UNIVERSITY OF CALIFORNIA SAN DIEGO

Impacts of biotic and abiotic stressors on benthic communities at Palmyra Atoll: investigating response to invasion and bleaching at multiple ecological scales

A dissertation submitted in partial satisfaction of the
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in

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by

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The Dissertation of Amanda Lockett Carter is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

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EPIGRAPH

For whatever we lose(like a you or a me)

it's always ourselves we find in the sea.

– E.E. Cummings

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Carter AL, Edwards CB, Fox MD, Amir CG, Eynaud Y, Johnson M, Lewis LS, Sandin SA, Smith JE (2019) Changes in benthic community composition associated with the outbreak of the corallimorph, Rhodactis howesii, at Palmyra Atoll. Coral Reefs 38: 1267-1279

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ABSTRACT OF THE DISSERTATION

Impacts of biotic and abiotic stressors on benthic communities at Palmyra Atoll: investigating response to invasion and bleaching at multiple ecological scales

by

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Doctor of Philosophy in Marine Biology

University of California San Diego, 2020

Professor Jennifer E. Smith, Chair

Understanding how relatively healthy coral communities respond to disturbance provides an important baseline to which the response and recovery of more degraded ecosystems can be compared. Here, Palmyra Atoll was used as a case study to examine the response of an intact coral reef community to biotic and abiotic stressors in the form of a species invasion and bleaching event. Techniques from the fields of spatial, chemical, and microbial ecology were used to investigate the response of benthic communities to these disturbances. A decade-long, spatially robust dataset found contrasting patterns in the invasion of a corallimorph, *Rhodactis howesii*, around the atoll. Decreasing cover of *R.*

howesii was observed at the epicenter of the invasion, but contrasted sharply with increased spread and cover of the corallimorph at additional sites. Sites with high coral cover and low levels of disturbance experienced lower levels of invasion, while sites with high wave energy and lower coral cover experienced increased corallimorph growth. Competitive mechanisms of the corallimorph were examined using an assay that found evidence of bioactive chemical compounds present in *R. howesii* tissue that caused coral mortality. To further investigate the impact of invasion on coral health, 16S rRNA amplicon sequencing of coral-corallimorph interaction zones showed shifts in coral-associated microbial communities with increasing proximity to *R. howesii*. Overgrown coral tissue was dominated by bacterial families strongly associated with stress and disease, suggesting that the corallimorph can directly impact coral colonies through microbial community alteration.

Evidence of acclimatization was found by studying the response and recovery of coral-associated bacterial communities to the global stressor of the 2015-16 bleaching event. Although microbial community composition shifted as a result of bleaching, healthy symbionts were maintained in coral tissue and pathogenic bacterial invasion was not observed. These data, in combination with the low mortality at Palmyra, support the hypothesis that natural temperature variability may lead to a more resilient community in the face of thermal stress. Collectively, the results of my dissertation provide valuable insight into the response of coral communities to invasion and their ability to adapt to increasing thermal stress events over time. This dissertation also highlights the importance of long-term and spatially robust data sets that allow for accurate quantification of reef trajectories in response to disturbance events.

INTRODUCTION

Understanding the impacts and consequences of both local and global stressors has become increasingly important across a variety of habitats. One of the most biodiverse and productive ecosystems on the planet, coral reefs, are increasingly threatened at a multitude of scales. To truly understand how humans are altering reefs, we must establish baselines from which we can measure change. To date, much research has been done on shifting baselines and on establishing static states on reefs used to measure change (Knowlton et al. 2008; Sandin et al. 2008). The resilience of these ecosystems, or their ability to resist change, has been the topic of much debate and has risen to the forefront in media with the most recent global bleaching event in 2015-16. Widespread bleaching and mortality in highly protected areas (e.g. the northern Great Barrier Reef; Jarvis Island) has fueled debate regarding the efficacy of local management and the apparent lack of resilience of these intact habitats (Hughes et al. 2017, 2018; Barkley et al. 2018). However, coral reefs are dynamic systems that do not exist in a static state. Their response to change and their ability to recover from it, rather than resist it, may be a better way of measuring the state of a reef.

To date, the majority of studies have focused on local stressors such as overfishing, land-based pollution, and habitat modification (Pandolfi et al. 2003; Bellwood et al. 2004). However, biotic stressors in the form of species invasions can also result in reduced ecosystem services and habitat degradation (Elton 1958, Simberloff & Rejma´nek 2011). Ecosystem response to invasion is frequently studied in areas with confounding anthropogenic impacts due to local human populations. In combination with the long-term, large-scale monitoring efforts needed to capture trajectories of reef response, these factors

make it difficult to accurately quantify the persisitence of invasions. Degraded reefs with lower biodiversity, and thus available niches, are hypothesized to be more at risk for invasion than a fully intact system (Elton 1958; Levine & D'Antonio 1999; Stachowicz et al. 1999; 2002). However, the biotic resistance of an otherwise healthy reef can also be lowered due to disturbances that result in large-scale mortality (Norstrom et al. 2009). Pulse-disturbances (e.g. storm events, shipwrecks, disease) frequently result in open space that is readily occupied by opportunistic, invasive species. Once established, these species may persist throughout time (Marraiffini & Geller 2015), particularly in the presence of other anthropogenic impacts that alter the natural competitive dynamics on a reef.

Palmyra Atoll, a Wildlife Refuge and part of the Pacific Remote Islands Marine National Monument, is an otherwise intact ecosystem (Sandin et al. 2008; Smith et al. 2016) that has experienced invasion as a result of a shipgrounding on the reef terrace in 1991 (Work et al. 2008). A bloom of the corallimorph, *Rhodactis howesii*, was oberved in the vicinity of the shipwreck and has since expanded to sites up to 6km away from the epicenter (Chapter 1, Carter et al. 2019). Portions of the reef that were previously dominated by hard coral have reached up to 100% cover of the corallimorph despite the atoll's high levels of protection and minimal human influence. Work et al. (2008) hypothesized that iron leaching from the shipwreck was releasing a limiting nutrient into the oligotrophic waters of the atoll and facilitating the invasion of the corallimorph. In light of this, the wreck was removed from the reef terrace in the winter of 2013, creating a unique opportunity to study the recovery of a reef post-disturbance and invasion without additional confounding stressors.

Chapter 1 examines the response of the benthic communities at Palmyra to the corallimorph invasion using a data set from 2010-2017. These long-term and spatially robust data also allowed for investigation of trajectory and persistence of the corallimorph population around the atoll. Interestingly, two distinct patterns in the benthic community response were found at the wreck site and other locations around the atoll. Significant decreases in the corallimorph were observed at the wreck site following removal of the shipwreck, resulting in open space that was quickly settled by turf and crustose coralline algae (CCA). Turf communities were grazed down by healthy herbivore populations at Palmyra (Hamilton et al. 2014), allowing CCA and juvenile corals to occupy much of the space that was once dominated by corallimorph. In contrast, some sites further from the wreck showed increasing cover of the corallimorph, particularly in areas with higher wave action (Gove et al. 2015). However, the corallimorph was unable to proliferate at sites with high coral cover, suggesting that intact reef communities are less at risk. This study was one of the first to track the long-term changes in a benthic community associated with an invasion in the absence of other stressors. Although there was an observed decrease in corallimorph cover at the wreck site, it's continued growth in areas with higher levels of natural disturbance warranted further investigation and potential removal and restoration efforts.

The ability of corallimorphs to proliferate and replace hard corals and other benthic competitors has been studied in a variety of locales including the Red Sea and Tanzania (Kuguru et al. 2004, 2007, 2010; Muhando et al. 2002). Although their aggressive nature and ability to overgrow corals has been documented, the actual competitive mechanism of the

corallimorph was unknown. In Chapter 2, I investigated the potential for allelopathy, or chemical warfare, in interactions between the corallimorph *R. howesii* and a reef-building coral, *Acropora yongei.* Chemical extracts from the corallimorph were placed in contact with *A. yongei* to look for evidence of coral mortality in the absence of the physical presence of *R. howesii*. Initial results showed clear evidence of corallimorph-derived allelopathy and paved the way for further work to investigate which compounds were responsible for the observed coral mortality. Non-polar extracts of the corallimorph were the most bioactive, similar to those found in a chemically-active macroalgae and speonges (Pawlik et al. 2007; Rasher et al. 2011). High performance liquid chromatography (HPLC) and proton nuclear magnetic resonance spectroscopy (^{1}NMR) were used to identify a compound present in the most bioactive fraction, a carotenoid Peridinin. The results from the Chapter 2 conclusively demonstrate the chemical activity of the corallimorph, *R. howesii*, and provide insight into the competitive mechanisms used in the invasion at Palmyra.

Exposure to chemical-rich seaweeds has been shown to alter the microbial composition of corals in prevous studies, at times resulting in coral tissue or colony death (Smith et al. 2006; Kline et al. 2006). Given the chemical activity of the corallimorph that I observed, Chapter 3 investigated how invasion impacted the coral microbial communities *in situ* across five different species of coral. Specifically, I looked for changes in the microbial communities in response to proximity and overgrowth of the corallimorph and examined differences in species response and survival over time. A large sampling effort was conducted in 2015-16 to track the changes in coral microbial communities during invasion. Individual coral colonies were tagged and samples were taken across their interaction with

the invader and tracked over time. I found that corals harbor species-specific microbial communities in their healthy tissue, but that the bacterial composition (as measured through increases in alpha and beta diversity) became more similar to one another with proximity to the corallimorph. In fact, invaded tissue across all coral species was no longer significantly different in bacterial diversity and composition. Interestingly, I also observed invasion by pathogenic microbes in coral species that demonstrated the lowest survivorship during the sampling time period. Coral species that suffered low mortality and showed the highest resistance to the corallimorph also had fewer changes in the microbial diversity and community. These data showed that the corallimorph directly impacted coral microbial communities during invasion, and at times, caused an increase in relative abundance of potentially pathogenic bacteria.

In Chapter 4, I shifted focus from the local stressor of the corallimorph invasion to the global stressor of the 2015-16 bleaching event and its impact on the coral microbial communities at Palmyra. Fox et al. (2019) found that coral mortality on Palmyra was surprisingly low despite widespread bleaching across both the reef terrace and fore reef. There was no change in coral cover on the reef terrace and less than 10% loss on the forereef despite ~20 weeks of temperatures above the bleaching threshold. Fox et al. hypothesized that natural temperature variability on the shallow reef terrace (~5m) had faciliated adaptation to thermal stress, explaining the minimal mortality observed. The coral probiotic hypothesis states that coral bacterial communities can rapidly respond to environmental conditions, providing a mechanism for rapid acclimatization in long-lived coral communities (Reshef et al. 2006). Physical stressors have been shown to alter this microbial community,

allowing for invasion by opportunistic and potentially pathogenic bacteria that can prove detrimental to coral health (Bourne et al. 2008; Thurber et al. 2009). I investigated microbial community response of five different species of coral at Palmyra both during and after the 2015 global bleaching event. Survivorship of all sampled colonies was tracked and no complete mortality was observed. Interestingly, the microbial communities of all sampled corals stayed significantly different from one another throughout time, although their composition did shift as a result of bleaching. However, all corals sampled maintained high levels of beneficial symbionts (*Endozoicomonas*) and resisted invasion by pathogenic bacteria such as *Vibrios*. Collectively, these data support the hypothesis that the natural temperature variability on the reef terrace has led to acclimatization of the coral community, potentially rendering it more resilient in the face of prolonged thermal stress.

The results of this dissertation contribute to our understanding of how an intact reef ecosystem can respond to stressors at both the local and global scale. These studies highlight the dynamic nature of coral reefs and show that intact habitats are not necessarily resistant to change in the face of stressors, but are more readily able to recover post-disturbance.

CHAPTER 1

Changes in benthic community composition associated with the outbreak of the corallimorph, *Rhodactis howesii*, at Palmyra Atoll

Amanda L. Carter, Clinton B. Edwards, Michael D. Fox, Corinne G. Amir, Yoan Eynaud, Maggie D. Johnson, Levi S. Lewis, Stuart S. Sandin, Jennifer E. Smith

ABSTRACT

Few studies have documented the spatial and temporal dynamics of highly invasive species in coral reef benthic communities. Here, we quantified the ecological dynamics of invasion by a corallimorph, *Rhodactis howesii* at Palmyra Atoll in the central Pacific. A localized outbreak of this species was first observed following a shipwreck at Palmyra in 1991 and has subsequently spread across hectares, reaching 100% cover in some areas. We examined the spatial and temporal dynamics of this invasion, and its impact on the benthic community, using a combination of permanent photoquadrats and large-scale photomosaic imagery. Our data revealed two distinct patterns in the spatial dynamics of *R. howesii* on the reef. First, following the removal of the shipwreck in 2013, the cover of the corallimorph in the immediate vicinity of the wreck has decreased markedly, with crustose coralline algae (CCA), an important reef-builder, now dominating the newly available substrate. However, in contrast to the decline at the epicenter of the invasion, the corallimorph has spread to additional sites around the atoll where increases in abundance have been associated with decreases in hard coral cover. Reductions in percent cover and corallimorph patch size near the epicenter of the outbreak, coupled with increases in cover and patch size and appearance of the corallimorph at other locations around Palmyra, demonstrate the dynamic nature of this "invasion." Further, we found that the corallimorph settled disproportionately often on patches of turf or CCA cover, but can then overgrow all benthic competitors following establishment. This study provides evidence that *R. howesii* has the capacity to be highly invasive on coral reefs and highlights the importance of large-scale, long-term monitoring efforts to capture the dynamic nature of such invasions.

INTRODUCTION

Species invasions can rapidly transform the structure and function of ecosystems, resulting in the loss of biodiversity and productivity, thus impoverishing ecosystems and the human populations that rely on them (Elton 1958, Simberloff & Rejma´nek 2011). While mobile, invasive predators such as fishes (Pringle 2011) and reptiles (Wiles et al. 2003) are known to transform communities via top-down mechanisms, sessile invaders such as mussels (Nalepa & Schloesser 2013), clams (Kimmerer et al. 1994), and algae (Boudouresque et al. 1995) transform ecosystems via bottom-up dynamics and direct habitat modifications. On coral reefs, invasion by fleshy, non-calcifying organisms directly undermines the structural integrity and complexity of the ecosystem's carbonate foundation, resulting in numerous indirect negative impacts (Perry et al. 2008). For example, reef habitat with high corallimorph cover can shift from net accretion to net dissolution, resulting in a loss of overall ecosystem function (Takeshita et al. 2016). Though the effects of fleshy algae have received the greatest attention on coral reefs, similar impacts of non-calcifying sessile animals (e.g., sponges, tunicates, and certain anthozoans) warrant equal attention (Nörstrom et al. 2009).

Community transitions are often catalyzed by large-scale coral mortality resulting from pulse disturbances (Nörstrom et al. 2009) or from species invasions (Conklin and Smith 2005). However, less is known about the stability and long-term effects of these transitions due to lack of spatiotemporal data needed to accurately quantify successional dynamics and changes in community composition. The persistence of species invasions (here defined as the over-proliferation of a species that may or may not be native to the region of study *sensu,* [Warren 2007]) or outbreaks is difficult to assess without long-term monitoring. Here, we make use of a long-term and spatially extensive dataset collected at Palmyra Atoll from 2010-2017 to examine

the spread of a corallimorph, *Rhodactis howesii*, and related changes in benthic community composition.

Corallimorph species are exceptional benthic competitors on coral reefs that can rapidly invade and colonize open space (Chadwick-Furman & Spiegel 2000; Muhando et al. 2002). They have a variety of life history strategies and behavioral characteristics that contribute to their competitive success. For example, corallimorphs are able to reproduce sexually and asexually via spawning, budding, clonal replication and fragmentation (Chadwick & Adams 1991; Chadwick-Furman & Spiegel 2000) and population sizes can double in as little as two months (Chadwick and Adams 1991). In the Red Sea, *Rhodactis spp.* can tolerate high temperature and irradiance, which affords an additional competitive advantage in shallow back reef environments (Kuguru et al. 2004; Kuguru et al. 2007). Additionally, the corallimorph *R. howesii* possesses large nematocysts that have been known to cause significant damage to living coral tissue, enhancing their capacity to outcompete corals and other benthic organisms (Work et al. 2008).

Corallimorphs, and *R. howesii* in particular, have been shown to be aggressive competitors and outbreaks have been documented on some coral reefs (Muhando et al. 2002; Work et al. 2008; Crane et al. 2016). Here, we focus on the outbreak of *R. howesii* at Palmyra Atoll, a National Fish and Wildlife Refuge and part of the Pacific Remote Islands Marine National Monument in the central Pacific. Due to its protected status, Palmyra's reefs are relatively intact, with high cover of reef-building organisms (Smith et al. 2016) and healthy herbivore populations (Sandin et al. 2008; Edwards et al. 2014). The reefs have had minimal human impact due to the lack of a permanent population, aside from a brief occupation by the US military during WWII. However, despite its high level of protection over the last 17 years, a portion of the reef on the shallow terrace has undergone a shift in community structure from a

coral to corallimorph-dominated state (Work et al. 2008). This shift was associated with a localized disturbance caused by the grounding of the *Hui Feng,* a longline fishing vessel, in 1991. *R. howesii* was initially observed in low abundances at the site of the shipwreck and was generally rare at other sites around the atoll (Work et al. 2008). However, by 2005, the corallimorph population at the wreck site (hereafter referred to as the "Longliner site") had rapidly expanded, covering over 2km^2 of the reef (Work et al. 2008). While there was no clear link between the wreck and the corallimorph outbreak, the rapid spread and alteration of the benthic community was of particular concern to management agencies. Work et al. (2008) hypothesized that iron leaching from the wreck facilitated corallimorph growth via nutrient input and that removal of the wreck would assist in recovery of the benthic community. In light of this, the U.S. Fish and Wildlife Service removed the shipwreck in the winter of 2013 in an effort to reduce the impact of the corallimorph and facilitate reef recovery. This provided a unique opportunity to study the changes in the spatial and temporal trajectories of the corallimorph and recovery of the benthic community associated with the wrecks removal.

The goals of this study were to characterize the spatial and temporal variability in corallimorph abundance and corresponding changes to the benthic communities over a nine-year time window. Specifically, we sought to 1) document the spatial distribution of the corallimorph around the island, 2) examine changes in corallimorph cover and overall benthic community composition, 3) investigate initial establishment and subsequent spread of corallimorph invasions, and 4) explore the large-scale spatial dynamics of corallimorph patches throughout time. The results shed light on the impacts of corallimorphs on benthic coral reef communities, their response to shipwreck removal, and temporal trends in corallimorph dynamics at permanent

sites around the atoll. This information can inform future management efforts aimed at monitoring and limiting the expansion of this coral reef invader.

MATERIALS AND METHODS

Distribution of R. howesii at Palmyra Atoll

We used records and images of the occurrence of *R. howesii* at Palmyra from 2009-2017 to document the spatial extent of the population. These data included 27 one-time surveys from a monitoring expedition in 2010, permanent sites established at Palmyra between 2009-2012 (details below), and towed-diver surveys following protocol outlined in Work et al. 2008. Further details of the monitoring sites and methods can be found in Table 1.1.

Changes in benthic community composition

Changes in benthic community composition around Palmyra Atoll were examined using permanent photoquadrat transects established in 2009 at 4 forereef sites (10 m depth; FR3, FR5, FR7, FR9) and 4 sites on the shallow reef terrace (5 m depth; RT1, RT4, RT10, RT13; (Figure 1.1). An additional backreef site (Penguin Spit Middle, PSM) was established in 2010. Transects were 50 m in length and parallel to shore with ten permanent 0.63 m^2 photoquadrats positioned every 5 m along the transect and marked with stainless steel eyebolts. Each site was established with one, 50 m transect. The permanent sites were surveyed annually, and the results reflect data collected between late August to early October for each year to minimize any seasonal variation in the benthic community. Percent cover was determined using the image analysis software *Photogrid 1.0* (Barrott et al. 2012; Smith et al. 2016). Photographs were analyzed by overlaying

100 stratified random points across each picture and identifying the substrate under each point to the finest taxonomic resolution possible (genus for corallimorph, hard corals and macroalgae, functional group for crustose coralline algae (CCA), turf algae, and other invertebrates). Points were then kept consistent from year-to-year to examine variability in percent cover of common benthic functional groups and the corallimorph over time.

To examine changes at the epicenter of the outbreak, additional study sites were established in 2013 around the Longliner wreck prior to its removal by the USFWS. Four permanent transects (as described above) were installed along the cardinal directions (N, E, S, W) radiating out from the Longliner wreck itself. Plots were photographed annually and analyzed as described above. To examine changes in the percent cover of corallimorph and other benthic organisms, data from the 4 transects were combined to provide site averages of the dominant functional groups (corallimorph, hard coral, CCA, macroalgae, and turf algae) during the monitoring period from 2013-2017.

Initial establishment of R. howesii on benthos

We examined the initial stages of corallimorph establishment to better understand the invasion process. To determine if the corallimorph settled on certain benthic functional groups more frequently than would be expected by chance (based upon the abundance of each group), we recorded the functional group that each individual corallimorph polyp settled upon (hard coral, CCA, macroalgae, turf algae, or soft coral) for every permanent, invaded plot. The proportions of expected settlement substrate were calculated using functional group abundance for each plot. We then used a chi-squared analysis (R 2.15.1 (R Core Team 2016) to compare the

observed and expected settlement substrate values to determine preference or avoidance of certain benthic functional groups.

We then assessed the ability of the corallimorph to overgrow different benthic functional groups. To accomplish this, we identified each of the randomly stratified points used in the photoquadrat analysis (point locations were held constant through time) that were recorded as corallimorph. We then tracked each point back through time to identify what the point was in the year prior to transitioning to corallimorph. This showed which taxa could be overgrown by the corallimorph and how often it successfully displaced each taxa.

Changes in corallimorph patch dynamics throughout time

Because of the clonal nature of *R. howesii,* we were interested in measuring how patch dynamics changed over time at a scale larger than what was observed in the photoquadrats. To investigate this, we used large-area imagery (photomosaics) to survey swaths of reef annually from 2012-2016. In 2012, a 200 m^2 site was established adjacent to the Longliner wreck. In 2013, 16 replicate 100 m^2 photomosaic sites were established along the 10 m isobath of the forereef habitat across the north and south shores of the atoll. All plots were established with two stainless steel pins marked by GPS to ensure that the same area of the reef was photographed at each survey. Briefly, a single mosaic consisted of approximately 5000 individual images taken over a 45-60 minute dive (Edwards et al. 2017). Analytical processing of the mosaic imagery was conducted using custom algorithms designed by Edwards et al. 2017 in R 3.2 (R Core Team 2016). Further details regarding the collection and processing methods can be found in previous publications (Gracias and Santos-Victor 2000, 2001; Lirman et al. 2007, Edwards et al. 2017).

