Goldstein for all the help in prepping and conducting the two field trips in addition to guiding me through every step of an occasionally convoluted project. I also want to thank my advisor for the past four years Dr. Joan Bernhard at Woods Hole Oceanographic Institution who was instrumental to the design of the fieldwork and laboratory experiments both in Woods Hole and at University of Georgia. Two others at Woods Hole Oceanographic Institution I need to thank are Marti Jeglinski and Jos Wit, both helped tremendously in the handling and care of my samples while I was absent. Marti has also been one of my closest friends/advisors since I first met her in New Bern, NC almost four years ago and she's proven to be one of my most ardent advocates; without her I doubt I have pursued ocean science research. I want to thank Dr. Samuel Bowser for his time, advisement and expertise on the Scanning Electron Microscope at the Wadsworth Center at the New York Department of Health without which all of the amazing SEM images would not have been possible. I would like to thank all of the staff at the Keys Marine Laboratory for their help and guidance during our two field trips to the facility as well as Sam Bowser and Laura Von Rosk for their assistance during our second trip to KML. Awards from the JA Cushman Foundation for Foraminiferal Research, the Paleontological Society and the Watts Wheeler Fund (Department of Geology, University of Georgia) funded this research.

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CHAPTER 1

INTRODUCTION

This thesis is written as a manuscript and is intended for submission to the *Journal of Foraminiferal Research*. Chapter two contains the text of the manuscript and incudes an introduction reviewing the existing published work on *Homotrema rubrum* as well as the methods, results and discussion. Chapter 3 provides suggestion of important future work on *H. rubrum*.

This project aimed to better understand the biology and paleoceanographic, paleoecologic and paleoclimatologic potential of the important reef-dwelling encrusting foraminifer *H. rubrum*. The fieldwork was conducted on Tennessee Reef in the Florida Keys National Marine Sanctuary (FKNMS) with the full logistical support of the Keys Marine Laboratory (KML). The laboratory experiments were maintained at Woods Hole Oceanographic Institution and University of Georgia. *In situ* transects using half-meter quadrats were analyzed to resolve *H. rubrum*'s relative abundance and distribution on the reef. Specimens were also collected for laboratory analyses under controlled conditions to track morphology, calcification and feeding strategies. The collected specimens were fed four different food types and closely monitored for signs of active feeding (i.e., feeding cysts). This study used a combination of close observation under reflected light and calcein incubation to track any calcification during laboratory experiments. SEM images were taken by Dr. Sam Bowser (Wadsworth Center, New York State Department of

Health) and were instrumental in accurately assessing and documenting the active carnivory of *H. rubrum.*

The results of this study provide more insights into the distribution and abundance of *H. rubrum* as well as a more accurate assessment of its trophic mechanisms and morphogenesis. The consistent distribution and abundance patterns of *H. rubrum* in modern oceans and the exceptional preservation of encrusting organisms in the fossil record may allow for *H. rubrum* to be used as a paleo-proxy for past physical or ecological disturbances in tropical or subtropical shallow water regions.

CHAPTER 2

*HOMOTREMA RUBRUM (*LAMARCK): DISTRIBUTION AND BIOLOGY OF A POTENTIAL REEF BIOINDICATOR AND UNDERWATER ANGLER

Introduction

Marine ecosystems throughout the worlds ocean's are threatened today more than any other period in human history due to the direct and indirect consequences of anthropogenic activity (McCauley and others, 2015). A comprehensive study recently concluded that the increase in average sea surface temperatures (SST) and rising atmospheric partial pressure of carbon dioxide (pCO_2) is lowering the pH of the oceans and causing unprecedented damage to marine species (McCauley and others, 2015). Furthermore, McCauley (2015) reports that coral reefs are among the most vulnerable marine ecosystems to defaunation because of a wide range of disturbances they endure (i.e., sedimentation, pollution, thermal stress, disease; Selig and others, 2012; Doney and others, 2009; Bruno and Selig, 2007; Roberts and others, 2002).

Coral-reef degradation is especially concerning because even though reefs occupy less than 0.1% of the ocean floor, they are associated with a quarter of all marine species (Spalding, 2001; Doney and others, 2012). The debilitated reefs rely on contributions of carbonate from other calcifying organisms including foraminifera, particularly when under environmental stress (Mallela, 2007). *Homotrema rubrum* and other encrusting foraminifera reinforce the entire coral framework by calcifying in cracks, crevices, marine caves and non-growing parts of reefs contributing up to 20% of the carbonate to

some Caribbean reefs (Mallela, 2007; Langer and others, 1997; Elliott and others, 1996). Moreover, *H. rubrum* may also be threatened by the same conditions plaguing many coral species because it is sessile, relies on a test that is high in Mg-calcite (Blackmon and Todd, 1959) and has no known endosymbionts (S Bowser, unpubl.) that can buffer some of the negative effects associated with ocean acidification (e.g., De Beer and Larkum, 2001; Kohler-Rink and Kuhl, 2000, 2005).

Lamarck (1816) first described *Homotrema rubrum* under the name *Millipora rubra*. Hickson (1911) then divided the genus *Polytrema* into *Polytrema*, *Homotrema*, and *Sporadotre* placing the species *rubra* into the genus *Homotrema*. Later, Cushman (1927) reclassified the foraminifera and placed *Homotrema* within the newly erected Family Homotrematidae. Emiliani (1951), in a detailed morphological study of *H. rubrum*, recognized five distinct morphotypes: encrusting, globose, branching, globose composite and pseudo-ramose test. Lowenstam (1967) reduced the number of morphotypes to three (globose, encrusting and aborescent) and suggested that morphotype is determined by microhabitat conditions. Lowenstam argues that exposed areas are dominated by the encrusting morphology, protected areas by globose forms and the arborescent forms in the least turbulent deepest cavities. However, Rooney (1970) did not find similar microhabitat restrictions on morphotype and instead conjectured that the morphotypes were stages in ontogenetic growth. Elliott (1996) most recently categorized *H. rubrum* into five morphotypes based on over 4,000 tests collected in Bermuda ranking them from most to least abundant: hemispherical, globose, knobby, encrusting and columnar. He found each of the morphotypes in all habitats he surveyed around the Bermuda platform (0-30 m; offshore, midshore, nearshore) and concluded that *H. rubrum*

is regulated by ontogeny and microenvironmental conditions (i.e., wave energy, light, sponge spicule availability; Elliott and others, 1996).

As early as 1903, Lister (1903) reported sponge spicules protruding from the test and suggested they may be used as "scaffolding" to support extended pseudopodia. Cushman (1922) also documented the presence of numerous sponge spicules projecting from the apertures of *H. rubrum* attached to dead coral. These observations were later corroborated by Rooney (1970) who identified the sponge as *Spirastrella* sp. Lipps (1983) subsequently suggested that *H. rubrum* uses these spicules to support reticulopodia during feeding and possibly to add structure to the newly formed test wall. Elliott (1996) later posited that the availability and acquisition of spicules may orient morphologic development towards the knobby morphotype.

Previous studies that assessed the abundance and relative distribution of *H. rubrum* concluded they are most often found in cryptic environments (e.g., Hepburn and others, 2015; Elliott and others, 1996; Rooney, 1970) and the highest population densities occur on newly formed coral rubble (e.g., Elliot and others, 1996). Nevertheless, Elliot (1996) found that the distribution of morphologies between substrates and microenvironments did not vary significantly (Elliot and others, 1996). Moreover, Elliot and his colleagues deployed five cement blocks on a ledge at the base of North Rock Rim reef, Bermuda for five years and found that the vast majority (93%) of *H. rubrum* settled inside the sheltered parts of the blocks (Elliot and others, 1996). He posited that *H. rubrum*'s overwhelming settlement on the underside of the blocks was the result of competition from filamentous algae, selective settlement and/or predation.

Benthic foraminifera are widely studied, easily fossilized and some species are used as proxies for specific marine physical and chemical conditions (e.g., Waelbroeck and others, 2002; Elderman and Ganssen, 2000; Bernhard and others, 1997). Encrusting organisms that secrete mineralized skeletons, such as *H. rubrum*, are well preserved in the fossil record and thus have the potential to be important in paleoecological analyses (Taylor and Wilson, 2003). *Homotrema rubrum* is ideally suited for use as a paleoproxy for past ecological or physical disturbances as it is prevalent in tropical reef systems throughout the world's oceans and predominately resides in cryptic habitats on these reefs (Poag, 1981). As previously noted, *H. rubrum* recruits to the undersides of coral colonies and other typically dead portions of the coral rubble. These areas are most common in the reef flat just shoreward of the reef crest where the largest pieces of coral rubble fall out of suspension (Hauser and others, 2008; Gischler and others, 2003; Gischler and Ginsberg, 1996; Streeter, 1963). Moreover, *H. rubrum* are often out competed by other epibionts that thrive on living coral, but often die soon after the coral (Greenstein and Pandolfi, 1997). These conditions suggest that the ideal substrate for *H. rubrum* is large, uninhabited, recently deceased coral rubble that is deposited on the reef flat, a substrate that is most profuse following a disturbance event (Greenstein and Pandolfi, 2003). Thus, relative abundance of *H. rubrum* in the fossil record could be a valuable proxy for disturbance events of all types and spatial scales.

Furthermore, *H. rubrum* has proven to be a dependable proxy for other conditions including sediment transport from shallow-reef environments (Pilarczyk and others, 2014; Mackenzie and others, 1965). Mackenzie understood that living *H. rubrum* are limited by depth and examined deep-sea cores for fossilized pink *H. rubrum* tests that are

easily identified because they retain their pigment post mortem. Another more recent study investigated the possibility of using detrital *H. rubrum* found in sediments of marine ponds as an indicator of overwash from tsunamis and hurricanes in the British Virgin Islands (Pilarczyk and Reinhardt, 2012). However, Pilarczyk and Reinhardt were unable to effectively connect *H. rubrum*'s presence in the record as a result of a tsunami or hurricane because their taphonomic analysis was limited by the lack of comparative data. These studies illustrate two of many possible applications for this important species that is abundant in the tropical Atlantic, Pacific and Indian Oceans.

While *H. rubrum*'s contribution to reef structure is well documented, most basic biological functions of this species remain equivocal. The only previous report on trophic mechanisms in *H. rubrum* suggests that photosynthetic endosymbionts serve as a primary source of nutrition (Strathearn, 1986). However, his study relied entirely on shell chemistry and an analysis of pigment contained in the shell without distinguishing between living or dead specimens and did not include any direct observation of symbioses.

This study aims to document the distribution, relative abundance and morphological variation of *H. rubrum* along Tennessee Reef in the Florida Keys National Marine Sanctuary (FKNMS). This project incorporated field data, observations made on living specimens in static and flow-through aquaria, feeding and calcification experiments, and light-, epifluorescence, and scanning-electron microscopy to better understand *H. rubrum* morphotypes, feeding strategies, calcification and viability under controlled laboratory conditions. In addition, attempts were made to examine recruitment and reproduction through the deployment of a settlement array and close monitoring in

laboratory experiments, however, these proved unsuccessful over the limited duration of the study.

Methods

Field Data

The study site for this project is located in the Florida Keys because the occurrence of *H. rubrum* was previously recorded there (e.g., Bock, 1971; Hallock and Peebles, 1993). *In situ* observations, sample collection, settlement plate deployment and collection were made during two sampling trips to the Tennessee Reef, in May and September of 2014. All field sampling and data collection was performed using SCUBA. During the first trip we collected abundance data and samples along three distinct transects, one 342 m transect perpendicular to the reef crest extending from the reef flat to just seaward of the reef crest (CR1) and two 80 m transects parallel to the reef crest (LI and SS; Fig. 1). In September, another 280 m transect across the reef crest was completed over the course of two dives (CR2). A 0.5 m^2 quadrat was laid down approximately every 9 m (distance measured with kick cycles) and a single compass heading was followed (Fig. 2). At each station the area within the quadrat was sampled on the surface and in the subsurface sediment down to approximately 15 cm for coral rubble and *H. rubrum*. Abundance, relative vitality (i.e., coloration and extent of test erosion), predominant morphology and depth data were recorded on an underwater slate. We used a Sony Cyber-Shot DSC-W100, with an Ikelite case for all underwater photography to visually

illustrate the substrate and sampling procedure. The images were edited for exposure in Adobe Photoshop to account for reduced levels of red and green spectra.

During the first trip in May we deployed 12 terracotta settlement plates on the reef flat landward of the reef crest at a depth of ~7 m (Figure 1). The plates and supporting apparatus (Fig. 2) were secured to the reef, and modeled after a design deployed by Dr. Mary Alice Coffroth (written communication, 2014) from the University of Buffalo that complied with guidelines of the FKNMS. The plates were carefully collected in early September and inspected using a stereo-dissecting microscope for the recruitment of large or small individuals of *H. rubrum*. Plates were then air dried and stored for transport back to University of Georgia.

Feeding Experiments

The first part of the feeding experiments began in the field with the collection of healthy *H. rubrum* determined *in situ* by color, the lack of visible erosion and presence of newly formed apertural mounds. The specimens that were dark red and least eroded were collected into resealable plastic bags and brought to the surface. Once at the surface the bagged *H. rubrum* were carefully placed in a cooler for transport by boat to Keys Marine Lab. After arriving at the lab, specimens were quickly transferred into 10 L containers filled with filtered seawater and aerated with aquarium bubblers (Fig. 3). After 24 hours the specimens were divided evenly into four smaller containers and fed approximately 50 mL of one of four varieties of food: *Isochrysis galbana* (Parke), Grell's *Dunaliella* sp., mix of marine diatoms (Carolina Biological) or live *Artemia* sp. Each container was monitored closely under the microscope over the next 48 hours for signs of feeding

including the construction of feeding cysts (e.g., Heinz and Hemleben, 2005; Jepps and others, 1956) or prey (*Artemia* sp.) capture. Still photographs and videos were taken at regular intervals.

Calcification

Approximately 10 of the living *H. rubrum* that were collected in either May or September were incubated in 10 mg/L calcein. The May experiment lasted five weeks and the September experiment for 27 days. Previous studies have shown that calcein incubation is an effective non-lethal method to stain calcifying foraminifera maintained under laboratory conditions (Bernhard and others, 2004; Bernhard and others, 2009). The *H. rubrum* in both rounds of the experiment were fed 30-50 mL of concentrated living *Artemia* sp. (approximately 200-300 individuals) every four to five days. Every two weeks, approximately 2,000 *Artemia* sp. cysts were hatched in 1L of aerated 35‰ Instant Ocean. The water in both treatments was replaced every seven to ten days and salinity was checked and adjusted as needed back to 35‰ every three days. The specimens for the May treatment were placed in a 1 L container filled with 35‰ Instant Ocean aerated with an aquarium bubbler and kept in an incubator at 25°C equipped with a 12-hour dark-light cycle. The September experiment was also performed in 35‰ aerated Instant Ocean, but it was maintained at room temperature (22°C) in the laboratory and approximately three meters from the window (only natural light source). At the end of the incubation the rubble pieces containing *H. rubrum* were preserved with 10% paraformaldehyde buffered with borax in individual 10 mL or 50 mL conical tubes.

The samples from the University of Georgia were then transported to Dr. Joan Bernhard's laboratory at Woods Hole Oceanographic Institution. The specimens were stored in 10% paraformaldehyde solution and imaged while submerged in 35‰ Instant Ocean. The reflected light and epifluorescence images were taken with an Olympus Fluoroview Camera mounted on a Leica MZ10 F epifluorescence microscope. The preserved and labeled samples were imaged using the appropriate epifluorescence optics (480 nm excitation, 520 nm emission; Bernhard and others, 2006). Specimens with parts of the test that fluoresced were separated and brought to Dr. Sam Bowser's laboratory at Wadsworth for SEM. A control group of unlabeled specimens was preserved to ensure neither *H. rubrum* nor any organic material within it autofluoresced under the aforementioned excitation and emission filters.

Experimental Design of Laboratory Analyses

The goal for this part of the project was to determine the optimum controlled conditions to maintain a living *H. rubrum* population in the laboratory. Previous studies observed and recorded *H. rubrum* populations *in situ*, but there was no published research focused on experimental analyses of *H. rubrum* in the laboratory. Thus, a wide array of experimental set ups were devised using a combination of the *in situ* conditions of *H. rubrum*'s preferred habitat, established benthic foraminifera culture designs and the facilities available in Dr. Joan Bernhard's laboratory and the Environmental Systems Laboratory (ESL) at the Woods Hole Oceanographic Institution. The two aquaria designs installed at the ESL had the advantage of natural filtered seawater and constant water replacement, but the limited range of temperatures (20°C maximum) kept the seawater in

those aquaria below the temperature recorded *in situ* at the time of collection (23°C). The three treatments at the Bernhard laboratory were kept closer to the recorded temperature, but the seawater was artificial (Instant Ocean) and only replaced once a week. The results from close monitoring of these five independent systems with specimens collected from the first trip in May were vital as they informed many of the decisions for the second round of laboratory experiments in September.

After the May field trip the live samples were divided, half were shipped overnight in small sealed containers (less than 0.5 L) to Dr. Bernhard's laboratory and the other half were transported by land (via personal vehicle) over the course of three days. Once the shipped samples arrived at WHOI they were immediately placed into one of five aquaria, each with a unique experimental design. The three aquaria in the laboratory all contained 35‰ Instant Ocean seawater and were aerated with bubblers. One of the three was placed in an incubator set at 25°C and the other two at room temperature in the laboratory (~22°C). Of the two kept at laboratory temperature one was placed adjacent to the window while the other remained on the interior of the laboratory and was exposed to little natural light.

The other two aquaria were in the Environmental Systems Laboratory at WHOI and were filled with filtered seawater pumped in from Vineyard Sound, MA and warmed to 20°C with salinity fluctuating between 33‰ and 35‰ (Fig. 3). One of the designs allowed for a fast flowing turbulent microhabitat (~3 L/min) and the other was a slower overflow system (~0.25 L/min). The coarse calcareous sand (>2 mm) that was collected from the sampling site was washed with dH_2O and air dried twice and introduced into two of the four overflow containers and the flow-through aquaria. As a result of the

constant seawater turnover/replacement of these two systems the flow was ceased an hour before feeding and remained off until two hours after feeding (e.g., Hintz and others, 2004). All five of the treatments were fed live *Artemia* sp. weekly and were closely monitored for feeding, algal competition for substrate, pigment retention and calcification. After five weeks the experiments were terminated and the specimens were archived by preservation in 70% ethanol. Specimens were later examined to determine algal cover on the *H. rubrum* test.

Microscopy

Electron Microscopy

The living specimens were transferred from the aquaria into 50mL conical tubes underwater and then were fixed in a chilled solution of 3% glutaraldehyde and 0.15% ruthenium red with a 0.1 M cacodylate buffer and 0.1 M sucrose (after Goldstein and Barker, 1988) at the University of Georgia. The fixative was introduced at the bottom of the conical tube as the seawater was simultaneously pipetted off from the top. This was done to prevent the surface tension from damaging the reticulopodia or dislodging the captured prey. The conical tube was then filled completely with fixative and placed in the refrigerator. The top 5-10 mL of the conical tube was siphoned off and replaced with the aforementioned fixative three times over the next 10 days to remove as much of the remaining seawater as possible. The specimens remained in the fixative for 20 more days then were carefully transported to the Wadsworth Center. Next, the specimens were dehydrated through a graded series of acetone and critical point dried (Bowser and Travis, 2000) to prevent damage to the reticulopodia. The samples were then carefully

mounted on SEM stubs with Tacky Glue (Gaunt Industries, Inc.) and sputter coated with Au-Pd for 45 seconds. The high-resolution Scanning Electron Microscopy (SEM) used to illustrate carnivory, morphology and calcification was all done at the Wadsworth Center (New York State Department of Health) in the laboratory of Dr. Samuel Bowser using a Zeiss NEON 40EsB.

Reflected Light Videos and Images

All of the reflected light images and videos were taken either with an iPhone 4S camera manually positioned on the optic of a standard Zeiss reflected light microscope or with the Olympus DP70 Camera mounted on the Leica MZFLIII. In some instances dim reflected light was used in combination with the fluorescent filters to reveal the entire specimen as well as the calcein labeled portion (akin to a double exposure).

Results

Field Data

Within the study area, the distribution and relative abundance data suggest that *H. rubrum* is most abundant on Tennessee Reef attached to coral rubble 40 m to 100 m leeward of the living reef (80%; Fig. 4). However, the single highest abundance of *H. rubrum* (230 specimen/m^2) occurred in a 15m wide sand strip between two living portions of the reef, approximately 110m north of the reef crest (Fig. 1, CR1: station 9). This quadrat only contained two pieces of rubble with *H. rubrum* present, but the most populated piece was over 20 cm long and had the vast majority attached (83%). Transects

1 and 2 were taken at two different times of the year on different parts of the reefs and a Pearson's Chi-squared test does show a significant difference between the two (x^2 = 157.7193, df = 120, *p = 0.0119*). However, the transformed count data distributions (Fig. 4) show that for both transects the vast majority of the *H. rubrum* abundance was recorded on the reef flat leeward of the living reef. The two transects parallel to the reef crest (SS and LI) indicate that the *H. rubrum* populations were not at all uniformly distributed laterally across the reef, but instead are very patchy with large differences in relative abundance along both transects (Appendix).

The settlement plates and supporting apparatus were retrieved on September 16, 2014, 144 days after deployment. The "arms" of the array that initially kept 12 of the plates suspended approximately 0.5m above the seafloor had broken away from the center support and were laying adjacent to the center post partially submerged in the sediment (Fig. 2). At the time of collection four of the plates remained supported several centimeters above the sediment, four were partially covered in sediment and four were completely buried under sediment. Close inspection under the microscope revealed that no *H. rubrum*, juvenile or adult, had recruited to any of the plates. Nevertheless, there was substantial colonization by various marine invertebrates (i.e., sponges, bivalves) and one very abundant foraminiferan, *Planogypsina acervalis* (Brady, 1884), which is commonly found attached to sea grass blades leeward of the reef flat (e.g., Richardson, 2006). The two plates buried in the sediment but still attached to the support, did not have any discernable epibionts, living or dead.