Changes in corallimorph patch size (defined here as spatially continuous aggregations of corallimorph polyps) were digitized in Adobe Photoshop and data were analyzed for the Longliner site and the forereef site (FR5) where initial invasion and subsequent growth was observed (as described above). For each survey year (2012-2016), imagery was uploaded to Adobe Photoshop Creative Cloud and the boundaries of all contiguous corallimorph patches were digitized by hand using a Wacom pen-tablet (model # CTH-470). The digitized patches were then exported from Photoshop as a single .PNG image file for each year, from each site. The total number of patches, the size of each patch, and total spatial cover were calculated using R 2.15.1 (R Core Team 2016). Significant changes in patch size across years at each site was analyzed using a Kruskal-Wallis Rank Sum test as the strongly right skewed data prevented the use of standard parametric approaches. All statistical analyses for the study were conducted in R 2.15.1 (R Core Team 2016).

RESULTS

Distribution of R. howesii at Palmyra Atoll

Based upon atoll wide surveys from 2009-2017, the corallimorph was widely observed across the shallow reef terrace, particularly around the Longliner wreck site, with continued spread south across the channel toward the back reef site (PSM) and across the southern forereef (Fig. 1a-b). Data from photomosaic sites across the atoll showed distant populations of the corallimorph on the forereef up to 6 km away from the invasion epicenter. *R. howesii* was virtually ubiquitous along the southern forereef, where it was observed at 7 of the 9 sites. In contrast, *R. howesii* was observed at only 4 of 13 sites along the northern forereef and was completely absent at the 2 eastern most (162º0) and 3 western most sites (162º9) (Figure 1.1).

However, due to logistical difficulties reaching those sites, they have not been re-surveyed since 2010 and it is possible that the corallimorph has now reached the tips of the atoll.

Changes in benthic community composition

The forereef site FR3 had 1 % cover of *R.* howesii when first surveyed in 2009. Corallimorph cover at the site peaked in 2014, reaching a max of 15 % cover that has since dropped to 6 % in 2017 (Figure 1.2c). Hard coral cover has remained high at FR3, with an average of 56.1 ± 1.7 % (mean ± SE) (Figure 1.2a) from 2009-2017. Percent cover of CCA remained relatively stable, with an average of 15.2 ± 1.2 % at the site from 2009 - 2017 (Figure 1.2b). In contrast, percent cover of turf algae has decreased at FR3, dropping from a high of 14.9 ± 2.7 % in 2015 to a low of 7.3 ± 1.5 % in 2017 (Figure 1.2d).

In contrast to FR3, the corallimorph was not recorded in the first survey of site FR5 in 2009, but was present in 2010. *R. howesii* cover peaked at 27.6 ± 8.3 % in 2016 and dropped to 24.6 ± 8.3 % in 2017 (Figure 1.2c). Hard coral cover steadily decreased at the site from a high of 24.2 ± 4.4 % in 2009 to a low of 11.7 ± 3.1 % in 2016. A slight increase in hard coral cover was observed in 2017, reaching 13.4 ± 3.8 %. CCA and turf algal cover also decreased throughout the monitoring time period (Figure 1.2f). CCA peaked at 44.1 ± 3.2 % in 2010 and has since dropped to 27.4 ± 5.9 % in 2017. Turf algae decreased from 16.4 ± 2.5 % to 8.1 ± 3.1 % from 2009-2017.

Initial invasion of corallimorph in established plots was observed at the reef terrace site RT10, which was established in 2009 with 0 % cover. *R. howesii* invaded a single plot in 2014, peaked with 22 % cover in 2015, and has since decreased to 9 % cover in 2017. Hard coral cover

was highest at RT10 for all sites, with an average of 59.1 ± 1 % from 2009-2017. CCA and turf algae decreased at the site, dropping from 13.3 ± 2.2 % to 9.6 ± 2.3 % and from 29 ± 6.4 % to 15.7 ± 4.2 % respectively (Figure 1.2). In contrast, macroalgae increased in percent cover at the site from 6.6 ± 1.7 % in 2009 to 18.3 ± 3.7 % in 2017.

The back reef site, PSM, was the only site with a continual increase in corallimorph cover throughout time. Initial percent cover was 19 ± 6.7 % in 2010 and increased to 43.3 ± 11.1 % in 2017. Hard coral cover remained consistent at the site, with an average of 20.1 ± 0.7 %. CCA increased in percent cover from 9.2 ± 1.9 % in 2010 to 27.6 ± 6.5 % in 2017. In contrast, turf and macroalgal cover declined over the monitoring period. Turf algae had an initial cover of 42.4 ± 7.3 % in 2010 and a final cover of 6.9 ± 2 % in 2017. Macroalgae decreased from 27.7 ± 6.3 % in 2010 to 11.8 ± 2.4 in 2017.

At the Longliner site, cover of the corallimorph decreased drastically from an average of 68.5 ± 2.3 % in 2013 to 1.5 ± 0.3 % in 2017 (Figure 1.3c). Cover of CCA increased the most during the monitoring period, starting at 8 ±1.2 % in 2013 and rising to 49.3 ± 1.6 % by 2017 (Figure 1.3b). Turf and macroalgae showed a general increase until 2016 where they both peaked at 30.1 ± 3 % and 28.8 ± 1.1 %, respectively (Figure 1.3d-e). Since 2016, both turf and macroalgae have dropped in percent cover to 17.7 ± 1.5 % and 24.7 ± 1.1 %, respectively.

Initial establishment of R. howesii on benthos

Upon initial invasion of a given plot, *R. howesii* polyps disproportionately colonized CCA and turf algae, despite accounting for abundance of these functional groups (Figure 1.4). Out of the 23 instances where a polyp landed on previously unsettled substrate, 11 settled on

CCA, 8 on turf, and 4 on macroalgae. Further, the corallimorph significantly avoided settling on hard coral and macroalgae (χ^2 =16.94, p-value 0.0007). However, once the corallimorph was established in a plot, it overgrew all other functional groups. A total of 836 transitions were documented from one taxon to corallimorph between 2009-2017 in the monitoring plots. Of those transitions, hard coral and CCA were overgrown 15.6 % and 23.3 % of the time, respectively. Turf and macroalgae were overgrown 30.9 % and 30 % of the time. In contrast, soft coral represented only 0.24 % of the transitions, making it the least overgrown functional group in our data set.

Changes in corallimorph patch dynamics throughout time

The percent cover of corallimorph in the Longliner photomosaic site declined from 50 to 10% (Figure 1.5) between 2012 and 2016, while the number of patches increased by an order of magnitude (232 to 2398). In contrast, the mean patch size decreased from 0.6 m^2 in 2012 to 0.2 m^2 in 2016, with significant decreases between 2012 and 2014, 2014 to 2015, and 2015 to 2016 ($p < 0.001$, Table 1.2, Fig. 6). However, at the more distant forereef site FR5, corallimorph cover increased from 5 to 23% between 2012 and 2016. The increase in cover was accompanied by a significant decrease in mean patch size from 2012 to 2014 ($p<0.001$, Table 1.2), though patch size has not significantly changed since 2014. The number of patches increased 143 to 636 from 2012 to 2014 (Figure 1.5).

DISCUSSION

Ecological dynamics of marine invasive species and their impact on benthic communities are often difficult to assess, as they require data spanning large spatial and temporal scales. Previous studies have documented large-scale, rapid changes in benthic community composition from hard coral to macroalgal cover (Done 1992; Hughes 1994; McCook 1999; Côté et al. 2005). However, few studies have examined less common community transitions where corals are replaced by fleshy-invertebrate taxa such as soft corals, anemones, sponges, or corallimorphs (Nörstrom et al. 2009; Kuguru et al. 2010; Elliott et al. 2016). Additionally, data on the impacts of these outbreaks or invasions on the associated benthos are limited because pre-invasion community structure is often unknown. Here, we present detailed information on the spatial and temporal dynamics of the invasive corallimorph *R. howesii* and associated impacts on the benthic reef communities of Palmyra Atoll. We show contraction of the population at the initial site of invasion, as well as expansion and spread of the corallimorph at more distant locations around the atoll. Further, while this species more readily invaded space occupied by CCA and turf algae, it appears able to overgrow all other benthic functional groups once established, highlighting *R. howesii's* invasive nature.

Interestingly, some sites with high coral cover were more resistant to invasion than others, despite proximity to the initial invasion epicenter,. For example, the forereef site just south of the Longliner wreck (FR3, Figure 1.1, mean hard coral cover: 56.1 ± 1.7 %), was established in 2009 and had a single corallimorph polyp in one quadrat along our transect. By 2014, 15 polyps were present in the quadrat and CCA was the only functional group to decline as the corallimorph increased at the site (14.6 ± 1.6% to 11.4 ± 2%). However, by 2017, the corallimorph had dropped back down to a single polyp and CCA had rebounded to 20.7 ± 2.8%, higher than its initial cover (Figure 1.2). A similar pattern was observed at RT10, the reef terrace

site with the highest coral cover (mean 59.1 ± 1 %). *R. howesii* first invaded a single quadrat in 2014 at 12% cover, increased to 22% (in the single quadrat) by 2015, and declined to 9% by 2017. The only functional group to suffer a concurrent decrease was turf algae, which decreased from 29 ± 6.4 % in 2009 to 15.7 ± 5.4% by 2017 (Figure 1.2). FR3 and RT10 represent the two sites with the highest initial coral cover, suggesting that spread of the corallimorph was constrained at sites with more intact benthic communities. Additionally, the concurrent declines in CCA and turf algae associated with increased corallimorph cover suggest that these functional groups were more susceptible to overgrowth.

While the growth of the corallimorph was suppressed at FR3 and RT10, it increased in abundance at the fore reef site (FR5) and back reef site (PSM). No corallimorph was recorded when FR5 was first surveyed in 2009, but cover was 1.5 ± 0.2% by 2010 (Figure 1.5e-h). However, the corallimorph steadily increased through time, reaching a site average of 27.6 ± 8.3% in 2016 (Figure 1.5). Unlike FR3 and RT10, where coral cover was unaffected as cover of the corallimorph increased, hard coral cover at FR5 decreased from 24.2 ± 4.1% in 2009 to 13.4 ± 3.8% in 2017. Turf algae cover also decreased by half during this time period (16.4 ± 2.5% to 8.1 ± 3.1%), suggesting that the expansion of the corallimorph at FR5 was mostly at the expense of hard coral and turf algae. The back reef site (PSM) was first surveyed in 2010 with 19% ± 6.7 % initial corallimorph cover. It increased to 43.3 ± 11.1% in 2017, more than doubling over 7 years and reaching 100% cover in some quadrats (Figure 1.2). In contrast to FR5, hard coral stayed relatively consistent (20.1 ± 0.7 %) throughout the monitoring period and CCA increased from 9.2 ± 1.9 % in 2010 to 27.6 ± 6.5 % in 2017. Turf and macroalgae showed the greatest declines over the monitoring period (42.4 ± 7.3 to 6.9 ± 2 % and 27.7 ± 6.3 to 11.8 ± 2.4 % respectively), suggesting that the corallimorph primarily overgrew algae at PSM. Overall, the

corallimorph most easily overgrew turf and macroalgae across all sites, with a significant decline in coral observed solely at FR5. This, combined with the suppression of corallimorph growth at FR3 and RT10, indicates that sites with minimal disturbance and higher coral cover were more resistant to corallimorph expansion.

These trends are likely due to coral defense mechanisms, including sweeper tentacles that can attack and defend against benthic competitors (Lapid et al. 2004; Chadwick et al. 2011), making it more difficult for the corallimorph to expand at sites with higher coral cover (e.g. RT10). Trends in initial invasion support this, with colonization occurring primarily on CCA and turf algae, with a strong avoidance of hard coral substrate (Figure 1.4). However, at sites with higher levels of disturbance and lower initial coral cover, the corallimorph overgrew hard coral as well, causing tissue necrosis and in some cases, death (Williams 1991; Langmead & Chadwick-Furman 1999; Work et al. 2008). In fact, the forereef site with the highest corallimorph cover (FR5), was characterized by the most consistent wave energy (Gove et al. 2015). These direct effects of the corallimorph are further exacerbated at sites with high *R. howesii* cover like FR5 and PSM by indirect effects. For example, the corallimorphs ability to rapidly colonize available space may result in less available substrate for juvenile coral recruits, thus preventing recovery of hard coral communities at these sites. Continued monitoring of the corallimorph at these sites is important and population control should be considered as a potential mitigation effort around Palmyra given the competitive abilities of *R. howesii*.

In contrast to the dynamics observed at FR5 and PSM, the corallimorph population at the Longliner wreck site decreased from 2013 to 2017. Prior to the wreck removal in 2013, *R. howesii* covered an average of 68.5 ± 2.3 % of the Longliner monitoring transects. Less than a year after the wreck was removed, the corallimorph decreased to 58.2 ± 2.8 % and dropped to

only 1.5 ± 0.3 % cover at the site by 2017 (Figure 1.3, Figure 1.5a). This startling decline in cover (Figure 1.6) was accompanied by an increase in CCA from 8 ± 1.2 % in 2013 to 49.3 ± 1.6 % in 2017 (Figure 1.3). This has important implications for recovery of the benthos because CCA is an active reef-builder and a preferred settlement substrate for juvenile corals (Harrington et al. 2004). Increased abundance of CCA also consolidated and cemented loose rubble that was dislodged during wreck removal, creating further substrate for juvenile corals to settle on, and assisting in the recovery of the reef as corallimorph cover declined (Bosence 1983; Littler & Littler 1984; Price 2010).

Turf and macroalgae at the Longliner site also increased after wreck removal, although less drastically than CCA (from 10.5 ± 1 % to 17.7 ± 1.5 % for turf and 7.9 ± 1.2 % t 24.7 ± 1.1 % for macroalgae). Turf, macroalgae, and CCA frequently dominate early successional communities given their ability to rapidly colonize available space (Steneck & Dethier 1994). Both turf and macroalgae can have varying impacts on coral communities through both indirect and direct methods of competition. Both can compete with juvenile and adult corals for space on the benthos via physical (shading, abrasion), biological (microbial- mediated feedbacks) or chemical (allelopathic) mechanisms that can influence competitive dominance (Barott et al. 2009; Barott & Rohwer 2012; Vermeij et al. 2009; Birrell et al. 2008; Box & Mumby 2007; Smith et al. 2006; Rasher et al. 2011). However, given the high biomass of herbivores at Palmyra (Hamilton et al. 2014), the turf and macroalgal assemblages are likely controlled via grazing, allowing for continued settlement and growth of CCA and juvenile corals (Cheal et al. 2010).

To further examine the different trends in corallimorph cover between FR5 and the Longliner site (Figure 1.6), the spatial dynamics of *R. howesii* were investigated using large-scale photomosaic images collected from 2012-2016 (Figure 1.5). Corallimorph cover in the

Longliner photomosaic decreased from 51.9 % in 2013 to 5.9 % in 2016 (Figure 1.5a-d, j) and was accompanied by a significant decrease in mean patch size (Figure 1.5k). The number of patches at the site showed an increasing trend until 2015, when an four-fold increase was observed in the course of a year (Table 1.3). Collectively, these data show that the decrease in percent cover of the corallimorph was directly related to the breakup of its patches and the reduction in the overall patch size. These trends may suggest that density-dependent mortality (either resource limitation or disease) contributed to the decline. In contrast, the percent cover of the corallimorph in the photomosaic at FR5 increased from 3.2 % to 19.1 % cover from 2012 – 2016. This growth was associated with an increase in the number of patches, and a slight but significant increase in mean patch size (Table 1.2). These data suggest that larger patches of *R. howesii* are better able to hold and subsequently increase territory through lateral expansion via budding and fragmentation. In contrast, smaller patches may be more susceptible to edge effects and more prone to further fragmentation and decline. Further monitoring of the large-scale dynamics of the corallimorph at FR5 may provide continued insight to the spatial trends of corallimorph invasion and its patterns of expansion.

The rapid decline in the corallimorph following the wreck removal strongly suggests that some aspect of the removal contributed to the population breakup of the corallimorph. The physical disturbance associated with the wreck removal may have caused scouring, sedimentation, and physical disruption of the habitat directly adjacent to the former wreck, facilitating break up of corallimorph patches (Figure 1.6). Severe physical disturbances have been shown to disrupt invasion dynamics in other systems, potentially altering the dynamics of the invader through large-scale mortality and facilitating recovery of the reef by opening up

substrate for settlement by reef-builders (Nyström & Folke 2001, Nyström et al. 2008; Nörstrom et al. 2009; Graham et al. 2013).

Many other factors could have contributed to the initial outbreak of *R. howesii* on Palmyra's reefs. For example, Work et al. (2008) suggested that a limiting nutrient (e.g. Fe) may have seeped from the wreck and released the corallimorph from bottom-up control. However, this mechanism does not explain the continued spread of the corallimorph around the island post-wreck removal. Rather, the overall distribution of the corallimorph around the atoll mirrors water flow models (Rogers et al. 2017), suggesting that propagule delivery from the Longliner site may have facilitated the expansion to the southern forereef (Work et al. 2018). Furthermore, Palmyra has had an extensive amount of metal debris on land and in the water since military occupation in the 1940-50s, making the outbreak at the wreck site curious. Previous studies found that iron content in corallimorph tissue was not significantly higher at the Longliner site when compared to other locations around the atoll (Carter 2014). It is also doubtful that *R. howesii* was brought in as an introduced species on the shipwreck, as it is native to the central Pacific and there were reports of it around the atoll before the wreck occurred (J. Maragos, pers com.). In light of this, it seems likely that an invasive strain may have been brought in on the *Hui Feng* or that the corallimorph was native to the atoll, but that the disturbance associated with the shipwreck facilitated its growth and expansion and the subsequent disturbance from the ship removal contributed to its current decline.

Although this study focused on the dynamics at Palmyra Atoll, *R. howesii* has been observed expanding at other sites around the Pacific such as Fagatele Bay National Marine Sanctuary, Helen Reef, and Ulithi Atoll in Micronesia (unpublished data, Crane et al. 2016). Although the decline in corallimorph cover associated with the shipwreck removal is

encouraging, the continued growth of the corallimorph at sites like FR5 and PSM (Figures 2, 5, 6) suggest that the outbreak may continue at more distant sites. However, sites such as RT10 and FR3 have shown decreases in their corallimorph population, suggesting that high coral cover may help prevent the rapid expansion of the corallimorph. These data suggest that sites with lower coral cover and initial stages of corallimorph invasion may benefit from removal and restoration efforts the most. The strong negative impacts of *R. howesii* merit continued long-term monitoring and conservation efforts to document and mitigate the continued growth and impact on the benthic communities of Palmyra Atoll.

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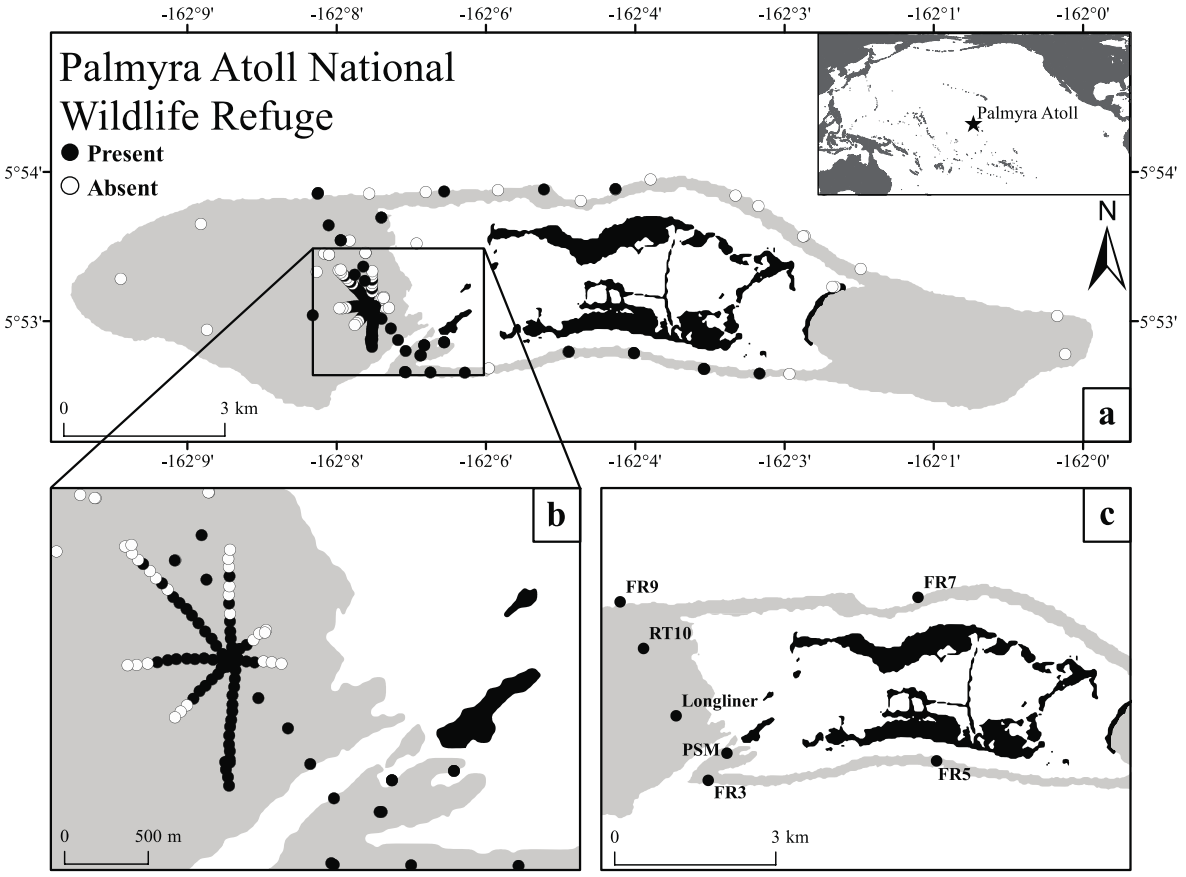

Figure 1.1. A spatial display of the historical observations of *R. howesii* around Palmyra Atoll. Reef communities are shown in grey, sand flats in white, and land mass in black **a** Observed (black circle) and Not Observed (white circle) of the corallimorph *Rhodactis howesii* at all sites surveyed at Palmyra Atoll from 2009-2016. **b** Higher resolution of the spatial cover of the corallimorph at the Longliner site across the western reef terrace from surveys spanning 2013-2017. **c** Site names for all long-term monitoring transects discussed throughout manuscript.

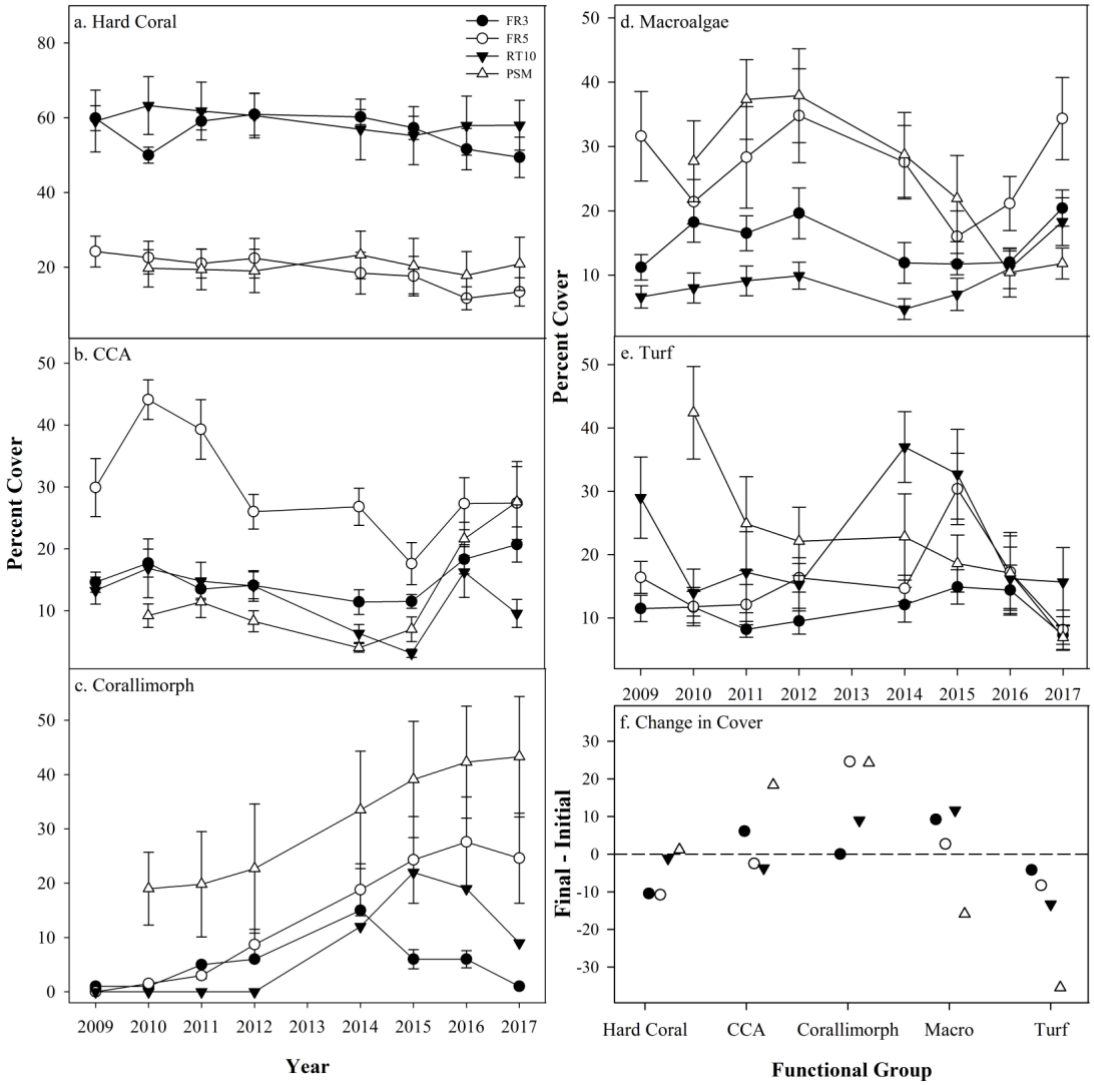

Figure 1.2. Change in mean functional group cover ± SE at four permanent monitoring sites around Palmyra Atoll from 2012 – 2017 (a-e). Two forereef (FR3, FR5), one reef terrace (RT10), and one back reef (PSM) are shown. **f** Final-initial change in cover for each functional group by site from 2009-2017. The dashed line represents the removal of the shipwreck from the site.

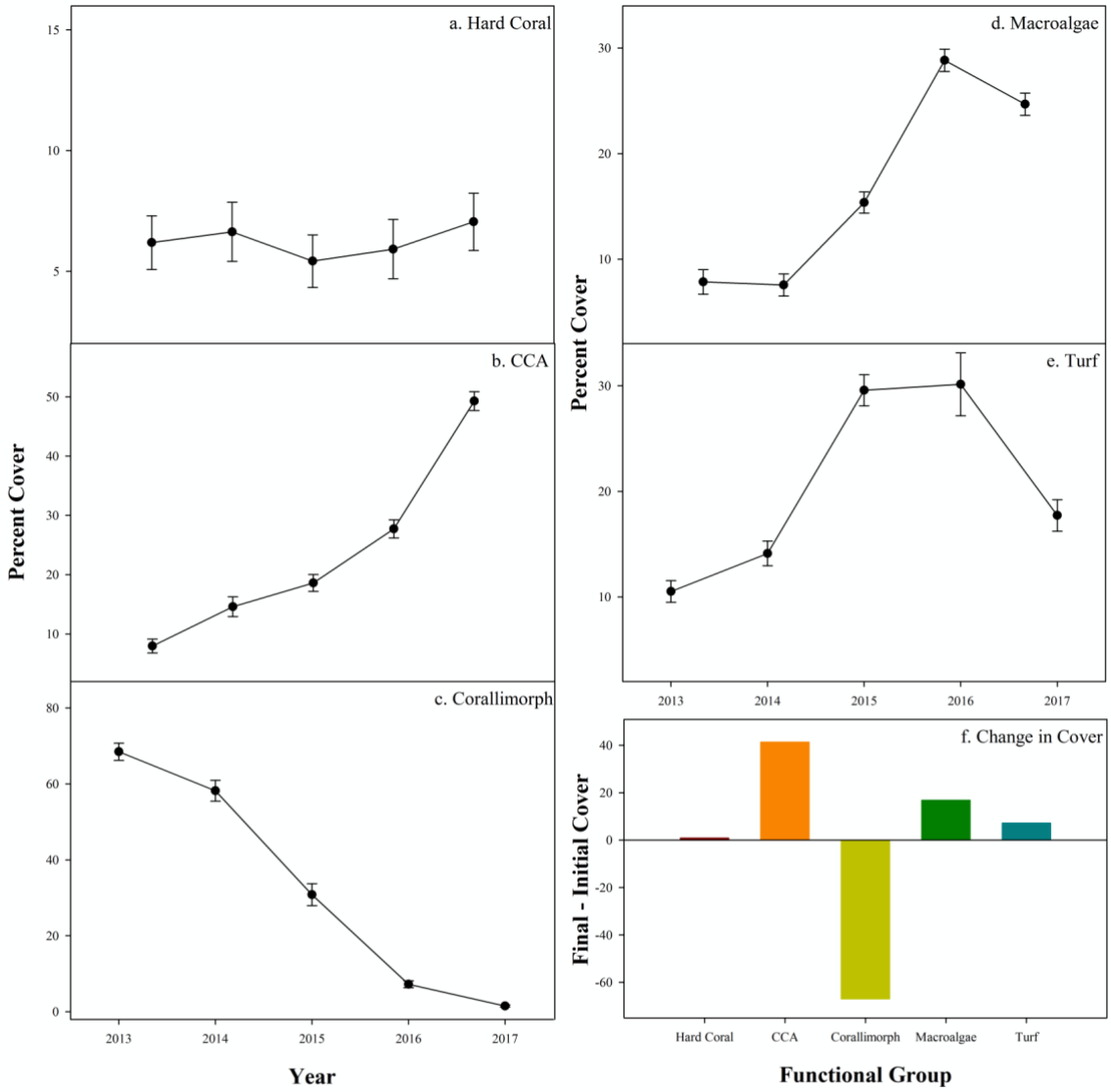

Figure 1.3. Change in mean functional group cover ± SE at the Longliner site from 2013 – 2017 (a-e). Shipwreck removal occurred in Winter of 2013. **f** Final-initial change in cover for each functional group at the Longliner site (2013 – 2017). The dashed line represents the removal of the shipwreck from the site.

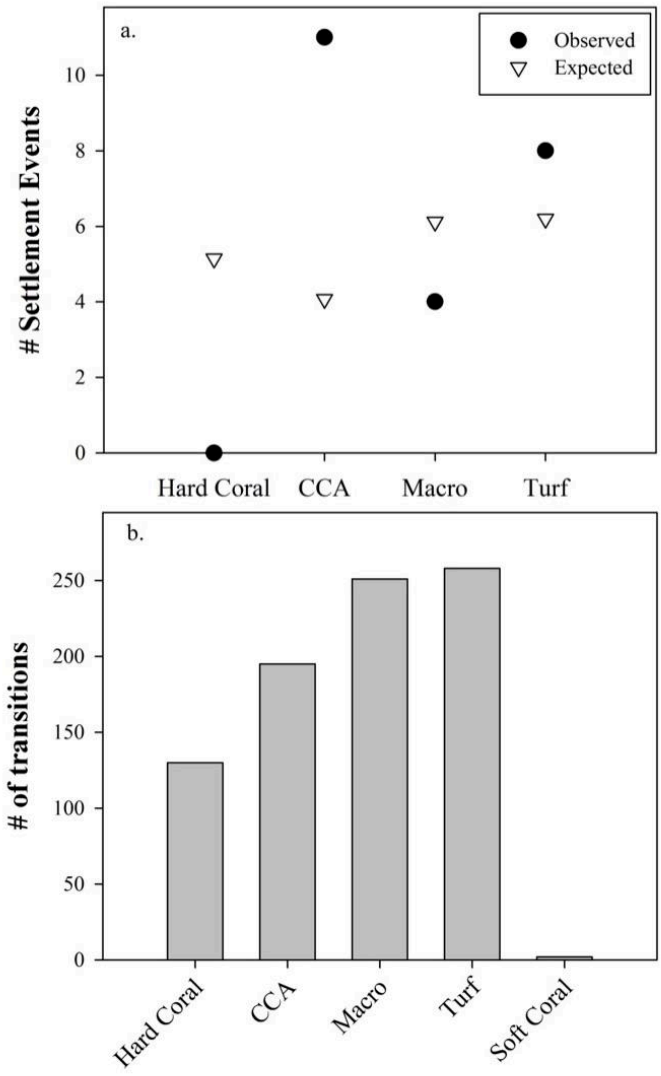

Figure 1.4. Substrate preference of initial corallimorph settlement in non-invaded plots and subsequent overgrowth of function groups post-invasion. **a** Number of observed and expected settlement events for each functional group. Observed values higher than expected demonstrate preference for habitat type, observed values lower than expected demonstrate avoidance of habitat type. Chi-squared test of observed frequencies indicated significant avoidance of hard coral and macroalgae and selectivity of CCA ($\chi^2 = 16.94$, $p < 0.01$). **b** Number of transitions from benthic functional group to corallimorph from 2009-2017 in all plots.

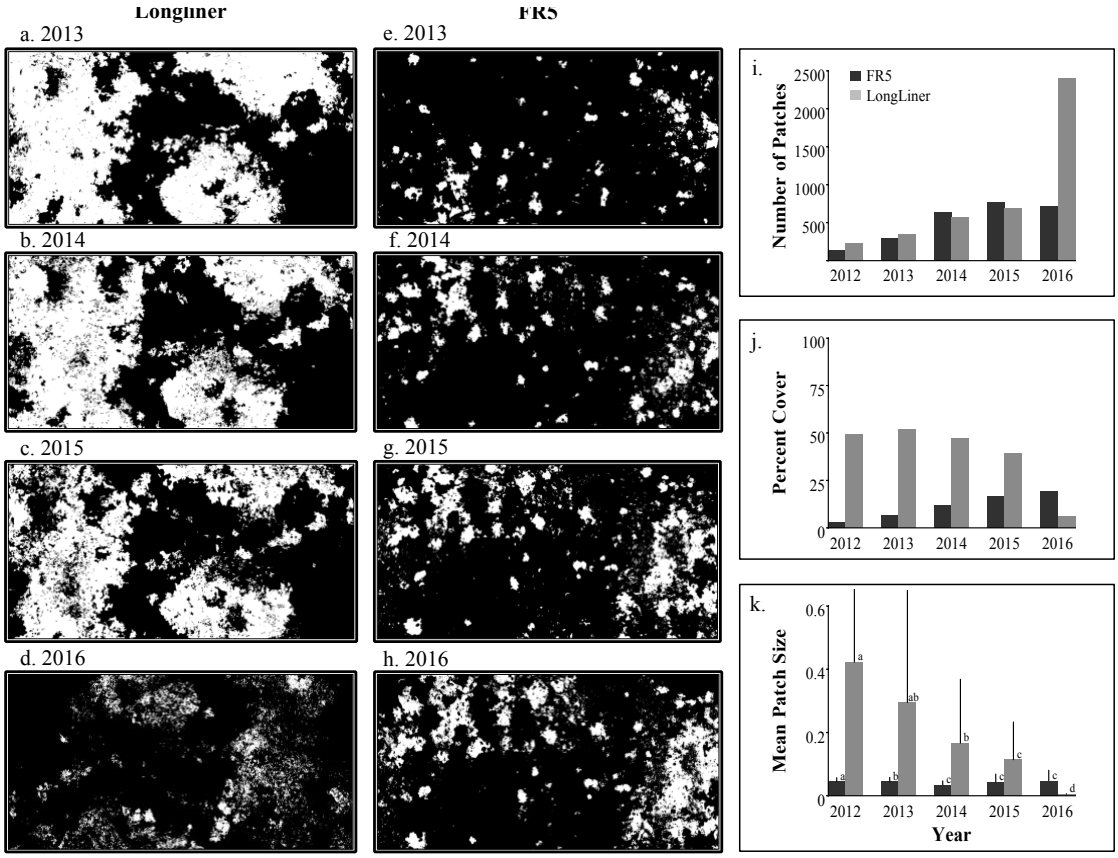

Figure 1.5. Contrasting spatial dynamics at the initial epicenter (Longliner) and a forereef site where initial invasion and subsequent expansion were observed (FR5). Photomosaics of the Longliner plot (**a-d**) and FR5 (e-h) from 2012-2016 with corallimorph patches shown in white and the remainder of the benthos in black. The number of corallimorph patches from 2012-2016 are shown in **i**. Percent cover of corallimorph (out of 200m^2) is shown from 2012-2016 for both sites (j). Change in mean patch size from 2012-2016 is shown in **k**. Letters denote significant changes in patch size from year to year with a $p<0.001$.

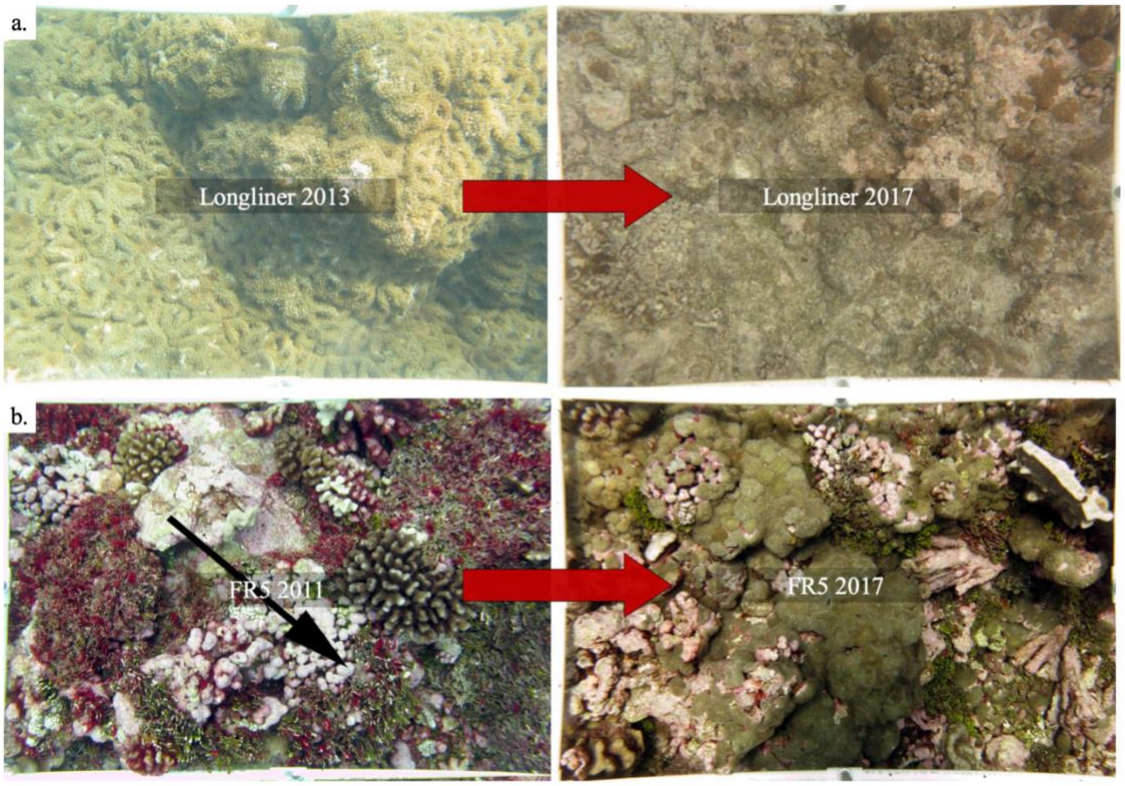

Figure 1.6. Initial and final corallimorph cover at the epicenter of the invasion contrasted with initial colonization and subsequent overgrowth of corals on the forereef. Initial and final photoquadrat from the Longliner North transect (a) in 2013 and 2017. Initial invasion and subsequent growth of the corallimorph in a quadrat from FR5 on the forereef in 2009 to 2017 (b). Black arrow shows initial corallimorph polyps at early stages of invasion. Images show the difference between sites that experienced a decline vs. invasion during the monitoring time period. It is important to note that not all plots at these sites are experiencing the same corallimorph dynamics.

Table 1.1. Metadata for all metrics in the paper including site, survey type, year, and method.

Metric	Site	Survey Type	Year	Method
Observed/Not Observed	FR3	Permanent Transect	2017	Photoquadrat
	FR5	Permanent Transect	2017	Photoquadrat
	FR7	Permanent Transect	2017	Photoquadrat
	FR9	Permanent Transect	2017	Photoquadrat
	RT1	Permanent Transect	2017	Photoquadrat
	RT4	Permanent Transect	2017	Photoquadrat
	RT10	Permanent Transect	2017	Photoquadrat
	RT13	Permanent Transect	2017	Photoquadrat
	PSM	Permanent Transect	2017	Photoquadrat
	Longliner North	Permanent Transect	2017	Photoquadrat
	Longliner East	Permanent Transect	2017	Photoquadrat
	Longliner South	Permanent Transect	2017	Photoquadrat
	Longliner West	Permanent Transect	2017	Photoquadrat
	RH_1	One-time Transect	2016	Photoquadrat
	RH_2	One-time Transect	2016	Photoquadrat
	RH_3	One-time Transect	2016	Photoquadrat
	RH_4	One-time Transect	2016	Photoquadrat
	RT4_Merrifield	One-time Transect	2016	Photoquadrat
	Tortogonia	Permanent Transect	2017	Photoquadrat
	Penguin Spit Inner	Permanent Transect	2017	Photoquadrat
	Penguin Spit Outer	Permanent Transect	2017	Photoquadrat
	North Barren	Permanent Transect	2013	Photoquadrat
	FR13	Wave Mosaic	2017	Mosaic
	FR132	Wave Mosaic	2017	Mosaic
	FR14	Wave Mosaic	2017	Mosaic
	FR239	Wave Mosaic	2017	Mosaic
	FR36	Wave Mosaic	2017	Mosaic
	FR37	Wave Mosaic	2017	Mosaic
	FR38	Wave Mosaic	2017	Mosaic
	FR39	Wave Mosaic	2017	Mosaic
	FR4	Wave Mosaic	2017	Mosaic
	FR40	Wave Mosaic	2017	Mosaic
	FR Strawn	Wave Mosaic	2017	Mosaic
	DRT1	One-time Transect	2010	Photoquadrat
	DRT2	One-time Transect	2010	Photoquadrat
	UVB	One-time Transect	2010	Photoquadrat
	PA25	One-time Transect	2010	Photoquadrat
	PA2	One-time Transect	2010	Photoquadrat
	WT8	One-time Transect	2010	Photoquadrat
	WT7	One-time Transect	2010	Photoquadrat

Table 1.1. Metadata for all metrics in the paper including site, survey type, year, and method, continued.

	PA14	One-time Transect	2010	Photoquadrat
Observed/Not Observed	NBW	One-time Transect	2010	Photoquadrat
	PA22	One-time Transect	2010	Photoquadrat
	ET1	One-time Transect	2010	Photoquadrat
	ET3	One-time Transect	2010	Photoquadrat
	PA17	One-time Transect	2010	Photoquadrat
	UVP	One-time Transect	2010	Photoquadrat
	RT22	Permanent Transect	2013	Photoquadrat
	RT30	Permanent Transect	2013	Photoquadrat
	GBR	Permanent Transect	2013	Photoquadrat
	RT25	Permanent Transect	2013	Photoquadrat
	RT6	Permanent Transect	2013	Photoquadrat
	RT23	Permanent Transect	2013	Photoquadrat
	N Tow	Diver Tow Survey	2013	Visual Survey
	NW Tow	Diver Tow Survey	2013	Visual Survey
	W Tow	Diver Tow Survey	2013	Visual Survey
	SW Tow	Diver Tow Survey	2013	Visual Survey
	S Tow	Diver Tow Survey	2013	Visual Survey
	SE Tow	Diver Tow Survey	2013	Visual Survey
	E Tow	Diver Tow Survey	2013	Visual Survey
	NE Tow	Diver Tow Survey	2013	Visual Survey
% Cover	FR3	Permanent Transect	2009-2017	Photoquadrat
	FR5	Permanent Transect	2009-2017	Photoquadrat
	RT10	Permanent Transect	2009-2017	Photoquadrat
	PSM	Permanent Transect	2010-2017	Photoquadrat
	Longliner North	Permanent Transect	2013-2017	Photoquadrat
	Longliner East	Permanent Transect	2013-2017	Photoquadrat
	Longliner South	Permanent Transect	2013-2017	Photoquadrat
	Longliner West	Permanent Transect	2013-2017	Photoquadrat
Mosaic Percent Cover, Patch Size, and Number	Longliner Mosaic	Permanent Mosaic	2012-2017	Mosaic
	FR5 Mosaic	Wave Mosaic	2012-2017	Mosaic

Table 1.2. Results from Kruskal-Wallis rank sum test examining significant change in mean patch size (m^2) by year at the Longliner and FR5 sites.