All five of the morphologies of *H. rubrum* described by Elliot (1996) were found during collection. However, only hemispherical, knobby and encrusting morphotypes

were found alive at the time of collection based on coloration, extent of erosion and feeding activity. Each of the specimens collected with the globose morphotype were intensely eroded and had lost most of the deep red coloration. Furthermore, the vast majority of specimens living and dead were buried in the sediment and not immediately visible without excavation (Fig. 2).

Feeding Experiments

Of the four food types offered to *H. rubrum*, *Artemia* sp. was the only one that was observed being actively collected and ingested. Over 80% of the 11 *H. rubrum* in the *Artemia* sp. fed aquarium caught either live *Artemia* sp. or unhatched cysts and ingested them through an aperture (Fig. 5 and 6). The *H. rubrum* that were fed the other three varieties of food were not observed collecting or ingesting the free-floating algae or diatoms during the experiments under low magnification microscopy (e.g., Heinz and Hemleben, 2005; Jepps and others, 1956). Furthermore, the apertures of multiple *H. rubrum* were closely inspected under high magnification SEM and though diatoms were present (Fig. 7), they did not seem to be corralled towards any particular aperture as would be expected during feeding. However, many other organisms living on or within the rubble did actively corral the other introduced food types. After six days, the *Artemia* sp. were introduced into the other three aquaria and within three hours 18 of the 30 *H. rubrum* had caught at least one of the live *Artemia* sp. or a cyst. Overnight another four specimens from these alternative treatments had successfully captured living *Artemia* sp. Furthermore, within two weeks of regular *Artemia* sp. feeding multiple encrusting specimens and one hemispherical specimen began forming new apertures (see below).

Direct observation under the microscope reveals that *H. rubrum* uses reticulopodial nets that cling to sponge spicules protruding from an aperture to catch *Artemia* sp. (Fig. 5 and 6). In nearly every observed instance the *Artemia* sp. swam into the end of one of the spicules protruding from the *H. rubrum* test and remained affixed even though the prey was still alive and actively swimming. After 30 to 45 minutes the *Artemia* sp.'s movement slowed and the *H. rubrum* would begin bringing the caught *Artemia* sp. toward the aperture (Fig. 5b). The combination of video and SEM images of the feeding clearly shows the reticulopodia wrapped around the *Artemia* sp. in the process of capturing and consuming its prey (Fig. 5B and 6). In this experiment *H. rubrum* was clearly exhibiting the capacity for carnivory and thus does not rely solely on photoendosymbionts for nutrition. (http://youtu.be/RMlyFB0e7BQ).

High magnification images from the SEM shows that the coral rubble that *H. rubrum* is attached to can be coated with small diatoms (Fig. 7). Moreover, the images also reveal that a copious amount of diatoms occur in the pores of the test of the *Artemia* sp.-fed *H. rubrum*. The images do not indicate whether the diatoms were collected in the pores for feeding purposes or simply amassed there because of local circulation patterns.

Calcification/Morphology

Close observations under the reflected light microscope during the feeding and laboratory experiments suggest that the *H. rubrum* with the encrusting and castle morphotypes were the most active calcifiers (Fig. 8). The calcification in hemispherical individuals seemed to be primarily focused on repairing damaged portions of the test, which restored the darker red color to formerly pink portions of the test. The calcification

of encrusting specimens in the laboratory was most notable because these individuals created “teepee-like” apertural mounds, commonly with associated sponge spicules, on the surface of the test (Fig. 9). As these “teepees” grow and more calcite (e.g., Milliman, 1974) is added to the base of the apertural mound the spicules are aligned vertically projecting out of the aperture. In less than three weeks one of the encrusting *H. rubrum* formed 16 “teepees” and eventually all of them were actively feeding on *Artemia* sp. (Fig. 10).

The uptake of calcein under laboratory conditions was observed in experiments with *H. rubrum* from both trips to KML. The first round of calcein incubation done in Dr. Bernhard’s laboratory at WHOI revealed darker red portions of one of the hemispherical *H. rubrum* to be labeled brightly with calcein. However, the results from the *H. rubrum* incubated in calcein at UGA suggests the calcein may in fact interfere with the red pigment in the test because the portions of newly formed tests that had either lost or never contained the red coloration were the only parts labeled (Fig. 11). Moreover, epifluorescent imaging proved especially difficult because the coral rubble that the *H. rubrum* is attached to autofluoresces under the same optics needed for calcein fluorescence. Nevertheless, SEMs of the calcein labeled specimens confirm that the fluorescent parts of the *H. rubrum* were in fact part of the test and suggest it may be a viable means to track calcification in controlled laboratory conditions.

Another unanticipated result of the calcein incubation was the presence and calcification of small foraminiferal epibionts within the pores of the *H. rubrum* test (Fig. 12). One of the incubated *H. rubrum* had harbored at least seven individual foraminifera ranging in size from 50 to 200 μm. Every chamber of the smallest epibionts (<100 μm)

was tagged with calcein, but for the two largest specimens only the final two chambers were tagged suggesting they were living *in situ* when the *H. rubrum* was collected.

Experimental Design of Laboratory Analyses

The five experimental designs all contained living *H. rubrum* at the end of the five-week experiment. However, the results varied markedly between treatments in overall vitality, algal growth and ease of replication. Of the three laboratory designs, the aquaria aerated at room temperature away from the window had the highest survival rate (over 50%) and least algal growth (~10%). The treatment in the incubator with the light-dark cycle had the second highest survival rate, but also had greater algal growth (~40%). The aquaria at room temperature placed by the window had only a few living *H. rubrum* left and had the greatest algae cover (~80%).

The *H. rubrum* in the two set-ups at the ESL facility exposed to 20°C water caught fewer *Artemia* sp. throughout the experiment, but also had a shorter window to eat due to the brief feeding period provided by the ceased flow (~2 hours). The success of the *H. rubrum* in the overflow system was almost entirely determined by the cover of sediment. The exposed *H. rubrum* (without sediment) within a week were covered in algae, whereas the *H. rubrum* buried under the sediment remained virtually algae free throughout the experiment (Fig. 13A). The exposed *H. rubrum* were all-dead by the end of the four week experiment, whereas almost half (~40%) of the *H. rubrum* covered in sediment retained their pigment and feeding regimen until the end. Moreover, the position of the *H. rubrum* in the flow through aquaria proved very important for survival (Fig. 13B). The rubble pieces in the center of the aquaria where there was the highest rate

of flow had three times the survival rate of the pieces towards the edge where the water was less turbulent. The pieces in the less turbulent water were also more prone to algal competition than the pieces in the flow, further tipping the advantage toward the center of the aquaria.

Discussion

The relative abundance and distribution data support previous reports showing that *H. rubrum* is most abundant on the reef flat shoreward of the reef crest attached to otherwise uninhabited coral rubble (Elliott and others, 1996; Rooney, 1970). Although all five morphologies defined by Elliot (1996) were found on Tennessee Reef, the most abundant living *H. rubrum* were the encrusting and hemispherical morphotypes. Every *H. rubrum* with the globose morphology, as described by Elliot (1996), were highly eroded and a lighter shade of pink suggesting that this morphotype is not living in significant numbers on the reef flat behind Tennessee Reef during the late spring and early fall. The distribution of *H. rubrum* on the reef flat itself is also interesting because the vast majority of *H. rubrum* were found between 40 m and 100 m from the living reef. This result suggests that there may be a critical distance from the living reef where *H. rubrum* thrives. This distance may be important because it minimizes the chance of overwhelming competition from epibionts while still being close enough to feed on small organisms or other food sources associated with the living reef. Moreover, the highest recorded abundance for any station was associated with a site in a sand strip between two living reefs. However, the results from the two transects parallel to the reef crest indicate

that *H. rubrum* populations are patchy even in the same substrate at the same distance from living reefs. This variability could be the result of local reproduction or may provide some support for the influence of microenvironments as others have suggested (Greenstein and Pandolfi, 1997, 2003; Elliot and others, 1996).

The limited *in situ* observational data did not reveal any major differences in distribution and relative abundance of *H. rubrum* between the two seasons (spring and fall) sampled. Only one piece of rubble was found seaward of the reef crest with *H. rubrum* attached and these individuals were intensely eroded with little coloration, indicating they were not living. Survey data taken across the living reef suggest that this region is devoid of *H. rubrum*, although this may in part be a consequence of our sampling technique. Previous studies have recorded *H. rubrum* living in cracks and within marine caves on living reefs (e.g., Elliott and others, 1996; Rooney, 1970), but we were unable to effectively sample in cryptic environments without damaging the reef. Living *H. rubrum* were not visibly present on the bases of living coral, and marine caves are not present in the study area.

Although the settlement array was reinforced with stainless steel bolts and commercial cable ties, it was not able to withstand the four months of deployment without damage. However, the position of the settlement plates and supporting PVC arms of the array on the seafloor should not have prevented recruitment of *H. rubrum*. Elliott (1996) left cinderblocks *in situ* for five years in an area with much greater *H. rubrum* abundances and was able to get *H. rubrum* to recruit and grow. A more general recruitment study deployed shells *in situ* east of Lee Stocking Island in the Bahamas with much higher *H. rubrum* abundance for five years, and *H. rubrum* was found to have

recruited in 15m and 33m of water after the first year (Walker and others 2011). Another more recent study deployed an innovative "cement breeze block" design for two years off the northern coast of the Yucatan Peninsula, Mexico (Hepburn and others, 2015). Hepburn (2015) and his colleagues were able to recruit a variety of encrusting organisms to their design including three *H. rubrum* morphotypes (encrusting, globose and knobby). A combination of competition and insufficient residence time may explain the lack of recruiting *H. rubrum* in the present study.

The feeding experiments in this study demonstrate that *H. rubrum* is an active carnivore that uses sponge spicules (e.g., Lister, 1903; Cushman, 1922; Reiss and Hottinger, 1984; Lipps and others, 1983; Rooney and others, 1970) to support reticulopodial nets to capture prey and ingest them through apertures. The other food resources offered to *H. rubrum* were not collected into feeding cysts typical of grazing foraminifera (e.g., Heinz and Hemleben, 2005; Jepps and others, 1942), nor were they observed being consumed. The apparent carnivory, absence of feeding cysts and SEM images showing diatom free apertures suggests that *H. rubrum may* prefer carnivory to other forms of nutrition. Furthermore, *H. rubrum* not only consumes living *Artemia* sp., but also ingests substantial quantities of free-floating cysts.

Strathearn (1986) contends that *H. rubrum* relies on photoendosymbionts, however this must be considered tenuous considering the results of this study. Strathearn based his conclusion entirely on the analysis of the red pigments preserved in the test and isotopic data of *H. rubrum* isolated from dredged material at a depth of 40m from an unknown location in the Gulf of Mexico. The two main pieces of evidence put forward in the analysis by Strathearn (1986) were that the red coloration of *H. rubrum*'s test might

result from the presence of the photosynthetic pigment pheophytin and not iron. Further, the carbon isotopic signature of *H. rubrum*'s test is 2‰ lighter than would be expected if calcification occurred in equilibrium with seawater, a result that can be explained by a number of vital and environmental effects. According to Strathearn (1986), the fact that each of these could be explained by *H. rubrum* harboring photosynthetic endosymbionts was enough to conclude that *H. rubrum* retained them. The presence of chlorophyll affiliated with certain photosynthetic organisms on or within the test is not sufficient evidence to claim reliance on a symbiotic relationship especially considering that no observations were made on living specimens. Furthermore, this study did not address vitality, morphology, or condition of the test or habitat. Rather, dead specimens with the darkest coloration were selected from dredged material. Additionally, numerous studies on *H. rubrum* have concluded that it prefers cryptic habitats such as marine caves, cracks and crevices (e.g., Gischler and others, 2003; Gischler and Ginsberg, 1996; Streeter, 1963; Poag, 1981; Pandolfi and Greenstein, 2003; Elliott and others, 1996; Rooney, 1970), and this study observed during collection that living *H. rubrum* were most abundant in subsurface sediment, living beneath the sediment-water interface. These preferred habitats are not conducive to an organism that relies on photosynthetic endosymbionts.

Observations and photomicroscopy throughout the laboratory experiments suggests that the most active calcifying morphology under the aforementioned conditions was the encrusting morphotype of *H. rubrum*. The encrusting specimens were initially flat, but during the six-week experiment they built multiple apertural mounds that grew into spicule fortified "teepees". The SEM images in this study reveal the evolution of

these apertures from an encrusting base. These images suggest that sponge spicules are first used as structural supports during the beginning stages of apertural-mound formation (Fig. 9a). However, once the calcite around the base is sufficiently robust, the spicules seem to be reoriented vertically out of the aperture where they ultimately aid in the feeding process (Fig. 9b). Other *H. rubrum* studies (e.g., Elliot and others, 1996; Lipps, 1983; Greenstein and Pandolfi, 2003) have posited that the spicules may be important for feeding or structure, and the results of this study indicate that they may be vital for both.

Observations during the laboratory experiments suggested that encrusting and knobby morphotypes were the most active feeders and most often procured spicules from the environment that were then used to extend the reach of the reticulopodia to catch free swimming *Artemia* sp. As previously noted, we were unable to collect any living globose specimen, and of the ten hemispherical specimens only one showed the ability to use spicules to catch *Artemia* sp. Initially, this study aimed to preserve a living *H. rubrum* for TEM to directly observe the presence or absence of photoendosymbionts, but removing the *H. rubrum* from large rubble pieces before sectioning without killing the organism proved difficult.

The results from the calcein incubation experiments suggest that calcein may be a useful tool to assess *H. rubrum* calcification under controlled laboratory conditions. In both the May and September incubation experiments multiple *H. rubrum* incorporated calcein into newly formed parts of its test, and this was easily detected with epifluorescent microscopy. However, these results seem equivocal. The results from the May incubation showed that the recently calcified dark red portion of the test was tagged with calcein, indicating that the pigment in the *H. rubrum* test did not interfere with the

calcein fluorescence. However, the labeled portions of the *H. rubrum* tests following the second attempt at incubation (September) all corresponded with regions without coloration. Further experiments with calcein incubation are needed to fully understand the effect that the *H. rubrum* pigment may have on the fluorescence of calcein incorporated into the calcite. Nevertheless, it is clear that the calcein did not in any discernable way harm *H. rubrum.* Those individuals incubated in calcein and seawater faired as well as, if not better than, those from the other treatments. In a recent study, Kurtarkar (2015) found increased rates of mortality for *Rosalina sp.* as a result of long-term exposure to calcein (Kurtarkar and others, 2015). However, that study found calcein incubation had no effect on vitality of specimens during short-term experiments operating on time frames similar to this study.

The results of this present study indicate that *H. rubrum* lives and grows in the laboratory under appropriate conditions. The collected *H. rubrum* were exposed to one of a variety of laboratory conditions to better understand the experimental design that would optimize the vitality of a *H. rubrum* population. The major observational conclusions that can be drawn from these different experimental treatments is that *H. rubrum* is not capable of withstanding significant competition from fast growing algae and survives better beneath a layer of coarse calcareous sediment than if it is completely exposed (i.e., epifaunal). This endobenthic lifestyle is further supported by the observation that *in situ* most of the living *H. rubrum* were found buried beneath a veneer of sandy sediment. The sediment most likely prevents fast-growing algae from overwhelming *H. rubrum*, while being sufficiently porous to allow prey organisms and water to circulate into the subsurface. However, the best experimental design for simplicity and survival is an

aerated aquarium maintained at room temperature with diffuse light. The distance from the light source, here a window, is vitally important because it limits algal growth sufficiently to render the sediment cover unnecessary which in turn allows the exposed *H. rubrum* to more easily catch freely swimming *Artemia* sp.

CHAPTER 3

FUTURE RESEARCH

The future of *H. rubrum* research should focus on maintaining *H. rubrum* in the laboratory for a longer period to monitor growth, morphological development, and possibly reproduction. In my seven-week experimental study I recorded nearly 50 newly formed apertural mounds and almost half were still actively feeding by the end of the experiment. A longer sustained laboratory experiment could resolve the debate over the influence of ontogeny verses microhabitat in determining *H. rubrum* morphology.

Future attempts at recruiting *H. rubrum in situ* should also be completed on a longer timescale than was permitted by this study. In addition to more time *in situ* the array should deploy different types of settlement strata (i.e., bleached coral rubble, carbonate shells, unglazed white porcelain) on the reef flat rather than the reddish terra cotta plates used here. Furthermore, this study has established that *H. rubrum* collected *in situ* can be maintained with relative ease under controlled laboratory conditions and thus is an ideal candidate for more involved experimental analyses.

The current and potential impacts of ocean acidification on many different marine species are well documented (e.g., Fujita and others, 2011; Andersson and others, 2009; Clark and others, 2009; Comeau and others, 2009; de Moel and others, 2009; Kuffner and others, 2008; Maier and others, 2011; Pandolfi and others, 2011; Reis and others, 2009; Semesi and others, 2009; Silverman and others, 2009). Further work should be conducted on important encrusting species such as *H. rubrum* because of its important contribution

to reef structure, presence in all in tropical oceans, high Mg-calcite test composition (Blackmon and Todd, 1959) and unmatched distinction and preservation in the fossil record.

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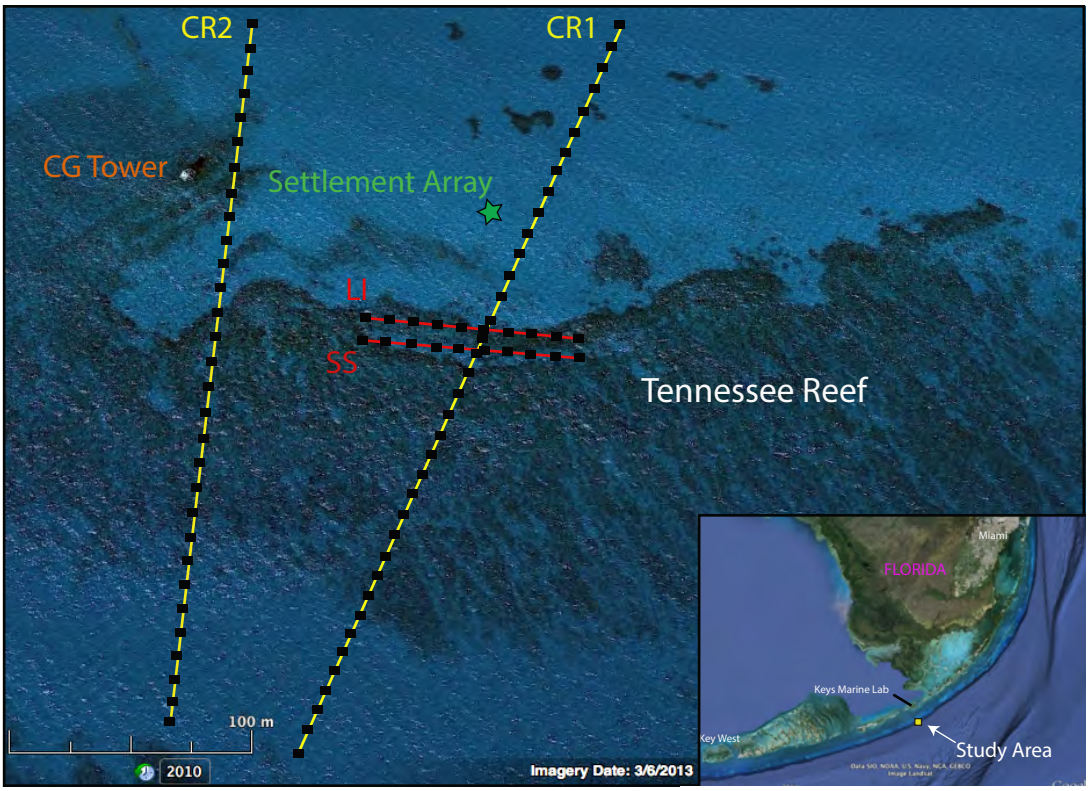

Figure 1. Study area, Tennessee Reef. (24° 44′ 42″ N, 80° 46′ 54.14″ W), is located approximately 7km southeast of Long Key, FL. The Keys Marine Lab (KML) provided labratory and field support for all of the transects and deployment/collection of the settlement array. Transect 1 (CR1), Live Island Transect (LI) and Sand Strip Transect (SS) were completed in May of 2014 and Transect 2 (CR2) was completed in September of 2014. (Maps from Google Earth)