Year	Longliner Mean Patch Size	Significance	FR5 Mean Patch Size	Significance
2012	2857.552	a	1788.476	a
2013	2587.432	ab	1518.674	b
2014	2495.768	b	1187.280	c
2015	2298.271	c	1277.246	c
2016	1838.020	d	1187.366	c
Chi-squared: 331.49, df=4, p-value<2.2e-16				

Table 1.3. Percent cover, number of patches, and mean patch size (m^2) from the Longliner and FR5 mosaics from 2012-2016.

Percent Cover

Site	2012	2013	2014	2015	2016
Longliner	49.02	51.93	47.35	39.02	5.96
FR5	3.17	6.71	11.66	16.68	19.08

Number of Patches

Site	2012	2013	2014	2015	2016
Longliner	232	352	570	685	2398
FR5	143	291	636	776	724

Mean Patch Size

Site	2012	2013	2014	2015	2016
Longliner	0.423	0.295	0.166	0.114	0.005
FR5	0.044	0.045	0.034	0.043	0.047

CHAPTER 2

Evidence of allelopathy in competitive interactions between the corallimorph, *Rhodactis howesii,* and scleractinian corals

Amanda L. Carter, Chambers C. Hughes, Jennifer E. Smith

ABSTRACT

Competitive success in benthic marine communities is frequently determined by an organism's ability to rapidly colonize available space, a form of exploitative competition. However, interference competition in the form of allelopathy can also play a role in determining competitive interactions between adjacent sessile benthic organisms. Here we examined the competitive interaction between scleractinian corals and the corallimorpharian *Rhodactis howesii,* a species that demonstrates highly invasive tendencies on disturbed reefs. Although the corallimorph's ability to overgrow corals has been documented, the competitive mechanisms used by *R. howesii* are not well known. Our study found that the corallimorph demonstrated strong chemical bioactivity through a series of bioassays using extracts derived from the tissue of *R. howesii.* Non-polar, hydrophobic fractions of the corallimorph caused significant tissue necrosis and a decline in the photosynthetic capacity of the branching coral *Acropora yongei*. In contrast, procedural controls mimicking physical abrasion of the tissue did not provide significant results. Bioassay-guided chromatographic mass spectrometry was used to identify the most common compound present in the active fractions, a light-harvesting carotenoid peridinin. Although peridinin is most commonly thought to be a harmless pigment, studies have investigated its role in contributing to disease pathogenesis in microbial interactions. These data demonstrate the corallimorph's ability to engage in chemical competition with corals and pave the way for future investigation into the chemical compounds present in their tissue.

INTRODUCTION

Benthic dynamics on coral reefs are frequently driven by direct and indirect competition for the limiting resource of space (Jackson & Buss 1975; Muko 2001). Indirect, or exploitative, competition includes shading, occupation of open substrate via rapid growth or recruitment, inhibition of juvenile settlement, and/or algal stimulation of microbial growth that negatively impacts coral health (Box & Mumby 2007; Nugues et al. 2004; Kuffner et al. 2006; Diaz-Pulido et al. 2010; Smith et al 2006; Kline et al 2006). Direct, or interference, methods of competition can include abrasion, overgrowth, and/or chemical warfare via release of allelopathic compounds (Tanner 1995; Box & Mumby 2007; Rasher & Hay 2014). Allelopathic compounds can directly affect competitors (e.g. injected via stinging nematocyst cells or released via surface-surface contact) or they can be released into the water column where they negatively affect the growth of competitors in the immediate vicinity or downstream (Sammarco et al. 1983; Aceret et al. 1995). Compounds isolated from various macroalgae demonstrate strong allelopathic properties that directly impact coral health (Rasher & Hay 2010; Rasher et al. 2011; Paul et al. 2011) and thus enhance algal competitive abilities.

Smith et al. (2016) found that benthic communities at uninhabited islands generally had more stony corals and calcifying organisms. In contrast, macroalgae and non-calcifying benthic competitors dominated the communities at more disturbed reef environments with higher levels of anthropogenic influence (Smith et al. 2016). Corallimorpharians, or corallimorphs, are an example of a non-calcifying, benthic competitor which has been shown to directly and indirectly affect hard corals. For example, the corallimorph *Corynactis californica* has been shown to negatively impact scleractinian corals by increasing their larval mortality, altering reproductive output, and affecting recruitment patterns (Chadwick 1987; Chadwick 1991). Corallimorphs are known to be weedy, fast-growing, stress-tolerant species that can be found in disturbed and

marginalized reef environments (Kuguru et al. 2007, 2010). Corallimorphs are aggressive competitors that can reproduce sexually and asexually through fragmentation and budding, allowing them to spread rapidly and colonize available substrate (Chadwick 1987, 2001; Chadwick and Adams 1991). Studies evaluating direct competition between the corallimorph *Rhodactis rhodostoma* and the coral *Acropora eurystoma* in the Red Sea found that *R. rhodostoma* responded to coral aggression through the formation of specialized bulbous marginal tentacles (BMTs). These BMTs damaged coral tissue and facilitated the corallimorph's overgrowth of the coral skeleton (Langmead & Chadwick 1999). This method of direct competition and aforementioned life history traits make the corallimorph an aggressive competitor with corals, particularly in disturbed environments. Though shifts towards corallimorpharian dominance have been recorded on a number of reefs (Kuguru et al. 2007, 2010; Work et al. 2008; Norström et al. 2009: Crane et al. 2016; Carter et al. 2019), there is a paucity of data about the specific mechanisms that provide the corallimorph with such aggressive capabilities in direct coral interactions.

The observed coral mortality at the interaction zone that is seen when corallimorph BMTs interact with coral tissue (Figure 2.1) suggests the possibility of an allelopathic interaction, similar to those that have been observed in macroalgal-coral interactions (Rasher & Hay 2010; Rasher et al. 2011; Morrow et al. 2011). However, it is unclear if coral tissue necrosis is due to a chemical produced by the corallimorph or the physical abrasion and contact between the two species. Work et al. (2008) hypothesized that the presence of large nematocyst cells in the corallimorph tentacles caused tissue necrosis through physical damage, resulting in the corallimorphs ability to overgrow and outcompete hard corals. However, these observations are also consistent with a chemical interaction between the two species. Photomicrographs taken of

Acropora sp. tissue experiencing *R. howesii*-induced tissue loss and fragmentation could support either hypothesis (Work et al. 2008).

To test for an allelopathic interaction between hard coral and *Rhodactis howesii* in the absence of direct physical disturbance, we developed an effective bioassay for assessing the toxicity of various fractions of chemical extract isolated from the corallimorph tissue on the health of a reef-building coral, *Acropora yongei*. Assessments of the impacts of these chemical fractions on coral health were conducted using two parameters, 1) percent tissue necrosis on corals post-exposure, and 2) measurements of coral photosynthetic efficiency using a pulse-amplitude modulated (PAM) fluorometer. Bioassay-guided chromatographic mass spectrometry was then used to identify the most common compounds present in the bioactive fractions of the corallimorph tissue.

METHODS

Corallimorph Collection

Individuals of *Rhodactis howesii* were collected by divers on Palmyra Atoll in September of 2013 and 2015. All samples were collected from the back reef habitat in approximately 5 m depth on the southwest portion of the atoll at a site referred to as Penguin Spit Middle. To prevent tissue damage, individuals were collected from loose substrate such as sand or small rubble. Samples were kept in seawater while transported to the lab, where they were sorted, cleaned of debris, and stored at -80°C. They were then transported in a dry shipper to Scripps Institution of Oceanography (SIO) where they were stored until initial extraction.

Experimental Design

A series of chemical extractions and subsequent fractionations were performed on the corallimorph tissue for the following purposes: 1) to look for preliminary evidence of chemical activity of *R. howesii* and 2) if activity was observed, to identify the most active component of the chemical extract through fractionation. To address these questions, a series of 3 extractions were performed in the following order:

1) Crude extraction of the corallimorph using Methanol (MeOH) and Ethyl Acetate (EtOAc) to remove the polar and non-polar compounds from the corallimorph tissue. These compounds were then combined into a bulk crude extract to test for evidence of any chemical activity (Experiment #1).

2) MeOH and EtOAc extracts were repeated, but kept separate to examine which compounds were the most bioactive, i.e. polar or non-polar (Experiment #2).

3) EtOAc extraction was repeated after evidence that it contained the most active compounds. Fractionation of the EtOAc extract was performed, resulting in 5 separate fractions (Experiments #3-4).

The 5 EtOAc fractions are described in Table 2.1. All extractions and experiments are outlined in Figure 2.2. Procedure for chemical extraction and subsequent fractionation are described below.

Chemical extraction and fractionation

Chemical extraction for each experiment was performed using the following procedure: 8 individual corallimorphs were freeze-dried, then ground into a fine powder to create 250g of dry tissue. Crude extracts (the base for each experiment) was made by adding 500ml of EtOAc to the dried tissue to remove any non-polar compounds present. After 24 hours the liquid was removed and dried *in vacuo* to provide a concentrated non-polar EtOAc crude extract. MeOH

was then added to the dry tissue to remove any polar compounds present. After 24 hours, the remaining liquid was removed and dried *in vacuo* to create a concentrated polar MeOH extract.

The bulk crude extract (Experiment #1, Figure 2.2) was made by combining the polar and non-polar extracts to test for any evidence of chemical activity in the corallimorph. The chemical extract for Experiment #2 was made following the procedure outlined above, but the polar and non-polar extracts were kept separate from one another to determine which extract was more active. After evidence that the non-polar extract was the most chemically active, Experiments #3-4 were run with only EtOAc extract. The EtOAc extract was then run through reverse-phase column chromatography using 3g of silica. This was used to separate the non-polar EtOAc extract into 5 fractions by running first EtOAc and then MeOH through the column to remove all active compounds (Figure 2.2, Table 2.1) (Rasher & Hay 2010; Rasher et al. 2011; Morrow et al. 2011).

Chemical suspension and strip design

To test for bioactivity of the corallimorph, extracts were suspended in Phytagel strips for application to the coral as follows (Rasher et al. 2011; Morrow et al. 2011). First, 0.98g (approximately half of an individual corallimorph) of each extract was dissolved in 1ml of MeOH to suspend the chemical in liquid. Then 10g of Phytagel (Sigma Aldrich) were dissolved in 10ml of water and heated slowly. The 1ml MeOH + 0.98g of extract were then added to the Phytagel solution just before boiling and stirred rapidly to distribute the chemical solvent throughout the liquid. The resulting mix was then poured into a mold measuring 12.7 cm long x 1.27 cm wide. The gel was allowed to cool, forming a pliable but firm strip containing the extract. The strip was cut into 10 equally sized (1.27x1.27cm) treatment (Tx) strips. Procedural

control (PC) strips were made following the same methodology, but without addition of the chemical extract (1ml MeOH + 10g Phytagel + 10ml water). No strips were applied to controls (C). This procedure was followed for all 4 experiments using the following extracts:

Experiment 1) bulk crude extract of MeOh + EtOAc (10 Tx strips, 10 PC, 10 C),

Experiment 2) MeOh and EtOAc extracts (10 Tx-MeOH, 10 Tx-EtOAc, 10 PC, 6 C),

Experiment 3) First two EtOAc fractions of 100 % Hexanes and 25% EtOAc (10 Tx-Hex strips, 10 Tx-25E strips, 10 PC, 6 C)

Experiment 4) Final EtOAc fractions of 100% EtOAc and 20% Meoh + 5% MeOH combined (10 Tx-Et100, 10 Tx-25MeOH, 10 PC, and 6 C) (Figure 2.2).

Bioassay Development

Chemical activity of the strips was tested using the following design in a series of 4 experiments (strip concentrations and contents for each experiment are described above). The hard coral, *Acropora yongei*, was used for all experiments because of its rapid growth rates and hardy nature in aquaria settings. *A. yongei* specimens were clipped to provide individual 2-3 inch fragments (n=30 for Exp #1, n=36 for Exp #2-4) that were then glued onto PVC (Exp #1) or carbonate (Exp #2-4) discs using BSI Cyanoacrylate IC-Gel Aquarium glue. Fragments were then acclimated and maintained at SIO in running seawater ~26°C seawater with full spectrum Aquaillumination UV lights (~400 E) for 5 days before experimental set up. Lights were set for sunrise at 6:30am PST and sunset at 5:30pm PST with an hour long ramp cycle for each. The *A. yongei* fragments were then placed in individual glass mesocosms with running seawater. Control, procedural control, and treated corals were dispersed randomly across a water table with

an additional jar containing an Onset pendant HOBO temperature and PAR data logger to monitor conditions throughout the experiment.

For each experiment the treatment and procedural control strips were applied to the individual coral fragments using a small zip-tie. The strips were left on for 72 hours in Experiment #1 and 48 hours in Experiments #2-4. Coral health measurements (as described in the section below) were taken before strip application and again following their removal.

Coral health measurements

Changes in coral health as a result of the experimental conditions were assessed using two different measurements. First, photographs were taken of all corals before and after removal of Phytagel strips to allow for quantification of tissue bleaching and loss. Using an in-frame scale, images were analyzed for two-dimensional tissue loss by circling bleached tissue on the corals using ImageJ software. The total bleached area was then subtracted from the starting tissue cover ($1.6cm^2$) to calculate the percent tissue loss that occurred. To assess experimental effects on coral photo-physiology, a diving Pulse Amplitude Modulation (PAM) fluorometer (Heinz Walz) was used to measure dark-adapted yield (Fv/Fm), a metric of photosynthetic capacity. Corals were dark-adapted for one hour before the PAM was used to measure the maximum quantum yield (MQY) of photosystem II of the zooxanthellae present in the coral tissue (Beer et al. 1998; Pawlik 2007; Rasher et al. 2011; Morrow et al. 2011). Dark-adapted yield was measured before strip application, after 72 hours of exposure, and seven days after strip removal for Experiment #1. Following bioassay proof of concept in Experiment #1, Experiments #2-4 did not include a recovery time point.

Identification of molecular compounds in active fraction

Following all experimental bioassays, the 100% EtOAc and 25% EtOAc fractions had significantly greater negative impact on the coral health when compared to the procedural controls and other fractions. In an attempt to identify the compounds present in the fraction, ^{1}H NMR spectral data and normal-phase High Performance Liquid Chromatography (HPLC) were used to look for look for individual compounds present.

Statistical Analysis

To look for evidence of chemical activity in the bulk corallimorph extract for Experiment #1, a Kruskal-Wallis analysis of variance (ANOVA) on ranked MQY values was used to look for significant differences between the control, procedural control, and treatment groups due to violations of the assumptions of parametric statistics due to heteroscedasticity. For post hoc comparisons, Dunn's test was applied. Changes in the percent coral tissue loss (Final – Initial) were analyzed using a two-sample t-test to compare the procedural control and treatment group (no tissue was lost in the control group).

For experiments #2-4, a one-way ANOVA with overall change in MQY as a factor was run for each experiment to look for significant differences between treatment groups. To probe the results of the ANOVA, planned comparisons of controls to procedural controls, procedural controls to treatments, and treatment to treatment were conducted. Tissue mortality was also analyzed for each experiment using a one-way ANOVA with percent tissue loss as a factor to look for significant differences across groups. A post-hoc Tukey HSD was used after significant results.

RESULTS

Evidence of chemical activity in corallimorph crude extract

The chemical activity of the crude corallimorph extract (Experiment #1) was clearly demonstrated by the bioassay design and showed significant declines in both coral photosynthetic efficiency as measured by the change in MQY (Kruskal-Wallis ANOVA, $p<0.05$, Fig. 2.4A) and in observed tissue loss post-exposure (t-test, $p <0.05$, Fig. 2.4B). The treatment was significantly different from the control and procedural control across all time points (Dunn's Test, $p<0.05$, Fig. 2.4A). Although both the procedural control and treatment corals showed a decline in MQY and tissue bleaching (Figure 2.3), the procedural control corals recovered all pigment and returned to their initial MQY measurements after 7 days of recovery (Fig. 2.3 & 2.4). In contrast, the treatment corals experienced full tissue mortality and turf algae had settled on exposed skeleton by the 7-day recovery time point (Figure 2.3).

Chemical activity in polar vs. non-polar extracts

Experiment #2 looked for a significant difference between the ethyl acetate crude extract (non-polar) and methanol crude extract (polar) to determine which exhibited higher levels of bioactivity. A significant difference between groups was observed (ANOVA, $p<0.001$) and Tukey's HSD test showed a significant difference in change in MQY between polar, non-polar, and procedural control strips ($p<0.0001$). The EtOAc extract was significantly different from the control ($p<0.0001$), procedural control ($p<0.001$), and the MeOH extract ($p<0.001$, Figure 2.5A). Significant differences in tissue loss were also observed across groups (ANOVA, $p<0.001$). Tukey's HSD test showed significantly greater tissue loss for the EtOAc group ($p < 0.001$). The

EtOAc extract resulted in significantly more tissue loss when compared to the procedural control ($p<0.0001$) and the MeOH extract ($p>0.001$) (Figure 2.5B).

Chemical activity of non-polar fractionations

Experiment #3 tested the chemical activity of the 100% Hexanes and 25% EtOAc fractions (Table 2.1) as compared to the procedural control strips. A significant difference in the change in MQY between groups (ANOVA, $p<0.0005$) was examined using a Tukey's HSD test. A significantly greater change in MQY was caused by the 25% EtOAc fraction when compared to both the control ($p<0.005$), procedural control ($p<0.01$), and 100% Hexanes fraction ($p<0.001$, Figure 2.6A). Significant loss in tissue (ANOVA, $p<0.05$) was analyzed using Tukey's HSD test, showing significantly higher percent loss associated with the 25% EtOAc fraction when compared to the procedural control ($p<0.05$) and the 100% Hexanes fraction ($p<0.05$, Figure 2.6B).

Experiment #4 tested the chemical activity of the 100% EtOAc fraction and the MeOH combination fraction (20%+5% MeOH) from the EtOAc crude extract (Table 2.1). A significant difference across groups (ANOVA, $p<0.01$) was observed and Tukey's HSD test showed that the 100% EtOAc extract was significantly more active than the procedural control ($p<0.05$), but not significantly difference from the MeOH combination ($p>0.05$, Figure 2.7A). However, the MeOH fraction was not significantly different from the procedural control ($p> 0.05$). Significant differences in percent tissue loss (ANOVA, $p<0.001$) were tested using Tukey's HSD test and the 100% EtOAc fraction had a significantly greater impact on tissue loss when compared to the procedural control ($p <0.001$) and the MeOH combination fraction ($p<0.001$, Figure 2.7B).

Because of this, we chose to focus on the 100% EtOAC fraction from Experiment #4 and the 25% EtOAc fraction from Experiment #3.

Isolation of corallimorph chemical compounds

Compound 1 (Figure 2.8) was the most abundant compound in the bioactive fractionation of 100% EtOAc. It was identified as peridinin, a light-harvesting carotenoid and important microbial pigment. Other compounds present in the fraction were primarily lipids, which have not been associated with allelopathic activity. However, additional compounds present in smaller quantities in the 100% EtOAC and 25% EtOAc fractions may be chemically active and require future investigation.

DISCUSSION

Corallimorpharians are strong benthic competitors with a variety of traits that allow them to proliferate and rapidly expand in disturbed environments; including the ability to actively overgrow visually healthy coral (Chadwick & Adams 1991; Langmead & Chadwick 1999; Kuguru et al. 2004). Our results suggest that the corallimorph *Rhodactis howesii* is able to directly interfere with coral health through the production of chemical compounds present in their tissue. Additionally, the physical abrasion caused by presence of procedural controls did not result in the same tissue necrosis and decline in photosynthetic abilities that were observed when corallimorph extract was present. These results make this is the first study to demonstrate that the coral mortality observed in corallimorph blooms has an chemical component, and is not the result of purely physical disturbance and abrasion.

Langmead and Chadwick-Furman (1999) described the development of bulbous marginal tentacles (BMTs) by the corallimorph *Rhodactis rhodostoma* (a close relative of *R. howesii*) when placed in contact with the coral *Acropora eurystoma*. However, in over half of their interactions, coral tissue necrosis was observed before the formation of BMTs and through tissue contact alone. Although they did not examine the exact mechanism employed by *R. rhodostoma,* these results were highly suggestive of a chemical compound that caused tissue degradation upon contact with the coral. Their findings agree with similar studies conducted on macroalgal compounds where hydrophobic extracts resulted in coral damage and, in some cases, mortality (Rasher & Hay 2010; McCook et al. 2001). Our findings agree with both of these studies, demonstrating that the more non-polar, hydrophobic fractions of the corallimorph extract resulted in significantly higher declines in coral health (Figure 2.5).

Although numerous studies have examined allelopathic interactions between corals and a variety of benthic organisms, few have identified the active compounds responsible for observed declines in coral health. Of those, Rasher et al. (2011) found that terpenes derived from several macroalgal species had significant impacts on coral health and caused tissue necrosis when in direct contact. Pawlik et al. (2007) found that extracts from sponges in the genus *Agelas* containing the alkaloid oroidin, an anti-predatory and antimicrobial compound, caused significant declines in coral photosynthetic capacity. Zidar et al. (2014) found that the large, hydrophobic molecules present in many marine alkaloids (e.g. oroidin) demonstrate high levels of antimicrobial activity. Similarly, the primary compound found in the 100% EtOAc fraction was peridinin, a carotenoid (Figure 2.8). Interestingly, previous work isolating peridinin-related norcarotenoids from the soft coral *Clavularia viridis* found that the pigment can have strong anti-cancer and antimicrobial activities (Suzuki et al. 2003). Liu & Nizet (2009) also found that

microbial pigments (e.g. carotenoids such as peridinin) can contribute to disease pathogenesis through alterations in host immune systems and cytotoxic properties. However, the peridinin found in the bioactive fraction of the corallimorph extract is a common carotenoid found in coral symbionts (Roth 2014). It is therefore difficult to determine if the peridinin is contributing to the activity of the fraction, or if it is simply the most abundant molecule present. Isolation of additional compounds present in the 100% and 25% EtOAc extract would shed further light on the corallimorph's active chemical component.

The evidence of chemical activity of *R. howesii* is of particular importance given the ability of corallimorphs to flourish in environmental conditions that are not ideal for hard coral growth (Muhando et al. 2002; Kuguru et al. 2007, 2010; Work et al. 2008; 2018). The chemical nature of the corallimorph may provide it with an additional competitive advantage over corals, particularly in high disturbance regimes or degraded environments. For example, the epicenter of the corallimorph invasion at Palmyra Atoll was located at the site of the Longliner shipwreck, an area that was previously dominated by stony corals and other calcifiers (Work et al. 2008; Carter et al. 2019). The corallimorph's ability to rapidly colonize available space resulting from the physical disturbance of the wreck, combined with a chemical advantage, may explain the invasion that was observed at the atoll. More broadly, the chemical competition between corallimorphs and corals may hinder recovery of reefs in disturbed environments, particularly when combined with other global and local stressors (e.g. nutrient pollution and thermal stress). In conclusion, our study provides the first empirical evidence of a chemical interaction between hard corals and the corallimorph *Rhodactis howesii* that is not purely the result of physical abrasion. Although this study was not able to definitively state that the isolated compound of peridinin is the cause of the observed tissue necrosis, it provides evidence that the corallimorph

is producing an allelopathic compound capable of resulting in coral mortality. This research sets the stage for further investigation into potentially novel compounds present in *R. howesii*. Additionally, there are important implications for managing a reef experiencing an invasion or bloom of *R. howesii*. The ability of the corallimorph to interfere with corals both directly and indirectly make *R. howesii* a strong competitor on reefs.

Chapter 2, in part, is currently being prepared for submission for publication of the material. Carter, Amanda L.; Hughes, Chambers C.; Smith, Jennifer E., Evidence of allelopathy in competitive interactions between the corallimorph, *Rhodactis howesii*, and scleractinian corals. The dissertation author was the primary investigator and author of this paper.

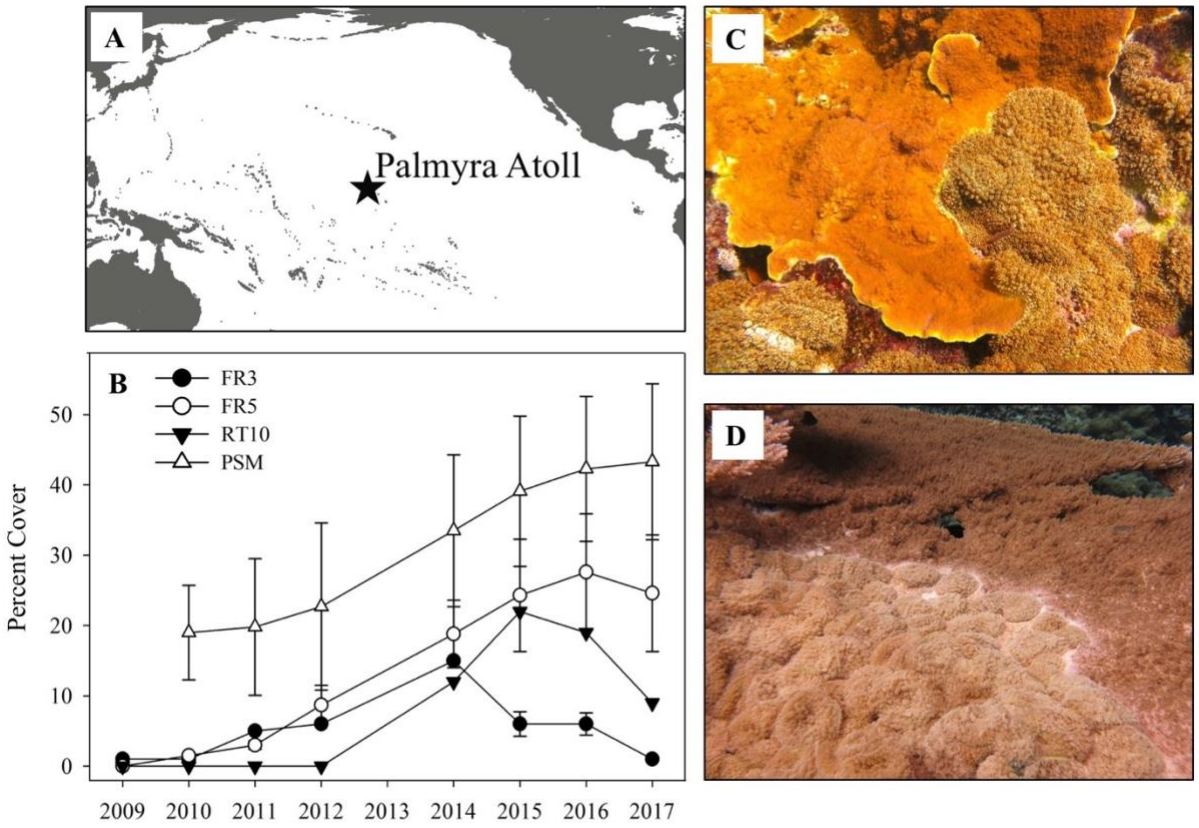

Figure 2.1. Corallimorph cover and images from the study location, Palmyra Atoll, Northern Line Islands. **A** World map showing the location of Palmyra Atoll where the samples were collected. **B** Percent cover of the corallimorph bloom at Palmyra Atoll from 2009-2017. All data points and error bars represent mean values ± 1SE. **C-D** Visual example of the corallimorph growing over *Montipora* and *Acropora* colonies at Palmyra Atoll. The white interaction band between the corals and corallimorph is visible.

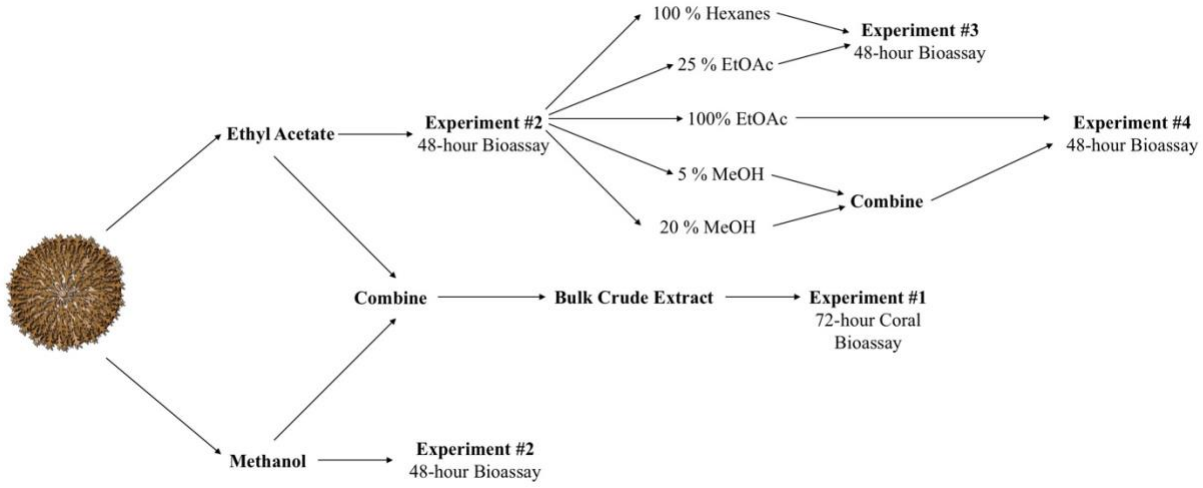

Figure 2.2. Schematic of corallimorph chemical extractions and fractionations with experiments. Starting tissue for each experiment was 250g freeze-dried corallimorph (~8 individuals). A total of 4 bioassays were run were run throughout the study. Each experiment had procedural controls and controls to test for strip effects.

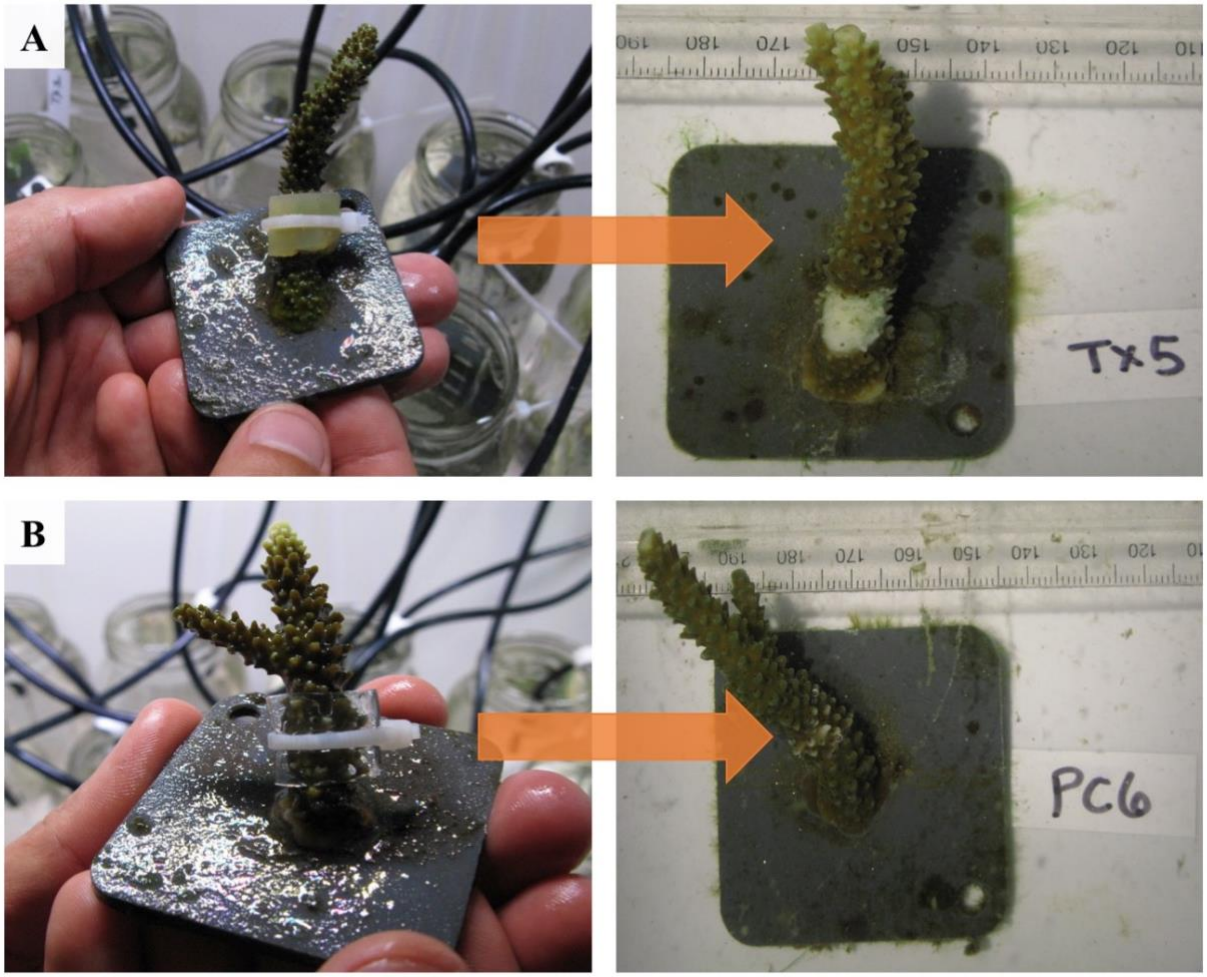

Figure 2.3. Impacts of corallimorph extract on coral tissue health following chemical bioassay. A Images showing example of initial application of a treatment Phytagel strip and the resulting coral tissue loss 48 hours later. **B** Images showing example of a procedural control phytagel strip and subsequent tissue loss 48 hours later.

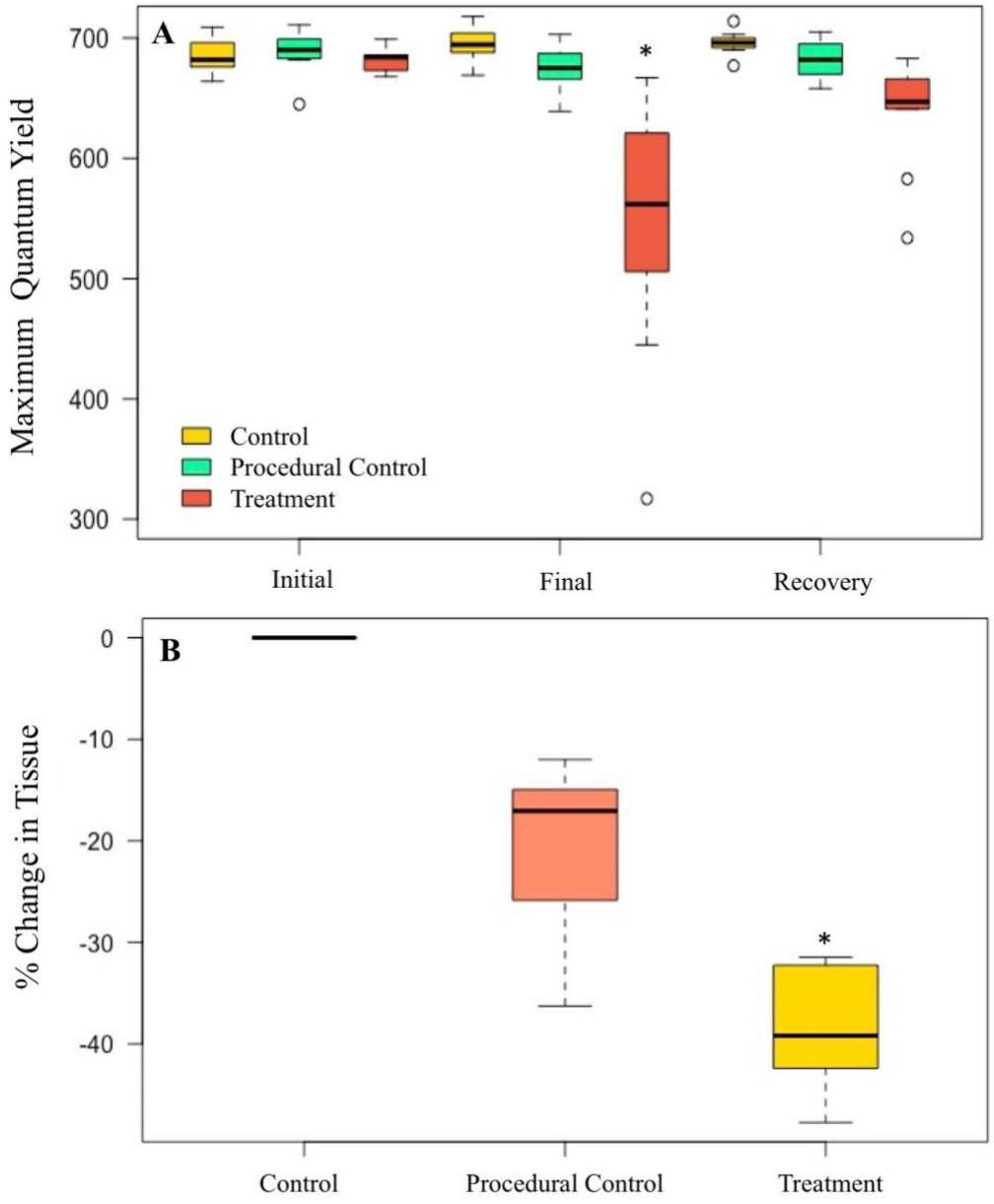

Figure 2.4. Treatment effects of corallimorph bulk crude extract from Experiment #1 (EtOAc + MeOH crude extract combination). *Acropora yongei* photosynthetic efficiency and tissue health after 72-hour bioassay. A Dark-adapted Maximum Quantum Yield measurements taken under the strip at the initial, final, and recovery time points. * denotes a significant difference (Kruskal-Wallis ANOVA, $p<0.05$). **B** Percent change (final-initial) of *A. yongei* tissue underneath the chemical strip. Exposure to the corallimorph chemical resulted in a significant loss in tissue (t-test, $p<0.05$). All data points and error bars represent mean values ± 1SE.

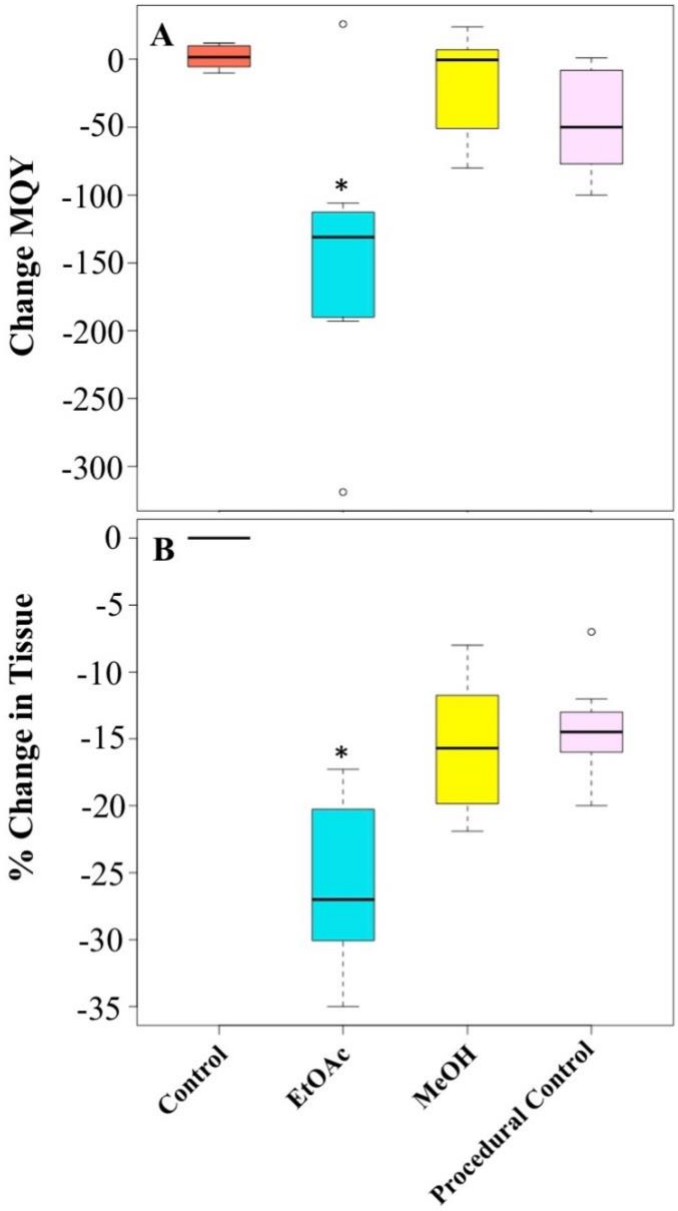

Figure 2.5. Experiment #2 treatment effects of Ethyl acetate vs. Methanol crude extracts. **A** Change in dark-adapted Maximum Quantum Yield (final – initial) measurements taken under the strip at the beginning and end of 48-hour bioassay. * denotes a significant difference (ANOVA, $p<0.05$). **B** Percent change (final-initial) of *A. yongei* tissue underneath the chemical strip. Exposure to the EtOAc extract resulted in a significant loss in tissue (ANOVA, $p<0.05$). All data points and error bars represent mean values ± 1SE.

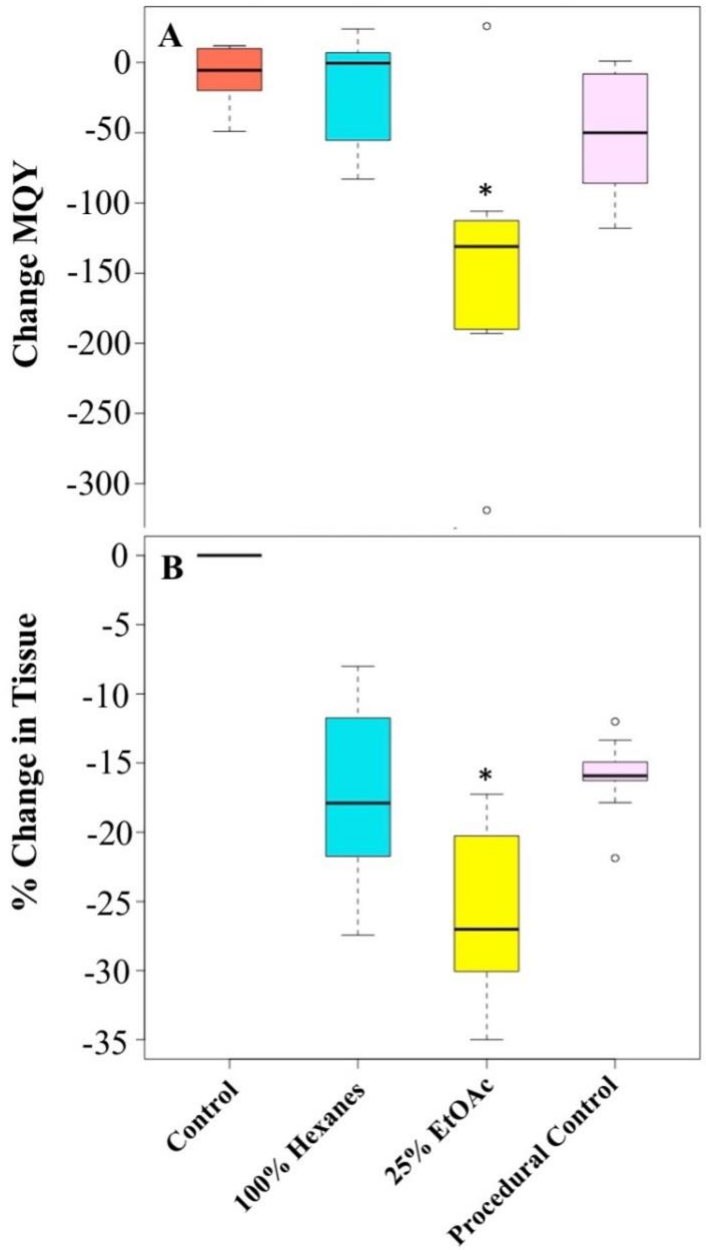

Figure 2.6. Experiment #3 treatment effects of 100 % Hexanes and 25% Ethyl acetate fractions of the EtOAc crude extract. A Change in dark-adapted Maximum Quantum Yield (final – initial) measurements taken under the strip at the beginning and end of 48-hour bioassay. * denotes a significant difference (ANOVA, $p<0.005$). **B** Percent change (final-initial) of *A. yongei* tissue underneath the chemical strip. Exposure to the 25% EtOAc extract resulted in a significant loss in tissue (ANOVA, $p<0.005$). All data points and error bars represent mean values ± 1SE.