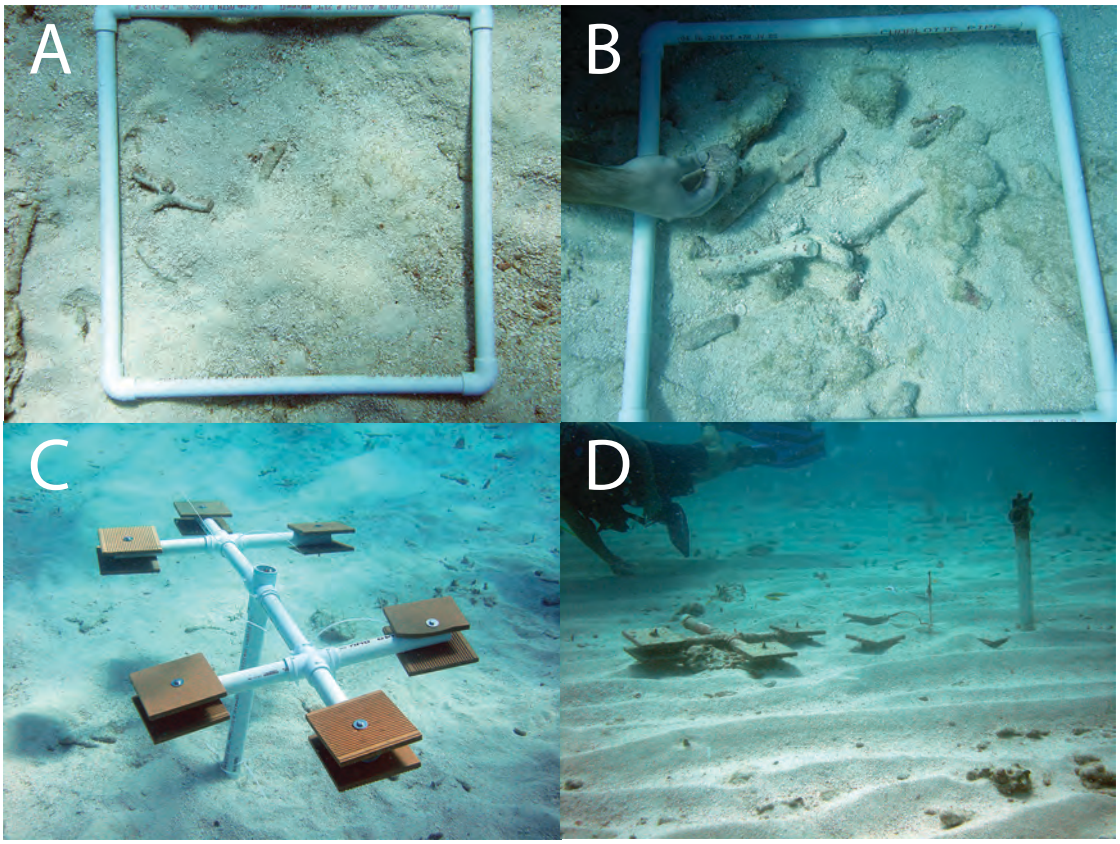

Figure 2. Photographs taken during sampling. From left to right, top row: (A)quadrat before excavation, (B) quadrat after excavation; bottom row: (C) settlement array after installation in May 2014, (D) settlement array during collection in September 2014.

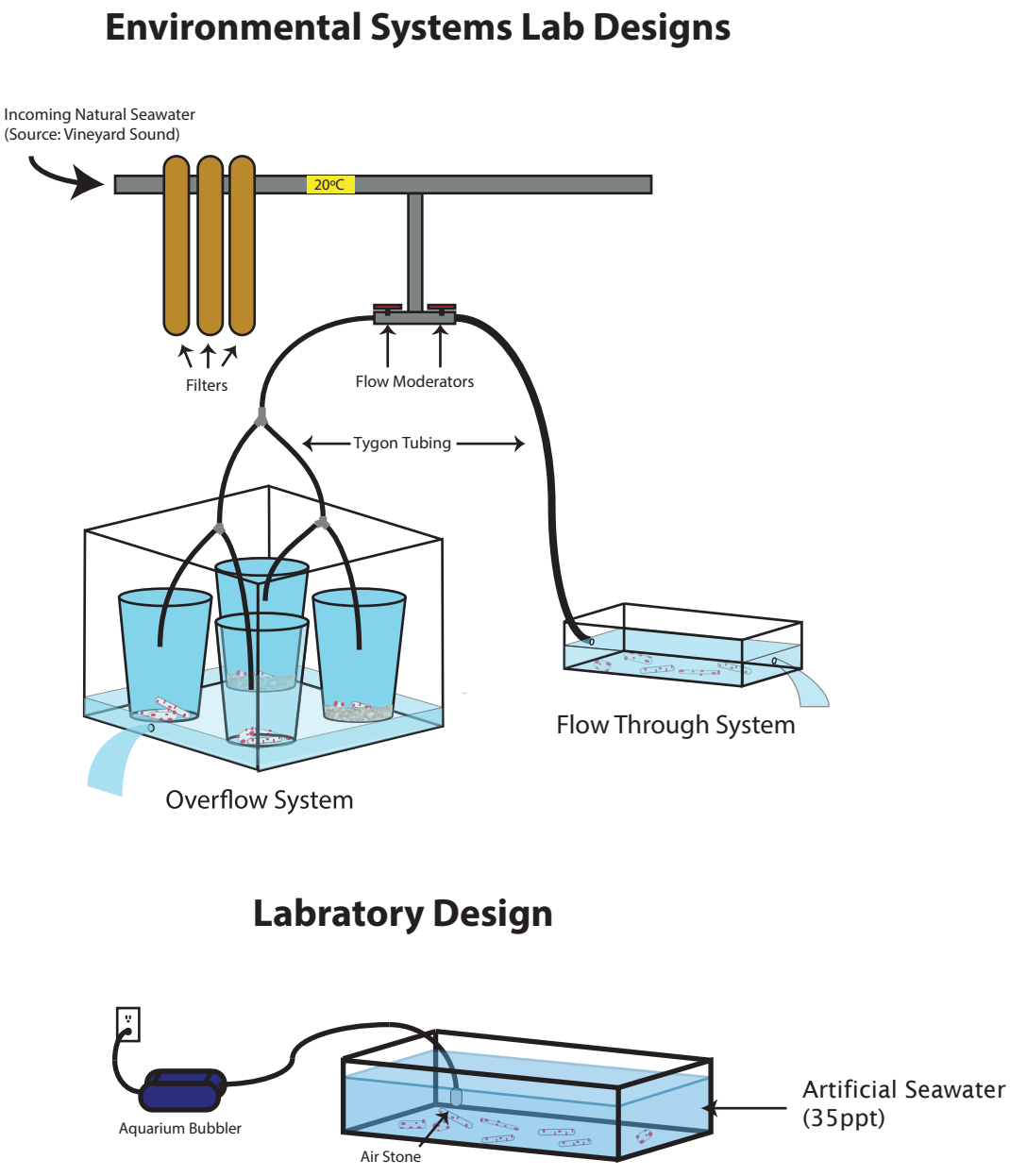

Figure 3. Three experimental designs used to maintain living *H. rubrum*. Top: two experimental set ups used at the Envinronmental Systems Lab (ESL) at Woods Hole Oceanographic Institution (WHOI) using constant replacement of natural seawater; bottom: design used in labs at WHOI and University of Georgia (UGA) kept at room temperature using artificial seawater (Instant Ocean).

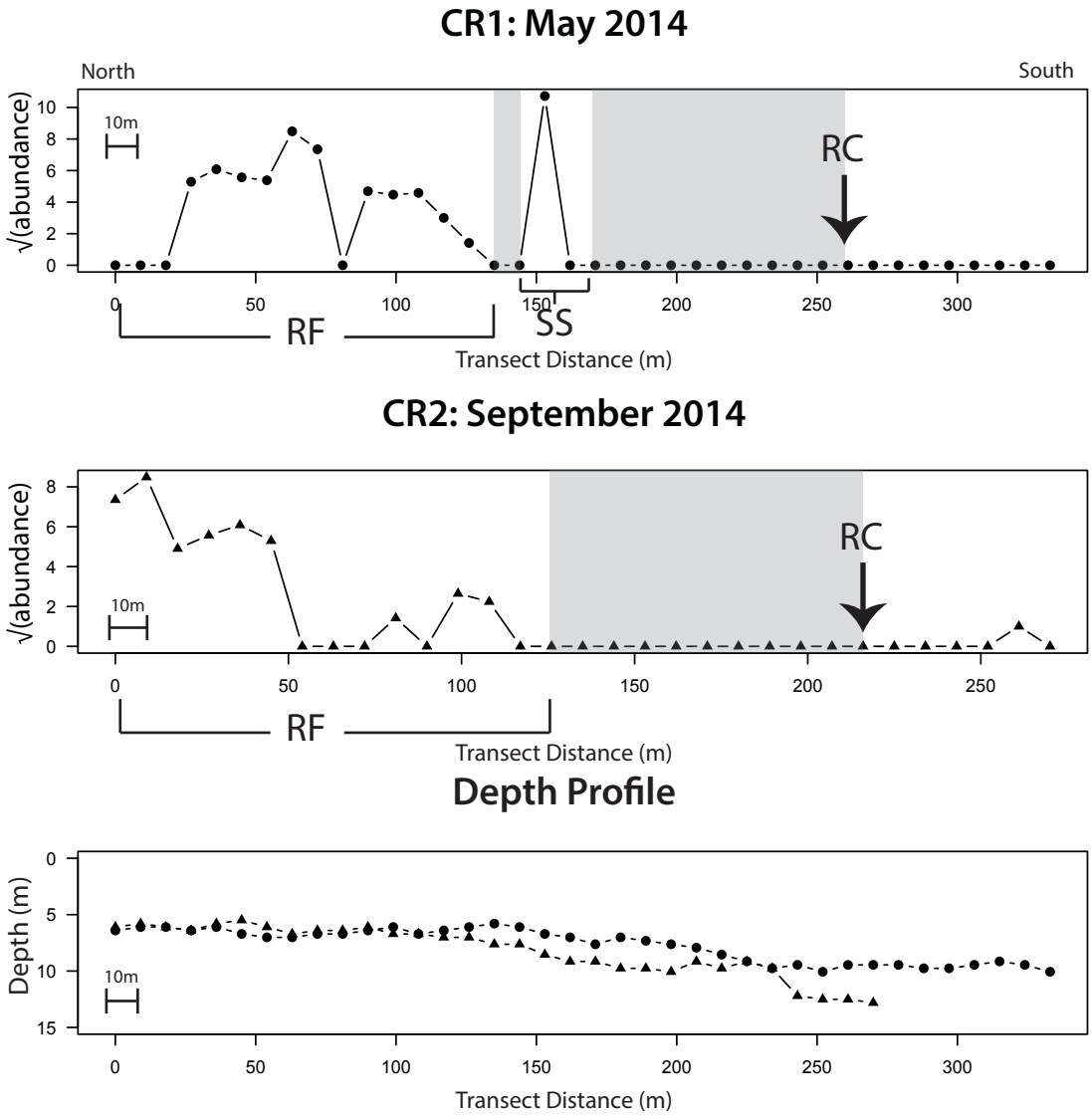

Figure 4. *In situ H. rubrum* abundance data. The square root of the *H. rubrum* abundance data for CR1 (●) and CR2 (▲) plotted against transect distance with the grey shaded area representing living reef. Bottom graph plots depth for each transect against station number. Each transect began on the reef flat shoreward of the living reef and progressed south until seaward of the reef crest. (RC) reef crest; (RF) reef flat; (SS) sand strip.