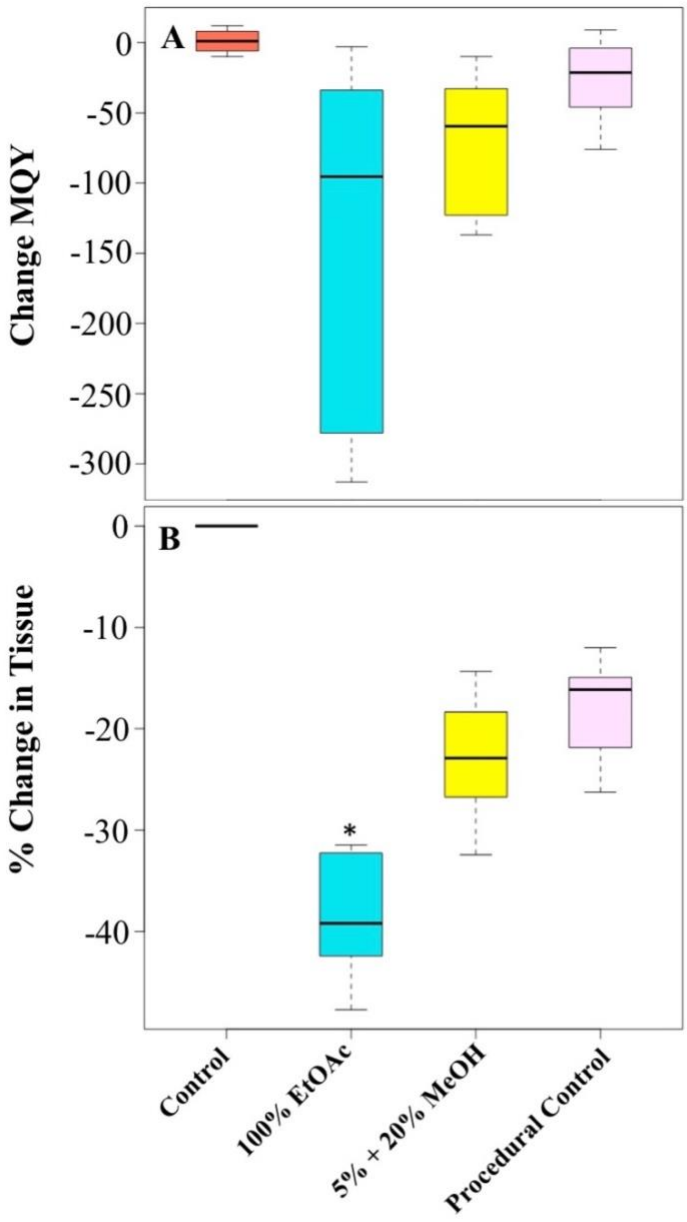

Figure 2.7. Experiment #4 treatment effects of 100 % Ethyl acetate and 5%-20% Methanol combination fractions of the EtOAc crude extract. A Change in dark-adapted Maximum Quantum Yield (final – initial) measurements taken under the strip at the beginning and end of 48-hour bioassay. **B** Percent change (final-initial) of *A. yongei* tissue underneath the chemical strip. Exposure to the 100% EtOAc extract resulted in a significant loss in tissue (ANOVA, $p<0.001$). All data points and error bars represent mean values ± 1SE.

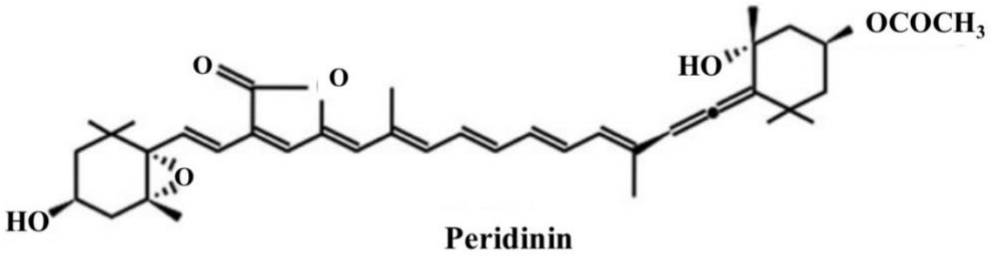

Figure 2.8. Peridinin, a light-harvesting carotenoid. Compound isolation from the 100% ethyl acetate fraction of the corallimorph tissue.

**Table 2.1. *Rhodactis howesii* ethyl acetate used for assessment of the most bioactive portion of the corallimorph chemical extract in Experiments #3-4. ***Due to low volume and concentration, the 5% and 20% MeOH fractions were combined in the final round of experiments.

Chemical Extract	Fractionation
Ethyl acetate (EtOAc)	100% hexanes
	25% EtOAc + 75% hexanes
	100% EtOAc
	5% MeOH*
	20% MeOH*

CHAPTER 3

Coral microbial community response to invasion by the corallimorph, *Rhodactis howesii*: implications for coral health

Amanda L. Carter, Luke R. Thompson, Jennifer E. Smith

ABSTRACT

Coral-associated microbial communities are a key part of the coral holobiont, which is comprised of the coral animal and its associated bacteria, archaea, fungi, and symbiotic dinoflagellates. These components play important roles in maintaining the physiological and biological functions of a coral. Shifts in these communities caused by physical or biological stressors can result in a decline in coral functions and pave the way for disease or mortality. We investigated the impact of a biological stressor on coral microbial communities at Palmyra Atoll in the Northern Line Islands. Five species of coral experiencing active invasion by the corallimorph *Rhodactis howesii* were sampled over two years across their interaction zones. Coral tissue not overgrown by corallimorph had unique microbial communities that were significantly different among species. However, microbial communities on tissues invaded by the corallimorph became more similar to one another and no longer showed significant difference among coral taxa. Opportunistic bacterial families such as *Enterobacteriaceae* and *Rhodobacteraceae* were more abundant on invaded tissue and contributed the most to increased similarity among coral species. Interestingly, microbial community composition shifted on the unexposed coral tissue in 2016, with *Enterobacteriaceae* and in some cases, *Vibrionaceae*, becoming more common. This suggests that long-term invasion can alter the microbial community on even the unexposed tissue, allowing opportunistic species to invade and possibly resulting in a reduction in coral health. This is the first studiy to examine the effects of corallimorph invasion on microbial community dynamics over a range of coral taxa.

INTRODUCTION

Corallimorphs are weedy, fast-growing benthic competitors that do well in disturbed and marginalized environments (Kuguru et al. 2007, 2010). Their ability to rapidly grow and proliferate through sexual and asexual reproduction allow them to quickly take up newly available space on a reef (Chadwick 1987, 2001; Chadwick and Adams 1991). This competitive advantage has led to observed shifts towards corallimorph dominance on reefs around the world, particularly after large disturbances (Kuguru et al. 2007, 2010; Work et al. 2008; Norström et al. 2009: Crane et al. 2016; Carter et al. 2019). Past studies have shown that corallimorphs compete with corals through indirect methods, such as colonizing available space, as well as direct methods, such as physical abrasion during overgrowth (Chadwick 1987, 1991). Previous work provided evidence that corallimorphs have a chemical component that impacts coral survivorship, even without the physical presence of the corallimorph itself (Ch. 2). These allelopathic interactions are not unique to corallimorphs; a number of macroalgal and sponge species also produce compounds that impact coral colony health and survival (Kline et al. 2006; Smith et al. 2006; Pawlik et al. 2007; Rasher & Hay 2010; Morrow et al. 2011). For example, compounds released from macroalgae can have indirect effects on corals by releasing dissolved organic carbon (DOC), which stimulates microbial growth and increases virulence (Smith et al. 2006; Kline et al. 2006; Nelson et al. 2013). Some species of macroalgae and sponges also produce secondary metabolites that can inhibit coral larval settlement or cause colony mortality through shifts in the coral-associated microbial communities away from beneficial symbionts and towards pathogenic bacteria like *Vibrio* (Thurber et al. 2009; Morrow et al. 2017).

The coral-associated microbial community (or coral microbiome), is just one part of the coral holobiont, which refers to the coral host and its complex assemblage of symbionts, bacteria, viruses, fungi, and other microorganisms that live on and within the coral tissue

(Rohwer et al. 2002; Thurber et al. 2009; Bourne et al. 2009; Ainsworth et al. 2008). These microbial communities play a role in overall coral function (e.g. protection from problematic bacteria) and metabolism (e.g., nutrient cycling), and are essential to maintaining healthy colonies (Lesser et al. 2004; Ritchie 2006; Wegley et al. 2007; Rypien et al. 2010; Krediet et al. 2013; Lema et al. 2012; Rosenberg et al. 2007, Ceh et al. 2013, Welsh et al. 2016; McDevitt-Irwin et al. 2017). It has been demonstrated that the coral microbiome is incredibly species-specific, to the extent that non-adjacent colonies from the same species will exhibit more similar microbial communities than adjacent colonies of different species (Rohwer et al. 2002; Ainsworth et al. 2015). However, stress can disrupt these unique microbiomes and causes them become more similar to one another across species due to a shift towards pathogenic bacteria (McDevitt-Irwin et al. 2019; Gibbin et al. 2019). This variation in the microbiome from coral to coral is known as 'beta diversity', and recent studies have shown that increasing beta diversity may indicate a stressed microbiome (Moeller et al. 2013; Zaneveld et al. 2016; 2017). Stressors can also affect the microbial richness, or 'alpha diversity,' of the coral microbiome (Bourne et al. 2007; Meron et al. 2011; Morrow et al. 2012; Vega Thurber et al. 2012; Santos et al. 2014; Zaneveld et al. 2016).

To date, studies investigating the impact of biological stressors on coral microbiomes have focused primarily on coral-macroalgal interactions. Here we aimed to investigate the role of corallimorphs as biological stressors, and the extent to which corallimorph overgrowth impacted coral microbial communities. Specifically, we looked for evidence that the chemical component of *R. howesii* discussed in Chapter 2 might drive negative changes in the coral-associated microbial community that could directly impact colony health. To do this, we took advantage of the corallimorph invasion at Palmyra Atoll to investigate the impacts of overgrowth by the

corallimorpharian *Rhodactis howesii* on coral microbiomes. We tagged individual colonies of 5 different coral species undergoing invasion by the corallimorph (*Acropora cytherea, Astreopora myriophthalma, Montipora aequituberculata, Montipora capitata,* and *Montipora monasteriata,* Fig. 3.1). We collected tissue samples across the corallimorph interaction zone in 2015 and then resampled the corals in the same location in 2016 (Fig. 3.1H). Corallimorph impacts on the coral microbiome were assessed using 16S rRNA sequencing. We hypothesized that 1) unexposed coral tissue would have unique microbiomes across coral species, and 2) invasion by the corallimorph would increase both beta and alpha diversity of the coral microbiome, indicating a stressed state.

METHODS

Study location and sampling sites

Samples were collected on the Western reef terrace at Palmyra Atoll in the Northern Line Islands (05°52' N, 162°06' W) in the Central Pacific (Figure 3.1A). The reef terrace of Palmyra has been experiencing a bloom of the corallimorph, *Rhodactis howesii*, following a ship-grounding that occurred in 1991 (Work et al. 2008, 2018; Carter et al. 2019). Although the corallimorph is native to the Indo-Pacific, it has demonstrated highly invasive tendencies at Palmyra Atoll, taking advantage of open or disturbed space, and in some cases, overgrowing hard coral (Carter et al. 2019). Aside from the corallimorph bloom, Palmyra is relatively free of other local, anthropogenic disturbances and has healthy fish and coral populations (Dinsdale et al. 2008; Sandin et al. 2008; Smith et al. 2016). Other than a brief military occupation during WWII, the atoll has remained uninhabited and was designated a U.S. Fish and Wildlife Refuge in 2001 and part of the Pacific Remote Islands Marine National Monument in 2009 (Dawson 1959;

Brainard et al. 2005). Samples were collected at two shallow sites on the reef terrace (~5m depth) which have experienced high corallimorph growth over the past 10 years (Figure 3.1B, Carter et al. 2019).

Coral sampling

To ascertain if coral microbiome alpha and beta diversity varied across the coral-corallimorph interaction zone, samples were collected along a 30 cm section spanning unexposed coral tissue, the interaction zone, and invaded tissue (Figure 3.1H). To assess species-specific responses to corallimorph interactions, we sampled 5 coral species and tagged 8 colonies of each species to allow for repeat sampling over time. The 5 species of coral spanned a range of morphologies as follows: *Acropora cytherea* (AC, plating), *Astreopora myriopthalma* (AM, mounding), *Montipora aquituberculata* (MA, foliose/plating), *Montipora capitata* (MC, corymbose), and *Montipora monasteriata* (MM, encrusting) (Figure 3.1C-G). Each colony had six replicates taken across the interaction zone: two from unexposed tissue, 2 from the interaction zone itself, and 2 from invaded tissue (Figure 3.1H). Fragments of coral were collected using bone cutters or a small chisel and placed into individual whirl-pak bags. Samples were stored on ice for transport back to the field station, where they were transferred into RNA-later in preparation for 16S rRNA amplicon sequencing. All colonies were re-sampled in September of 2016 following the same protocol with the exception of four *Montipora capitata* colonies, which were completely overgrown by corallimorph upon our return (n = 288 samples).

DNA extraction and 16s rRNA gene amplicon sequencing and processing

Coral fragments in RNA later were stored at -20°C at Scripps Institution of Oceanography prior to extraction. The coral fragments were processed using the Earth Microbiome Project protocols (www.earthmicrobiome.org), specifically for DNA extraction (Marotz et al. 2017) and amplification and sequencing of the 16S rRNA genes.

Primary data processing of the amplicon sequence data was prepared for bacterial community analysis using the following work flow:

> Sequence data was processed using QIIME 2 (Bolyen et al. 2018). QIIME 2 commands were executed using a Snakemake workflow (Köster et al. 2012) called Tourmaline (https://github.com/NOAA-AOML/tourmaline). The full workflow used is described at https://github.com/cuttlefishh/papers/palmyra-corals/tourmaline and included in the Zenodo archive. Briefly, demultiplexed fastq sequences were denoised using DADA2 (Callahan et al. 2016) in paired mode to generate a count table of amplicon sequence variants (ASVs). ASV sequences were assigned taxonomy using a naïve Bayes classifier with Release 132 of the SILVA 16S rRNA database (Quast et al. 2013) clustered at 97% identity with 7-level taxonomy formatted for QIIME. A phylogenetic tree was built by aligning ASV sequences with MAFFT (Katoh et al. 2013), masking the alignment, building a tree with Fasttree (Price et al. 2010), and midpoint rooting the tree. For downstream analyses, sequences assigned as "Archaea", "Chloroplast", or "Mitochondria" were filtered from the observation table.

Data analysis

Replicates were lumped into unexposed, interaction, and invaded zones as described above to examine changes across the interaction zone (n=288 samples). Sequences were

classified to the lowest taxonomic resolution possible and analyzed as described below. The relative abundance of bacterial families was observed using boxplots from ASV tables that were normalized to relative abundance, grouped and averaged by zone/year, and taxa (family level, "D5"). Taxa with average relative abundance >1% across all groups were plotted, and taxa not in these groups were placed in "Others".

Changes in alpha and beta diversity for each coral taxa

ASV counts were rarefied to 1000 sequence reads per sample. From these ASVs, alpha diversity (defined here as the number of unique species present in a sample) was examined for each species using a two-way ANOVA with region of the interaction zone (unexposed, IZ, IV) and year as the factors. Significant results were analyzed using a Tukey's Honest Significant Difference test (HSD) using the program R version 3.5.1 (R Core Team 2013 and related packages).

To assess how coral microbiome beta diversity varied across coral taxa along interaction zones and across years, we used a two-factor, multivariate permutation-based analysis of variance (PERMANOVA) on Bray-Curtis similarity metrics on log (x+1) transformed data of unique OTUs (partial sum of squares, 9999 permutations). Canonical Analysis of Principal (CAP) coordinates were used to visualize the differences among species across each zone over time. The dissimilarity for each coral taxa across zone and year was looked at with a Similarity Percentage (SIMPER) analysis. Multivariate analyses based on Bray-Curtis distances were conducted in PRIMER-E v6 (PERMANOVA +) software package (Clarke and Gorley, 2006).

Changes in alpha and beta diversity across zones

ASV counts were rarefied to 1000 sequence reads per sample. From these ASVs, alpha diversity (the number of unique species present in a sample) was examined for each zone using a two-way ANOVA with species and year as the factors. Significant results were analyzed using a Tukey's Honest Significant Difference test (HSD) using the program R version 3.5.1 (R Core Team 2013 and related packages).

To assess how coral microbiomes beta diversity varied across interaction zones among coral taxa and across years, we used a two-factor, multivariate permutation-based analysis of variance (PERMANOVA) on Bray-Curtis similarity metrics on log (x+1) transformed data of unique OTUs (partial sum of squares, 9999 permutations). The difference among zones across each species and over time was visualized using CAP ordinations. The dissimilarity for each zone across species and year was looked at with a Similarity Percentage (SIMPER) analysis. Multivariate analyses based on Bray-Curtis distances were conducted in PRIMER-E v6 (PERMANOVA +) software package (Clarke and Gorley, 2006).

RESULTS

Sequencing of microbial communities isolated from 5 coral species across interaction zones with the invasive corallimorph *R. howesii* resulted in 9,440,636 paired reads (18,881,272 reads) from 288 samples. The DADA2 feature tables contained 21,006 unique ASVs (median length: 253 bp) totaling 5,191,726 observations over 288 samples. Sequence data and metadata are available from the Qiita database (González et al. 2018) with study number 10798 and from the European Nucleotide Archive. Observation (ASV) and metadata tables are available from Zenodo.

Changes in alpha and beta diversity for each coral taxa across years

The two-way ANOVA showed significant changes in alpha diversity of *Acropora cytherea* (AC) for both zone and year, but no significant interaction was observed (Table 3.1). Coral microbiome beta diversity for AC was also significantly different across zone and year, but there was also a significant interaction between the two (two-factor PERMANOVA, Table 3.2). Interestingly, AC was one of the few corals to have Vibrios present in the top five bacterial families. However, the percent contribution to the similarity among AC IZ samples was only 3.55%, so still quite low. *Endozoicomonas*, Enterobacteriaceae and Rhodobactereaceae were also abundant for AC across all zones and years.

Changes in alpha diversity for *Astreopora myriophthalma* were significant across zone but not year (two-way ANOVA, Table 3.1). A similar pattern was seen in the microbial beta diversity, with a significant difference across zones but not for year (two-factor PERMANOVA, Table 3.2). Unexposed tissues and the interaction zone of AM were dominated by Flavobacteriaceae, Terasakiellaceae, and Enterobactereaceae for both years. The invaded zone for AM in 2015 was dominated by Enterobacteriaceae (37.52% similarity contribution) and Unclassified bacteria (12.92%). In 2016 the IV was still Unclassified bacteria (26.77% contribution), Rhodobacteraceae (15.72%), and Enterobacteriacae (11.44%).

In contrast, alpha diversity of *Montipora aequituberculata* was not significantly different across zone, but was for year (two-way ANOVA, Table 3.1). However, coral microbiome beta diversity was significantly different for both zone and year, although their interaction was not significant (two-factor PERMANOVA, Table 3.2). Unexposed MA tissues in 2015 and 2016 had high levels of Enterobacteriaceae, Flavobacteriaceae, and Rhodobacteraceae. The IZ changed between 2015 and 2016, maintaining Flavobacteriaceae and Rhodobacteraceae, but shifting from

unidentified gammproteobacteria in 2015 to Alteromonadaceae and Idiomarinaceae in 2016. Invaded tissue was similar in both years and was dominated by Enterobacteriaceae and Rubritaleaceae.

Montipora capitata showed no significant differences in alpha diversity for any factors (two-way ANOVA, Table 3.1). However, beta diversity was significantly different for both zone and year but not the interaction between the two (two-factor PERMANOVA, Table 3.2). All three zones of *M. capitata* (U, IZ, IV) were dominated by families associated with invaded tissue (Enterobacteraceae, Rhodobacteraceae, and unclassified bacteria), despite the significant difference between the zones.

Montipora monasteriata showed no significant difference in alpha diversity across any factors (two-way ANOVA, Table 3.1), although bacterial beta diversity across both zone and year were significantly different (two-factor PERMANOVA, Table 3.2). All three zones of MM were also characterized by Enterobacteraceae, Rhodobacteraceae, and unclassified bacteria, although there was significant difference between zones and across years.

Changes in alpha and beta diversity across zones across years

Alpha diversity of unexposed tissue was significantly different across species but not across year or species x year (two-way ANOVA, Table 3.3). Overall, unexposed coral tissue showed significantly different microbial beta diversity across species, year, and the interaction of the two factors (two-factor PERMANOVA, Figure 3.6; Table 3.4). Similarity Percentage analysis of unexposed *A. cytherea* tissue in 2015 was populated by Gammaproteobacteria (32.41%), Deltaproteobactera (9.41%), and two unclassified bacterial species (13.48%). The family Enterobacteriaceae, order Myxococcales, and family Endozoicomonadaceae were the

most abundant bacterial groups across all unexposed AC samples (Figure 3.4). Unexposed *A. myriophthalma* (AM) samples were primarily dominated by Alphaproteobacteria (similarity contribution: 26.66%) and Bacteroidia (16.12%) along with Gammaproteobacteria (20.55%). The families Terasakiellaceae, Flavobacteriaceae, and Enterobacteriaceae were the three most abundant bacteria in the healthy AM replicates. In contrast to the *Acropora* and *Astreopora* colonies, the *Montipora* species had 3-4x the number of bacterial species present, which was reflected in their high levels of unique OTUs/sample (Figure 3.5A,D). All three species of *Montipora* (MA, MC, and MM) were dominated by Gamma- and Alphaproteobacteria.