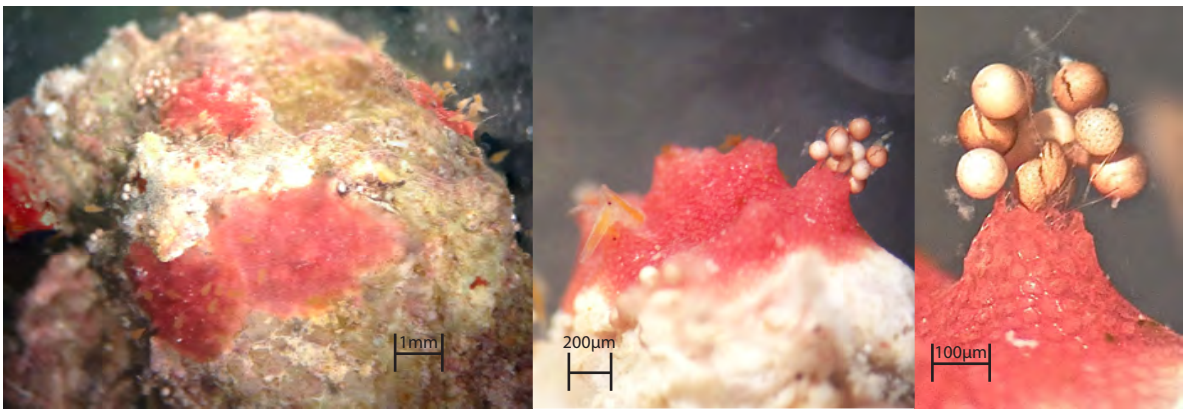

Figure 5a. Reflected light images of *H. rubrum* feeding on live *Artemia* sp. and *Artemia* sp. cysts. From left to right, five individual *H. rubrum* attached to the same shell actively feeding, knobby morphotype with one aperture (right) collecting and ingesting cysts while another caught and consumed three living *Artemia* sp. (left), fully developed teepee apertures using multiple sponge spicules in congruence with reticulopodia to trap and ingest free floating cysts.

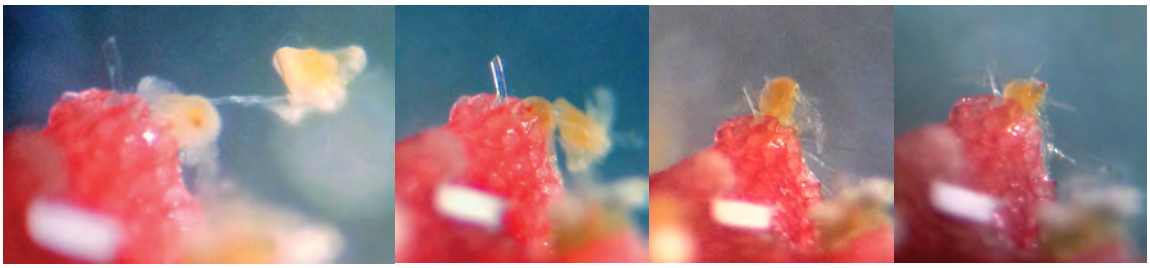

Figure 5b. Photomicrgraphs revealing the feeding process. *H. rubrum* uses sponge spicules to catch living *Artemia* sp. and reticulopodia to pull the prey into the aperture.

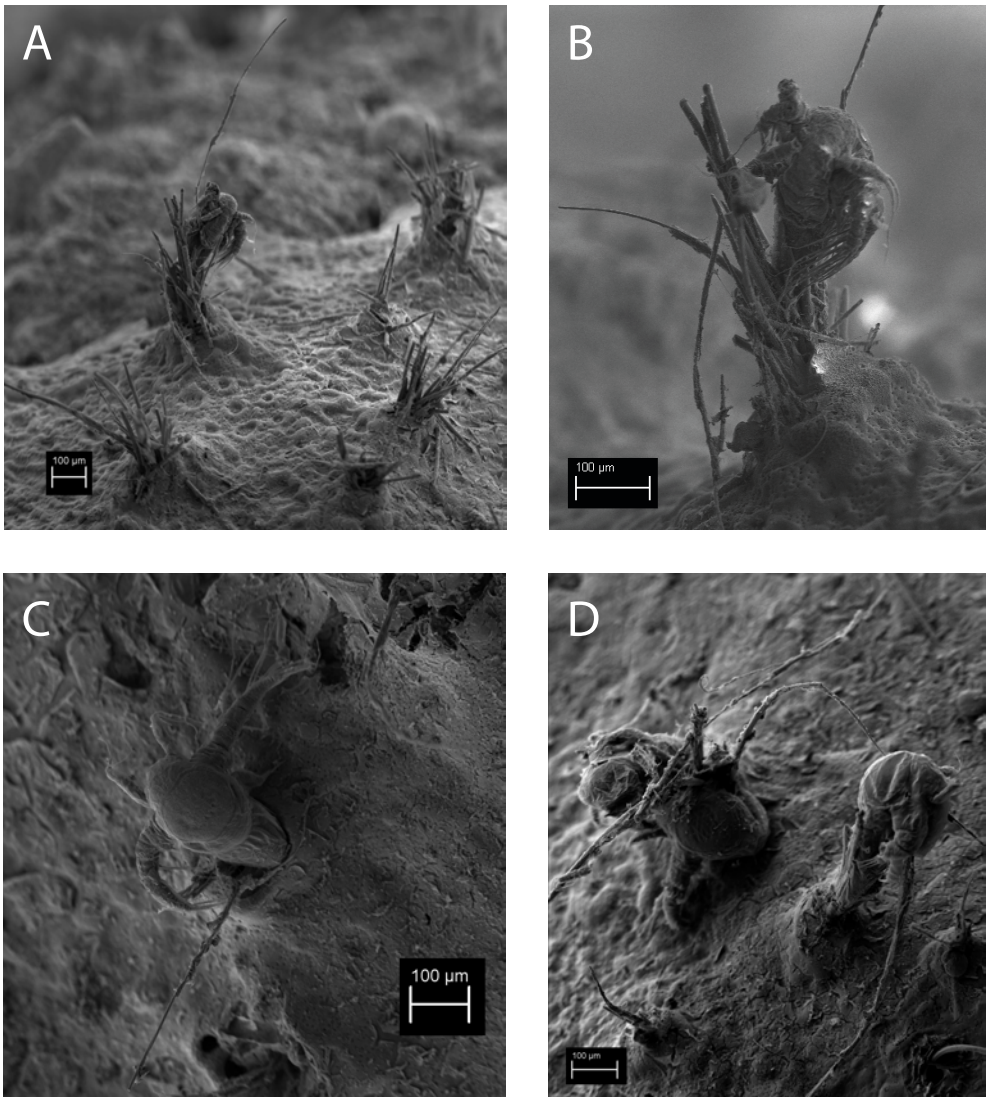

Figure 6. SEM images of feeding *H. rubrum*. In the process of catching and ingesting *Artemia* sp. From left to right; top row: (A) Multiple apertural mounds one with *Artemia* sp. caught in reticulopodial net deployed with the support of sponge spicules, (B) higher magnification image of captured *Artemia* sp.; bottom row: (C) half ingested *Artemia* sp. through newly formed aperture, (D) two *Artemia* sp. caught in close proximity in process of being ingested through separate apertures.

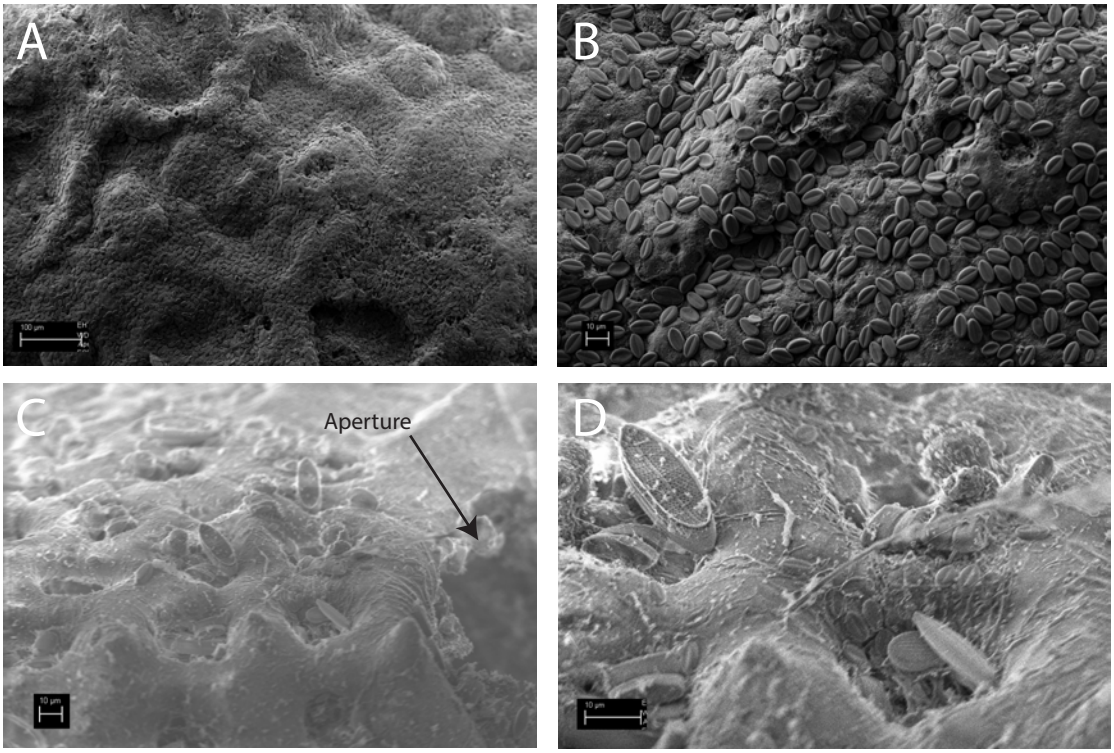

Figure 7. SEM images of the abundance of pennate diatoms. Present in large numbers both on the coral rubble and in the pores of the test of living *H. rubrum*. From left to right, top row: (A) low mag image of a collected piece of rubble coated with pennate diatoms, (B) a higher mag image of that same piece of rubble; bottom row: (C) image of the pores of a *H. rubrum* test at the edge of an aperture, (D) high mag image of pennate diatoms wedged into the pores of a *H. rubrum* test.