In 2016, there was greater variability in beta diversity across unexposed tissue, although coral species were still significantly different from one another (Figure 3.6) and the relative abundance of bacterial taxa shifted from the starting community observed in 2015 for all coral species. Interestingly, Enterobacteriaceae was the first or second most abundant species for AC, AM, MA, and MC in 2016. The bacterial community for MM shifted the most, with Idiomarinaceae, Vibrionaceae, and Altermonadaceae becoming the most abundant families present (Figure 3.4).

Overall, tissue from the interaction zone was highly significantly different across coral species, years, and the interaction between the two (two-factor PERMANOVA, Table 3.4, Figure 3.6). Samples from the interaction zones (IZ) were significantly different from those collected from tissue unaffected by the corallimorph for all coral species and across years (Table 3.4). While unique species-specific microbial communities were still evident, beta diversity increased and there was more variability and a larger spread in the similarity metrics at the interaction zones for all taxa (Figure 3.6). Pathogenic bacterial families also appear in higher relative abundance across coral species (e.g. Vibrionaceae on AC and Colwelliaceae on AM) and there

was a shift towards dominance by Rhodobacteraceae, which was the first or second most abundant family across all corals at the IZ.

In contrast, bacterial community beta diversity in the invaded zone (IV) was not significantly different across coral taxa, showing greater overall similarity regardless of species (Figure 3.6; PERMANOVA, $F = 0.95$, $p > 0.05$). Nor was there a significant difference between years or an interaction between the two factors. The similarity between coral species was largely driven by dominance of Enterobacteriaceae, which was either first or second most abundant bacterial family across all coral species in the invaded zone for both years. Rhodobacteraceae and unclassified bacteria were also present across species and contributed to the microbial community similarity across all species.

All species showed higher dissimilarity between healthy and interaction zones and healthy and invaded zones in 2015 vs. 2016 (e.g. AC 2015: H & IZ avg. dissimilarity = 79.92, H & IV Avg. dissimilarity = 81.91 vs. AC 2016: H & IZ avg. dissim. = 63.99, H & IV avg. dissim. = 77.38) (Figure 3.6). This reflects the lack of significant difference between species in invaded zones as discussed above, and is largely driven by Enterobacteraceae, Rhodobacteraceae, and unclassified bacteria within species (Figure 3.7).

DISCUSSION

Overall, diversity and composition of coral microbiomes varied across species, corallimorph interaction zone, and time. Our first hypothesis, that coral microbiomes would be unique across coral species on uninvaded tissue, was supported by our findings, and agrees with previous studies that have shown the ability of coral species to maintain unique microbial assemblages across space and time (Rohwer et al. 2002, Figure 3.6A). However, the species-

specific microbial associations began to break down with increased proximity to the corallimorph, *R. howesii* (Figure 3.6B,C). This supported part of our second hypothesis, that coral microbiome beta diversity would increase with corallimorph invasion. However, changes in alpha diversity varied broadly across species and between time points. Some species showed the expected increase in alpha diversity (AC, AM, MA 2015) with proximity to the corallimorph, while other species showed the opposite (MC 2016) (Figs. 3.2, 3.5). Overall, 2016 showed more variability in both alpha and beta diversity of the coral communities, suggesting that prolonged corallimorph invasion may lead to varied and lasting effects on both uninvaded and invaded tissue (Fig. 3.6D-F).

Unexpected variability in alpha diversity

Previous studies have shown that coral microbial alpha diversity increases when exposed to physical stressors (Meron et al. 2011; Lee et al. 2016; Ziegler et al. 2016b) and on diseased corals (Sunagawa et al. 2009). McDevitt-Irwin et al. (2017) suggested that increased alpha diversity was indicative of a coral's inability to regulate its microbiome when stressed. In light of this, we predicted that increasing stress of invasion in the zones of interaction and invasion would result in higher alpha diversity of the coral microbiome across species. This prediction was supported by significantly different and increasing alpha diversity for *Acropora* and *Astreopora*, but in contrast, all three *Montipora* species had higher levels of alpha diversity on uninvaded tissue which decreased across the interaction zone (Fig. 3.5). This pattern of decreasing alpha diversity has been seen in other hard corals when exposed to secondary metabolites from the macroalga *Lobophora variegata* (Morrow et al. 2017). It has been hypothesized that the anti-microbial properties of some algal-derived secondary metabolites may

reduce species richness, thus resulting in decreased alpha diversity with proximity to the competitor. Depletion of microbes can result in bleaching and tissue necrosis, as Glasl et al. (2016) observed after antibiotic treatment of healthy corals. Given the corallimorphs observed allelopathic abilities (Ch. 2), it is possible that the decrease in alpha diversity observed across *Montipora* may be a result of corallimorph-associated secondary metabolites. Interestingly, the compound peridinin that was found in the corallimorph tissue in Ch. 2 has anti-microbial properties (Suzuki et al. 2003; Liu & Nizet 2009), but it is difficult to ascertain if these properties would impact tissue health, as peridinin is a common carotenoid found in coral symbionts (Roth 2014). In particular, *Montipora capitata* showed significant declines in species richness when in contact with the corallimorph and was also highly susceptible to overgrowth, as half of the colonies sampled in 2015 were subsequently overgrown by the corallimorph and suffered complete mortality by 2016. Overall, these contrasting findings suggest that alpha diversity may not be the best metric for measuring stress in coral microbial communities, and interpretation of changes in species richness should be approached with caution.

Corallimorph invasion increases microbial beta diversity

Coral microbiome beta diversity of uninvaded coral tissue was significantly different across species in 2015 and 2016, supporting our hypothesis that coral species would maintain unique microbial communities (Figure 3.6, Table 3.4). However, the dissimilarity of the uninvaded microbiomes did increase in 2016, although not significantly, suggesting that prolonged competition with the corallimorph was stressing the corals ability to regulate its microbiome. Interaction zone tissue was significantly different from uninvaded tissue for all coral species and across both years (Table 3.4), and exhibited increasing beta diversity,

supporting the hypothesis that host capacity to regulate the microbial community diminishes with increasing stress (Zaneveld et al. 2017). Of interest was an increase in the relative abundance of pathogenic bacterial families at the interaction zone (e.g. *Vibrionaceae* and *Colwelliaceae*), which have been found to increase variation between coral colonies of the same species (Vidal-Dupiol et al. 2014) and can contribute to a decline in coral health (Thurber et al. 2009). Shifts towards *Vibrio* dominance have also been observed after exposure to allelochemicals derived from the macroalgae *Lobophora variegata* (Morrow et al. 2017), supporting our theory that chemical components from the corallimorph observed in Ch. 2 may contribute to changes in the coral microbiome. Bacterial community beta diversity in the invaded zone was not significantly different across coral taxa, supporting the hypothesis that stress drives increased similarity in coral microbiome beta diversity (Zaneveld et al. 2016; McDevitt-Irwin et al. 2019). The observed increase in bacterial community beta diversity (or increased similarity across samples) for all coral species at the invaded zone across both years (Figure 3.2C, 3.2F) was largely driven by the presence of an unclassified bacteria, Rhodobactereaceae, and Enterobactereaceae. It is possible that the unclassified bacteria may be a novel group associated with the corallimorph, *Rhodactis howesii*, but further investigation and classification would be required.

Varied responses in coral microbiome community composition

Although coral microbiome community composition was significantly different across species on uninvaded tissue, varying responses to corallimorph proximity were seen across coral species. Prolonged competition with the corallimorph lead to increased spread in beta diversity within coral species on uninvaded tissue in 2016, although they still remained significantly different across species (Fig. 3.6D). Enterobacteriaceae, a copiotrophic bacteria, was the first or

second most abundant species in uninvaded tissue for AC, AM, MA, and MC in 2016, and is commonly associated with increased DOC on macroalgal-dominated reefs (Haas et al. 2016). Enterobacteriaceae also drove the increased similarity in invaded tissue across coral taxa (Figure 3.6; PERMANOVA, $F = 0.95$, $p > 0.05$). It is possible that the corallimorph, a highly productive mixotrophic organism, may also release DOC that fuels bacterial growth in a similar manner to macroalgal compounds (Smith et al. 2006; Haas et al. 2016), resulting in the change in relative abundance of Enterobacteriaceae around the corallimorph.

All coral tissue in the interaction zone exhibited high relative abundance of Rhodobacteraceae, an opportunistic taxa that is commonly associated with unhealthy, diseased coral tissue (Thurber et al. 2009; Meron et al. 2011; Sharp et al. 2012; Röthig et al. 2016; McDevitt-Irwin et al. 2017). Potentially pathogenic bacterial families like Vibrionaceae and Colwelliaceae also increased in relative abundance at the interaction zones, while *Oceanospiralles*, a bacterial family strongly associated with healthy coral tissue, decreased in relative abundance across the interaction zones for all coral species with the exception of *A. cytherea*. These data support findings that stressors may shift coral microbiomes towards a more diseased state (Thurber et al. 2009; McDevitt et al. 2017, 2019), and support our hypothesis that corallimorph invasion would negatively affect coral microbial communities, whether through chemical compounds, DOC exudates, or other mechanisms.

In conclusion, our study provides evidence that corallimorph overgrowth can destabilize coral microbial communities as seen through shifts in alpha and beta diversity. Overall, relative abundance of potentially pathogenic and opportunistic bacterial families increased with proximity to the corallimorph and beneficial bacterial families decreased. However, species-specific responses to the corallimorph were observed. For example, *A. cytherea* was the only

coral species to maintain relatively high abundance of beneficial bacteria (*Oceanospiralles* and Endozoicomonas) over time and across the interaction zone. Interestingly, *A. cytherea* was the only coral to experience retraction of the corallimorph at the interaction zone when resampled in 2016. Future studies should build upon this work to look for impacts of biotic stressors on coral-associated microbial communities and to search for evidence of potential microbiome stability while under stress.

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Chapter 3, in full, is currently being prepared for submission for the publication of the material. Carter, Amanda L.; Thompson, Luke R.; Smith, Jennifer E., Coral microbial community response to invasion by the corallimorph, *Rhodactis howesii*: implications for coral health. The dissertation author was the primary investigator and author of this material.

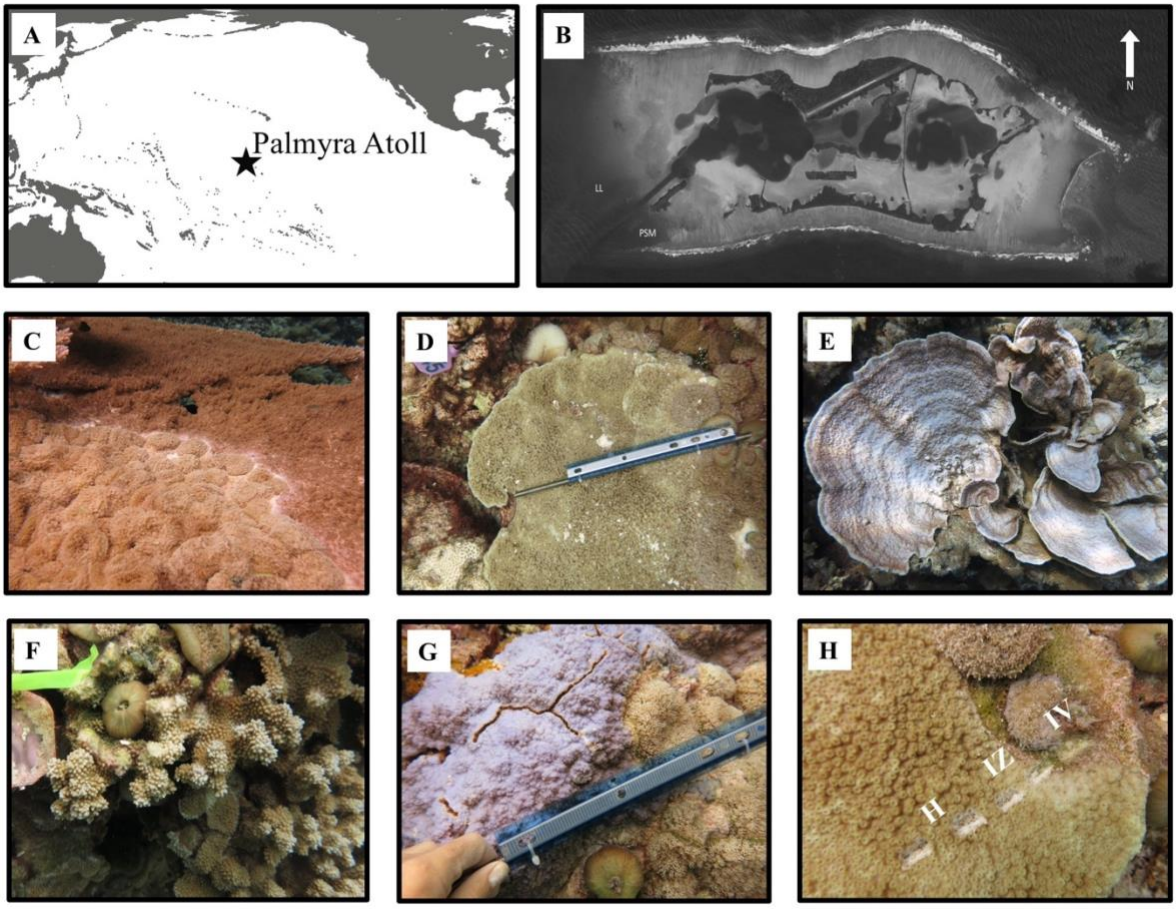

Figure 3.1. Map showing study location of Palmyra Atoll and sampling sites on Western reef terrace. Representative photos of each species sampled during study and of the sampling protocol. A Location of Palmyra Atoll in the Central Pacific Ocean. **B** Satellite imagery of Palmyra Atoll with sampling sites on western reef terrace labeled, Longliner (LL) and Penguin Spit Middle (PSM). **C-G** Species sampled in 2015 and 2016 as follows: *Acropora cytherea, Astreopora myriophthalma, Montipora aequituberculata, Montipora capitata,* and *Montipora monasteriata.* **H** Location of sample replicates taken across interaction zone of the coral species (n=2 replicates per zone).

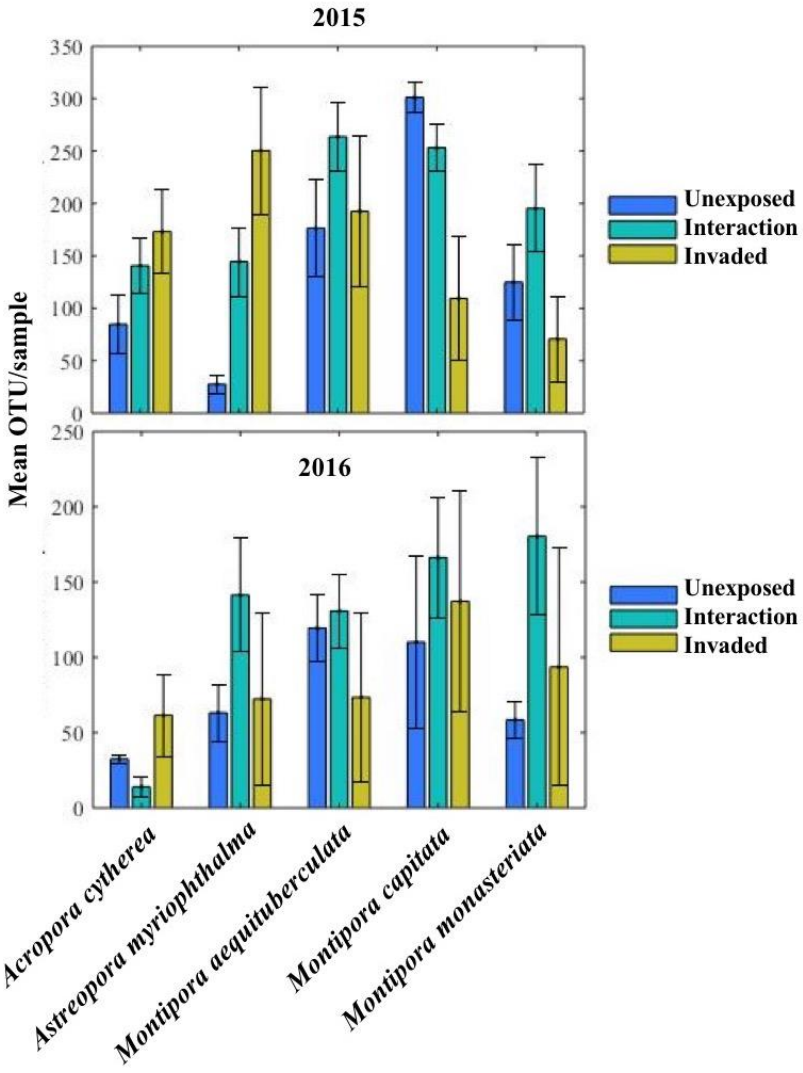

Figure 3.2. Bar graphs of alpha diversity as measured by unique OTUs/sample (means ± SE) for each species across zones by year. Species associated alpha diversity measurements 2015 (top graph) and 2016 (bottom graph) across healthy, interaction, and invaded zones. Each sample was rarefied to a minimum of 1000 sequence reads per sample before means ± SE calculation.

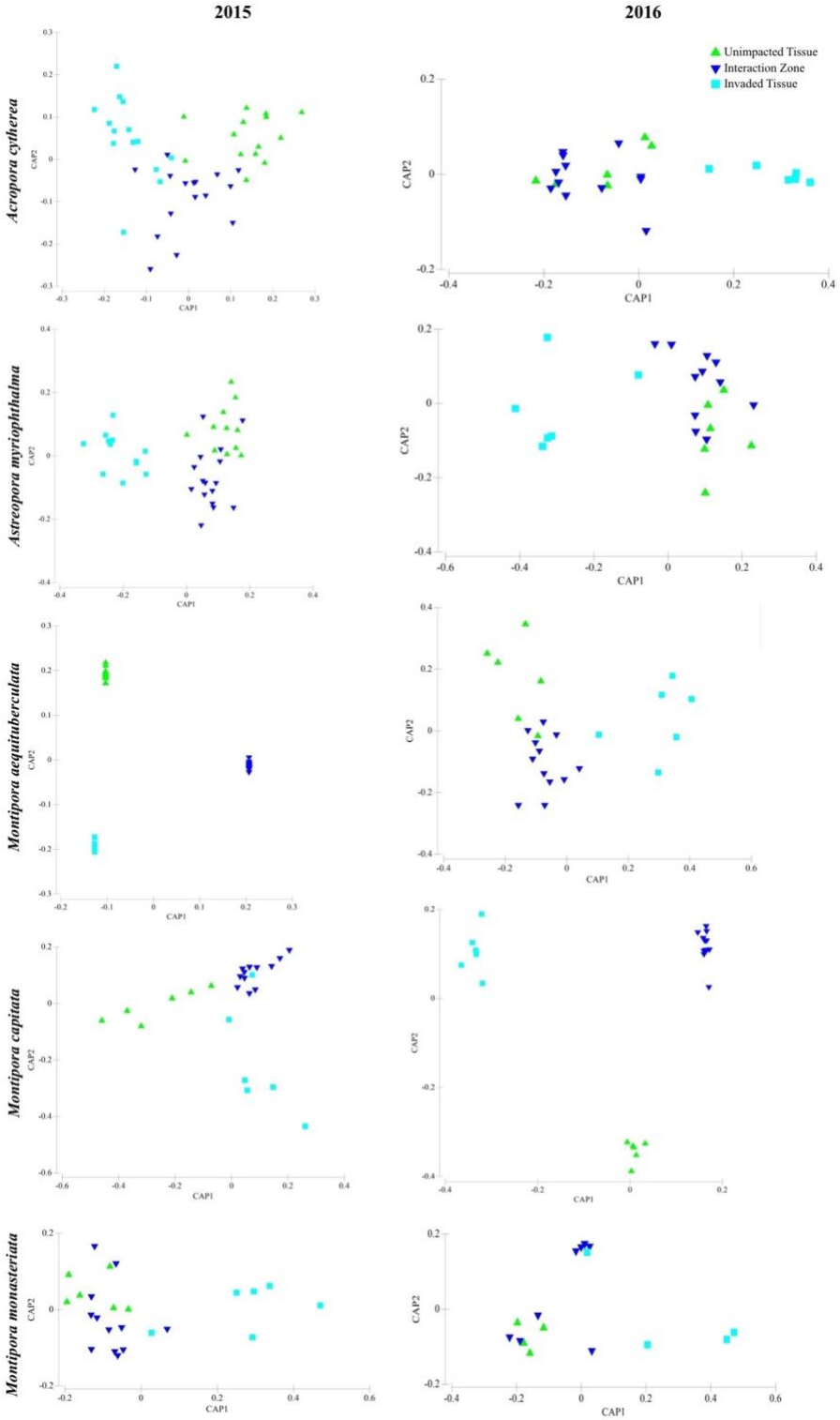

Figure 3.3 Canonical analysis of principal coordinates (CAP) of microbial community composition by zone (Unexposed, Interaction, and Invaded) for all coral species across years. Left column shows position of zone replicates for each species in 2015. Right column shows position of zone replicates for each species in 2016.