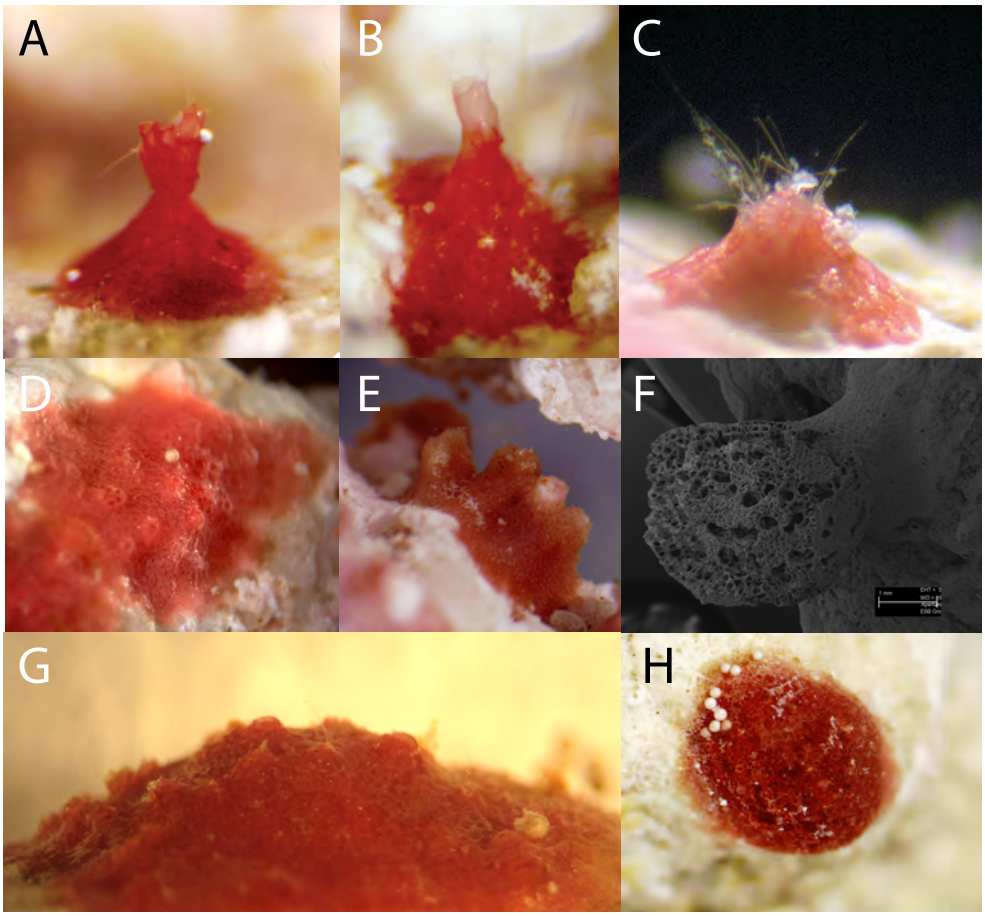

Figure 8. Reflected light and SEM images of *H. rubrum* morphologies. From left to right, top row: (A) fully developed teepee aperture, (B) newly formed translucent structure extending from an existing aperture, (C) early stage apeturial mound with loosly consolidated spicules protruding from test in multiple directions; middle row: (D) encrusting *H. rubrum* with apertures beggining to form, (E) knobby morphotype in a cryptic part of rubble piece, (F) SEM image of intensly eroded globose morphotype; bottom row: (G) an encrusting specimenwith early apertures developing becoming more hemispherical, (H) fully developed hemispherical specimen with *Artemia* sp. cysts colllected around the perifery.

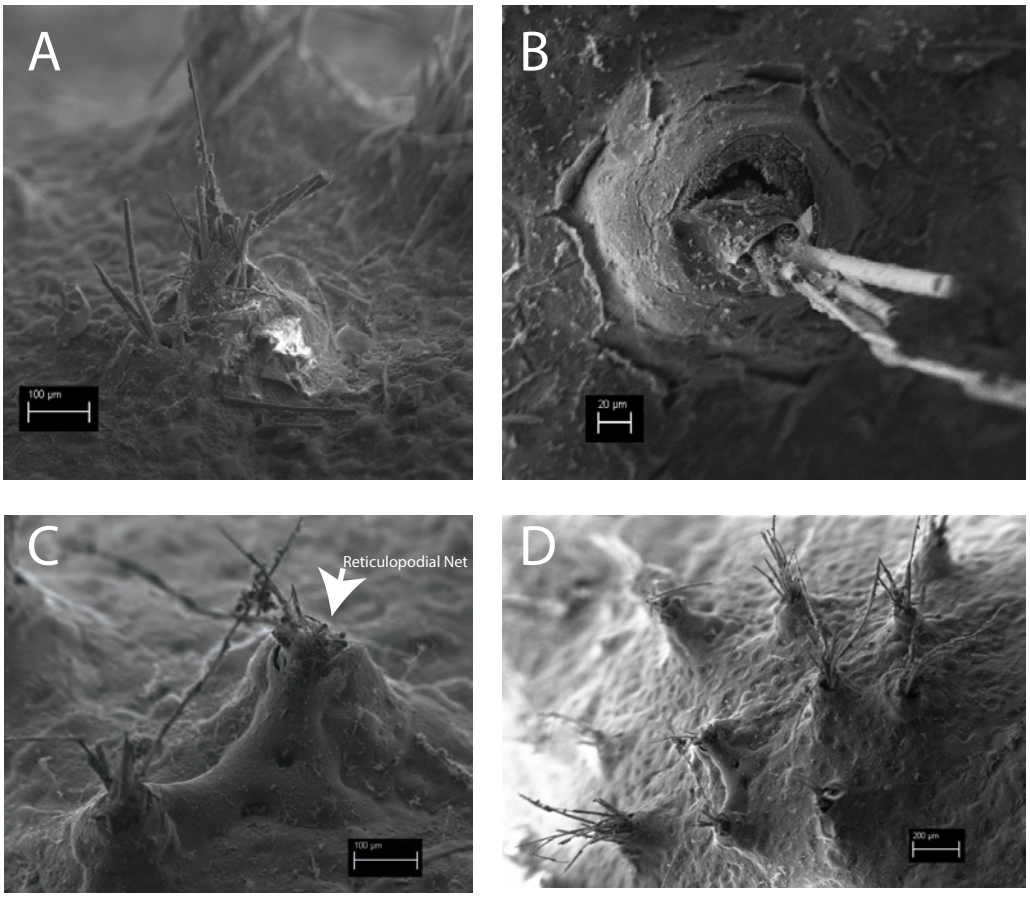

Figure 9. SEM images of newly formed *H. rubrum* apertures. From left to right, top row: (A) Early stage of teepee apertural development with spiclues loosley arranged, (B) fully developed teepee with spicules reoriented vertically; bottom row: (C) developed teepees with detritus trapped in reticulopodial net near opening, (D) 11 individiual apertures formed during the labratory experiment all from the same encrusting *H. rubrum* base.

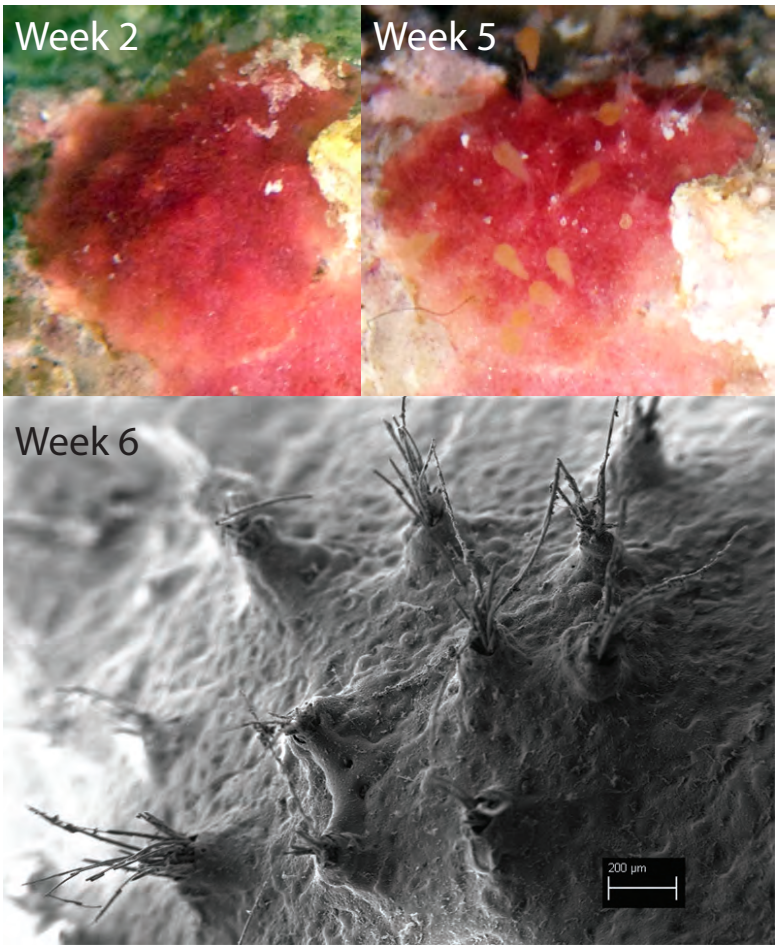

Figure 10. Photomicrographs and SEM image of one encrusting *H. rubrum.* This one specimen built numberous teepee apertures and used them to actively feed on living *Artmeia* sp. Clockwise from top left: circular apertures begin to appear on the test surface two weeks after specimen was collected from the Tennessee Reef, as many as 16 different aperturial mounds formed by week five and ten are actively feeding, SEM of fixed specimen at the end of the six week experiment with fully developed teepee apertures.

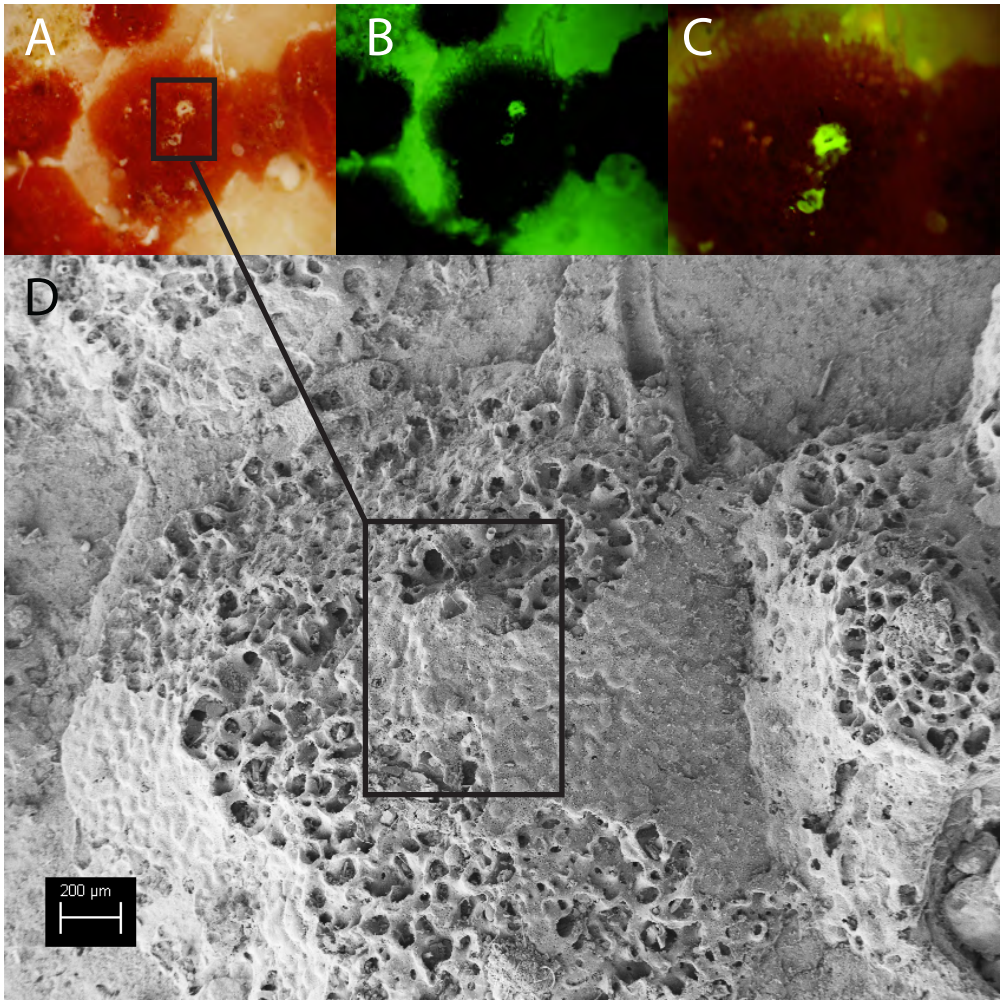

Figure 11. Calcein labeled *H. rubrum*. Clockwise from top left: (A) Low mag reflected light photomicrograph of specimen, (B) low mag image taken under epifluorescent light, (C) high mag image of specimen under epifluorecence and reflected light, (D) SEM image confirming the fluorecent calcite is part of the *H. rubrum* test.

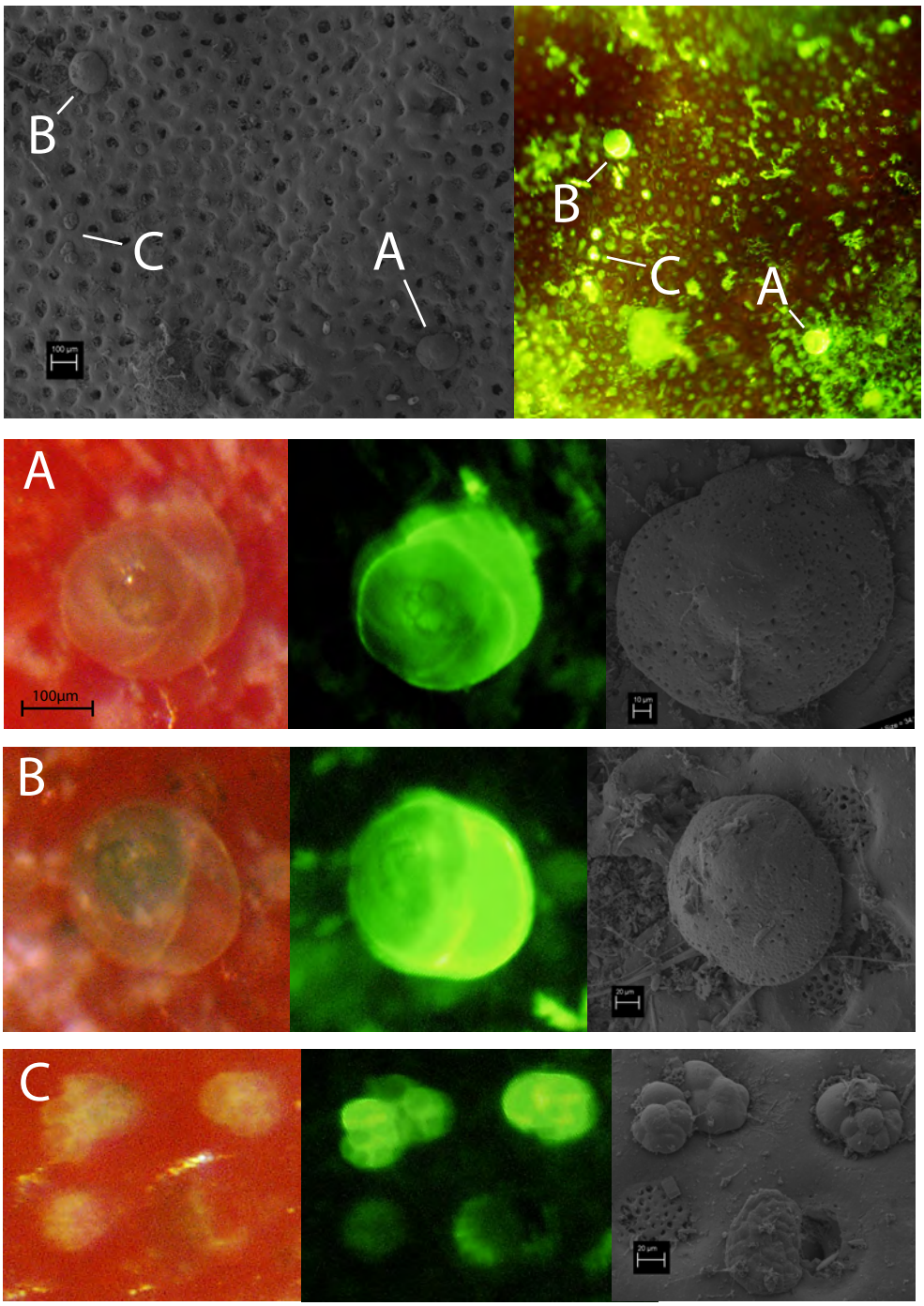

Figure 12. Reflected light, epifluorescent and SEM images of foraminiferan epibionts.From left to right, top: low mag SEM with labeled epibionts, low mag epifluorecent image of H. rubrum test with epibionts living in pores; bottom: highmag reflected light, epifluorecent and SEM images of corresponding epibionts with larger foraminifera (A and B) partially labeled and smaller (C) fully labeled.

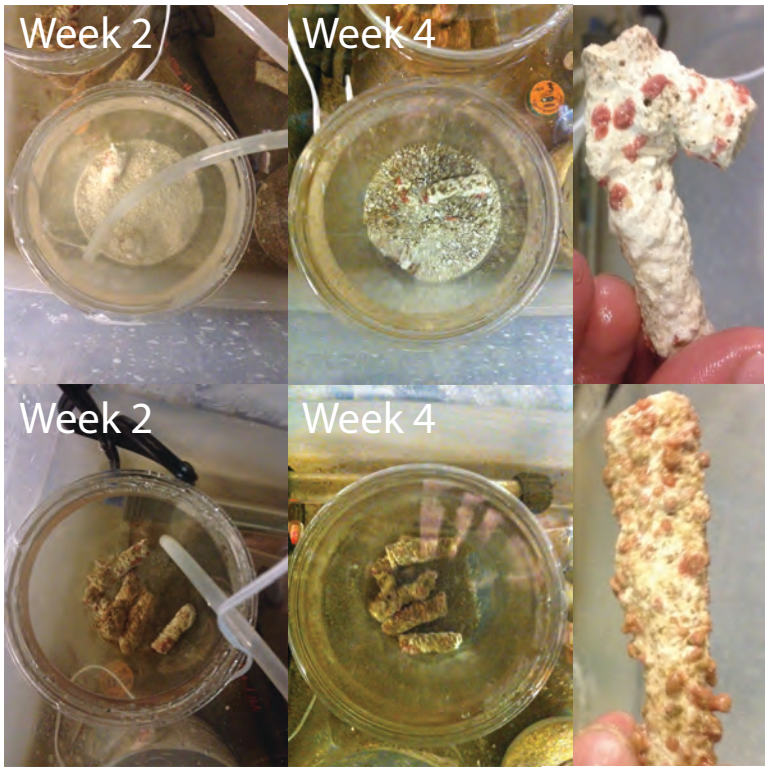

Figure 13a. Images of the two different treatments for the overflow system. Week two and week four show significantly more harmful algal growth occuring on the exposed rubble (bottom row) as opposed to the rubble buried in the sediment (top row).

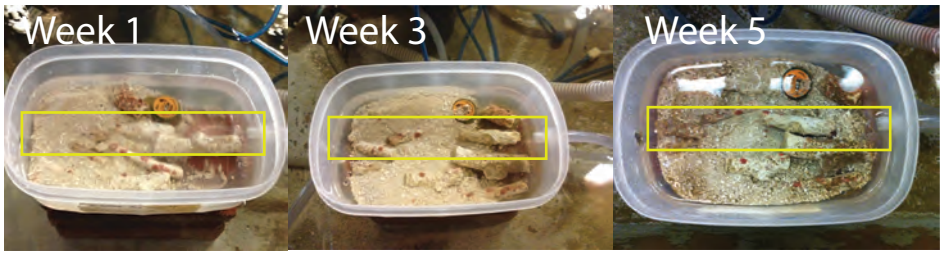

Figure 13b. Images of the flow through system. Revealing the difference in alagal growth between the rubble pieces in the areas of highest flow compared to the pieces in the less turbulent conditions. The area within the yellow box has the highest flow rate.

APPENDIX

CR 1						
Station #		Depth (ft)	Rubble	Rubble w/ Ho	Individual Ho	Notes
38	1	21	3	0	0	
37	2	20	12	0	0	
36	3	20	7	0	0	small fragmer
35	4	21	7	1	28	
34	5	20	8	3	37	Knobby and H
33	6	22	13	2	31	Live and deac
32	7	23	15	4	29	All dead (light
31	8	23	8	3	72	
30	9	22	6	2	54	From settlem
1	10	22	7	0	0	10 meters fro
2	11	21	11	3	22	
3	12	20	8	2	20	
4	13	22	2	2	21	All dead (light
5	14	21	2	1	9	
6	15	20	2	1	2	
7	16	19	10	0	0	
8	17	20	0	0	0	Live reef
9	18	22	6	2	115	Large coral ru
10	19	23	0	0	0	Live (boat rea
11	20	25	0	0	0	Live
12	21	23	0	0	0	Live
13	22	24	0	0	0	Live
14	23	25	0	0	0	Live
15	24	26	4	0	0	
16	25	28	0	0	0	Live
17	26	30	0	0	0	Live
18	27	32	17	0	0	small fragmer
19	28	31	0	0	0	Live
20	29	33	16	0	0	small fragmer
21	30	31	5	0	0	
22	31	31	0	0	0	live
23	32	31	18	0	0	
24	33	32	0	0	0	Live
25	34	32	0	0	0	
26	35	31	10	0	0	
27	36	30	0	0	0	Live
28	37	31	0	0	0	Live
29	38	33	0	0	0	

CR 2					
	Depth (ft)	Rubble	Rubble w/ Ho	Individual Ho	Notes
1	20	6	2	54	
2	19	8	3	72	
3	20	15	4	24	
4	21	12	2	31	Dead
5	19	8	3	37	
6	18	7	1	28	Live/Dead
7	20	7	0	0	Knobby and H
8	22	12	0	0	
9	21	3	0	0	
10	21	5	1	2	
11	20	5	0	0	
12	22	9	2	7	
13	22	7	1	5	Reef flat begi
14	23	0	0	0	Live
15	23	0	0	0	Live
16	25	7	0	0	
17	25	3	0	0	Live
18	28	0	0	0	Live
19	30	18	0	0	
20	30	2	0	0	Live
21	32	9	0	0	
22	32	0	0	0	Live
23	33	4	0	0	
24	30	0	0	0	Live
25	32	1	0	0	Live
26	30	3	0	0	Sand Strip
27	32	0	0	0	Live
28	40	6	0	0	Round rubble
29	41	2	0	0	
30	41	8	1	1	shell (living)
31	42	8	0	0	Round rubble

LI Transect				
Station #	Rubble	Rubble w/ Homotrema	Individual Homotrema	Notes
1	0	0	0	tiny spot of sand
2	3	1	5	
3	1	0	0	
4	0	0	0	
5	0	0	0	
6	2	0	0	one piece is a shell
7	0	0	0	
8	7	1	8	large depression with sand
9	0	0	0	
SS Transect				
1	60	10	77	
2	1	0	0	
3	4	1	12	
4	2	1	2	
5	3	0	0	
6	5	0	0	
7	3	0	0	