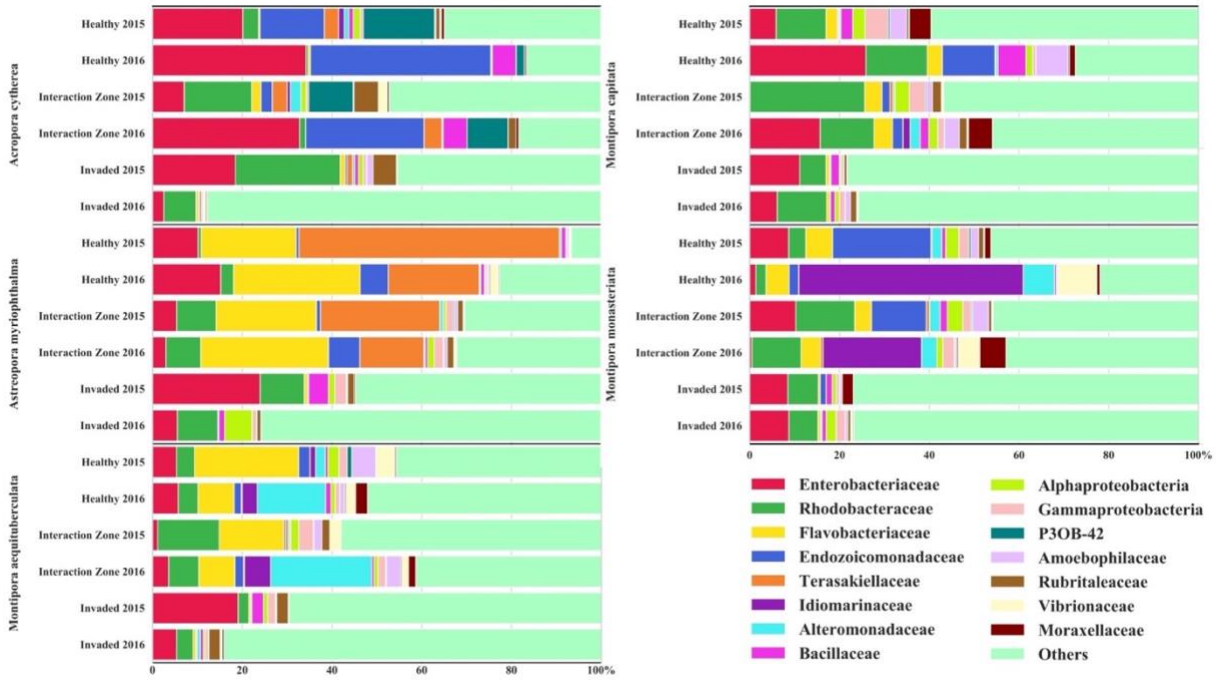

Figure 3.4. Mean proportions of bacterial families contributing to the microbial assemblage of 5 species of coral at Palmyra Atoll, Northern Line Islands. Species names (*n*=5 species) are shown along the left of the graph with each column corresponding to a specific zone and sampling year. The zones *healthy (unexposed)*, *interaction,* and *invaded* correspond to where the samples were taken along the coral colony experiencing overgrowth by the corallimorph, *Rhodactis howesii.*

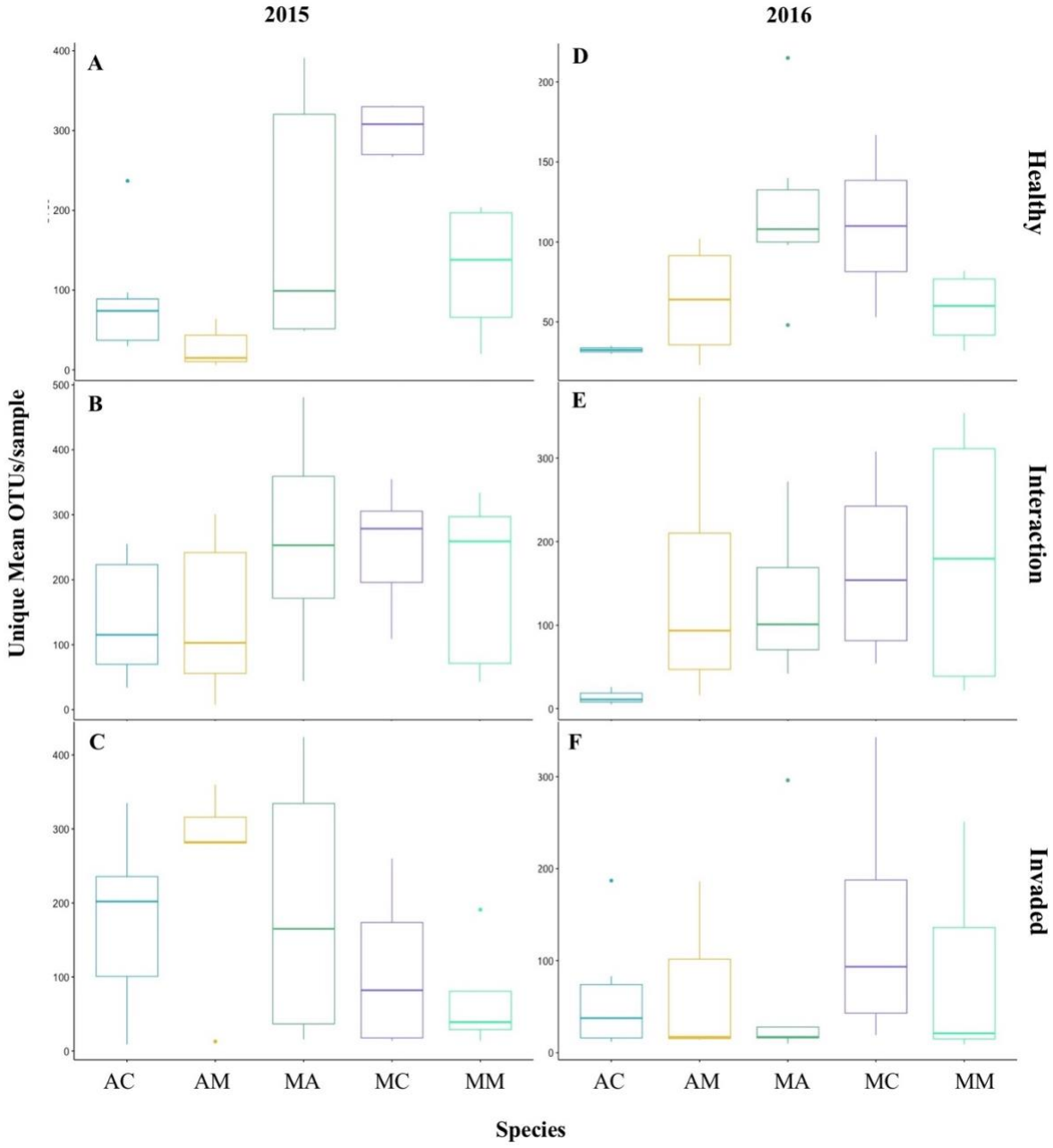

Figure 3.5. Plots of alpha diversity as measured by unique OTUs/sample (means ± SE) of coral species for each zone by year. A-C Species associated alpha diversity measurements for 2015 across healthy (A), interaction (B), and invaded (C) zones. **D-F** Species associated alpha diversity measurements for 2016 across healthy (D), interaction (E), and invaded (F) zones. Each sample was rarefied to a minimum of 1000 sequence reads per sample before means ± SE calculation.

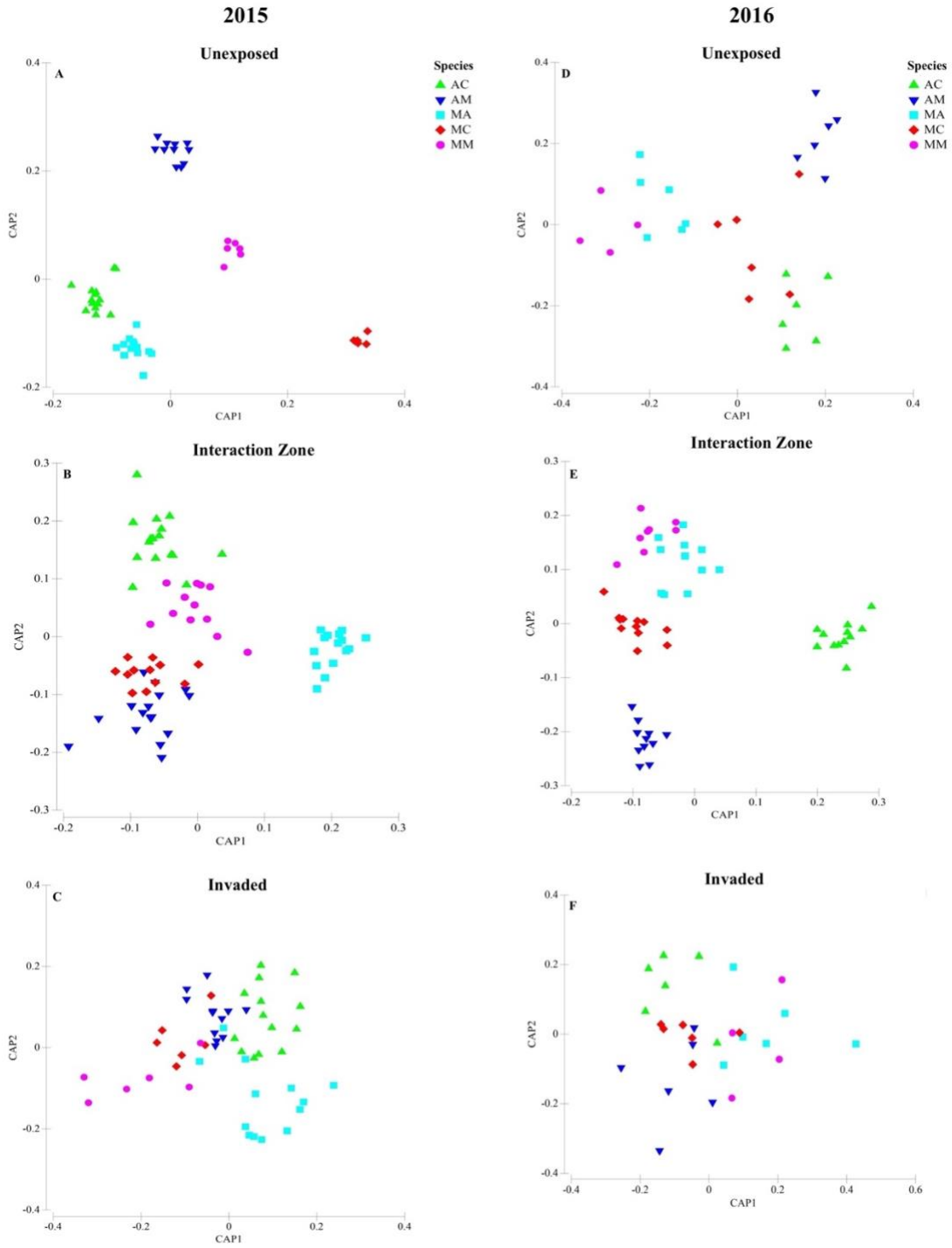

Figure 3.6. Canonical analysis of principal coordinates (CAP) for microbial community composition for each zone across species and by year. A-C CAP ordinations for 2015 for unexposed (A), interaction (B), and invaded (C) zones. **D-E** CAP ordinations for 2016 for unexposed (D), interaction (E), and invaded (F) zones. Coral species legend in top right corner of A & D for *Acropora cytherea, Astreopora myriophthalma* (AC)*, Montipora aequituberculata* (MA)*, Montipora capitata* (MC)*,* and *Montipora monasteriata* (MM).

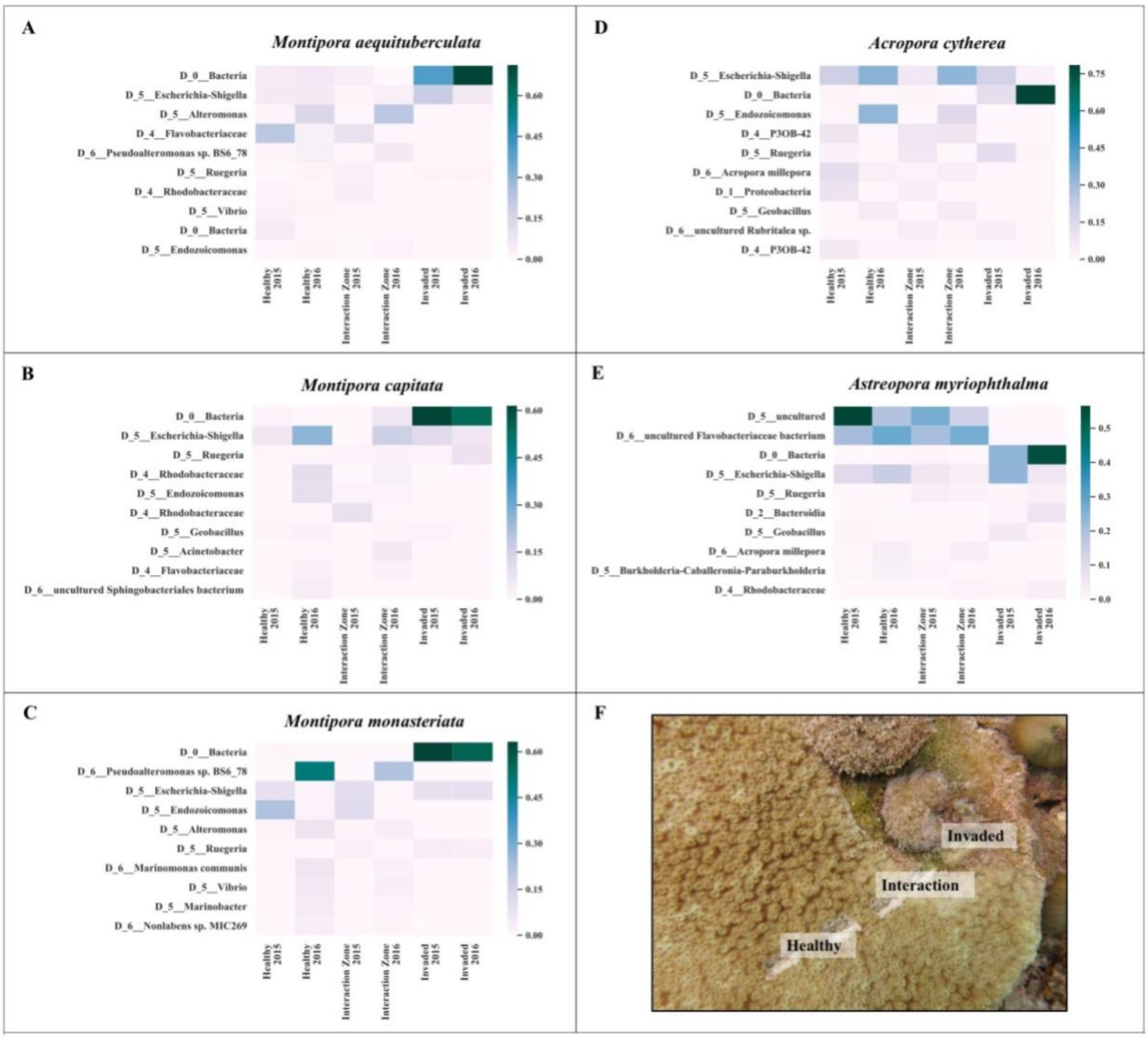

Figure 3.7. Heatmaps showing the top-10 amplicon sequence variants (ASVs) for each species with an example figure illustrating where each zone was taken from on a coral colony. A-E Heatmaps showing top-10 ASVs for all coral species. X-axis organized by zone/year combinations from healthy to invaded tissue. **F** Reference photo of zone locations sampled on a representative coral colony.

Table 3.1. Two-way ANOVA results examining change in alpha diversity across zone and year for each species.

Species	Factor	F	P value
AC	Zone	3.526	0.0430
	Year	15.606	0.0005
	Zone x Year	0.816	0.4523
AM	Zone	7.086	0.0031
	Year	0.990	0.3281
	Zone x Year	1.317	0.2834
MA	Zone	1.063	0.3546
	Year	7.534	0.0089
	Zone x Year	0.367	0.6953
MC	Zone	1.007	0.3809
	Year	1.113	0.3024
	Zone x Year	2.958	0.0719
MM	Zone	2.932	0.0726
	Year	0.189	0.6671
	Zone x Year	0.587	0.5636

Table 3.2. Results from a two-factor PERMANOVA examining changes in bacterial community composition as a result of zone and year for each coral species. Interactions between factors are listed as "Zone x Year."

Species	Factor	Pseudo-F	P value
AC	Zone	2.554	0.0042
	Year	4.316	0.0010
	Zone x Year	2.248	0.0095
AM	Zone	3.507	0.0001
	Year	1.687	0.0768
	Zone x Year	0.851	0.6370
MA	Zone	5.400	0.0001
	Year	4.846	0.0006
	Zone x Year	1.354	0.1469
MC	Zone	3.112	0.0016
	Year	4.900	0.0009
	Zone x Year	1.730	0.0658
MM	Zone	2.769	0.0018
	Year	3.164	0.0055
	Zone x Year	0.981	0.4431

Table 3.3. Two-way ANOVA results examining change in alpha diversity across species and year for each zone.

Zone	Factor	F	P value
Unexposed Tissue	Species	6.640	0.0004
	Year	3.086	0.0868
	Species x Year	0.588	0.67334
Interaction Zone	Species	1.785	0.1400
	Year	9.270	0.0032
	Species x Year	1.691	0.1603
Invaded Tissue	Species	1.304	0.2950
	Year	2.470	0.1280
	Species x Year	1.609	0.2020

Table 3.4. Results from a two-factor PERMANOVA examining changes in bacterial community composition as a result of species and year for each zone. Interactions between factors are listed as "Species x Year."

Species	Factor	Pseudo-F	P value
Unexposed Tissue	Species	2.841	0.0001
	Year	2.667	0.0076
	Species x Year	1.740	0.0039
Interaction Zone	Species	4.358	0.0001
	Year	7.096	0.0001
	Species x Year	3.663	0.0001
Invaded Zone	Species	0.944	0.5117
	Year	1.657	0.1082
	Species x Year	0.858	0.6441

CHAPTER 4

Coral microbial community response to a bleaching event at Palmyra Atoll, central Pacific Ocean

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ABSTRACT

Mass coral bleaching events are becoming more frequent and more severe, with the longest bleaching event on record occurring from 2015–2016 in conjunction with an El Niño Southern Oscillation (ENSO) event. Prolonged, record temperatures led to extensive coral mortality on reefs worldwide. In contrast, Palmyra Atoll experienced low mortality despite widespread bleaching, with less than 10% loss in coral cover on the fore reef and no change in overall coral cover on the reef terrace. The high level of survivorship across bleached colonies on the reef terrace may be indicative of coral acclimatization to high thermal variability in the shallow environment. Coral-associated microbial communities have been suggested as a mediator of rapid response and acclimatization to changing environmental stressors. To investigate this, we conducted a large-scale sampling effort across 5 species of coral at Palmyra Atoll, both during and after the 2015–16 mass bleaching event. All colonies survived the bleaching event and only partial mortality was observed between the two years. Coral microbial communities were significantly different from one another across species, although shifts in the dominant taxa changed within species, between bleached and unbleached tissue and over time. Of particular interest was the presence of *Endozoicomonas*, a coral symbiont, which was ubiquitous across all coral species and colony condition. Additionally, we did not see a dramatic increase in the abundance of *Vibrio*, a trend that is frequently seen in diseased and bleached tissues. Collectively, these data suggest that although we observed species-specific changes in coral microbiomes in response to the thermal-stress event, there was minimal disruption of the balance between the coral host and its associated bacterial community, which may have contributed to survivorship. This may be indicative of colony adaptation to variable temperatures on the shallow reef terrace at Palmyra Atoll.

INTRODUCTION

Historically, La Niña conditions are characterized by cooler waters in the central equatorial Pacific, and El Niño events result in warmer waters in the same region. However, current sea surface temperatures (SST) during modern La Niña conditions are warmer than they were during El Niño events 30 years ago (Hughes et al. 2018). This elevation in ocean temperature, combined with the increasing and prolonged frequency of global temperature extremes associated with ENSO events, created ideal conditions for one of the longest and most severe global bleaching events, recorded in 2015–16 (Heron et al. 2016). Coral bleaching, or the expulsion of symbiotic algae (*Symbiodinium spp.*) from their coral hosts, is frequently associated with elevated temperature and increased irradiance, although certain clades of symbionts are thought to be more thermally resistant than others (Rowan et al. 1997). These symbionts provide their coral host with an important source of fixed carbon and their loss can result in coral mortality, depending on the intensity and duration of the warming event (Brown 1997; McClanahan 2004). In addition to bleaching, elevated SST can disrupt coral-bacterial interactions, leading to a potentially detrimental shift in the coral microbiome (Thurber et al. 2009). For example, the genus *Vibrio* is common in coral microbiomes at low densities, but thermal stress can result in a sharp increase of *Vibrio,* leading to pathogenic infection and coral mortality (Tout et al. 2015; Gibbin et al. 2019). The 2015–16 global bleaching event led to mass coral mortality, with bleaching-associated death reaching highs of over 60% on the Great Barrier Reef (Hughes et al. 2017). Prolonged heat stress on Jarvis Island in the South Pacific led to areas with ~95% coral mortality and up to 100% bleaching of coral colonies in shallower depths (Barkley et al. 2018). The severe mortality observed at select sites during the last global

bleaching event emphasizes the importance of a deeper understanding as to how corals respond to thermal stress and how they might adapt to future ocean conditions.

Although reports of widespread bleaching and mortality were prevalent during the 2015-16 bleaching event (Hughes et al. 2017, 2018a,b; Brainard et al. 2018; Barkley et al. 2018), not all reefs suffered the same fate. Coral reefs of Palmyra Atoll, in the Central Pacific, experienced widespread bleaching but extremely low mortality following this bleaching event, despite the fact that sea surface temperature (SST) was at or above the bleaching threshold for 23 weeks (Figure 4.1, Fox et al. 2019). Fox et al. (2019) found that 89.9% and 92.6 % of the total coral surveyed on the fore reef and reef terrace, respectively exhibited some level of bleaching, although the severity of bleaching was variable. Despite this widespread bleaching, coral cover on the fore reef declined by only 9% and there was no overall change in hard coral cover on the reef terrace. The main differences between the regions of reef surveyed in these habitats include depth, hydrodynamics, and daily temperature range; with the fore reef (10m) experiencing regular internal waves that deliver cooler, nutrient rich water (Gove et al. 2015; Williams et al. 2018). In contrast, the reef terrace (5 m) experiences high levels of irradiance and daily temperature fluctuations of almost 1 ºC (Fox et al. 2019). Studies have suggested that corals growing in more variable conditions may be better adapted to surviving thermal stress associated with bleaching events (Oliver & Palumbi 2011). One such mechanism for rapid adaptation to variable temperature in corals lies in their community of microorganisms (Ziegler et al. 2017).

Scleractinian corals host billions of symbiotic algae, bacteria, archaea, viruses, and fungi. Collectively, these microorganisms and the coral are known as the coral holobiont (Rohwer et al. 2002). The Coral Probiotic Hypothesis posits that coral-associated bacterial communities can rapidly respond to environmental conditions, thus providing a mechanism for rapid

acclimatization in long-lived coral colonies (Reshef et al. 2006). Shifts in their microbial symbionts may help corals respond to environmental stressors in days or weeks, rather than over generations. Additionally, continuous exposure to variable environments has been shown to result in a more stable microbial community in response to stressors (Ziegler et al. 2017; Thomas et al. 2018). Reciprocal transplantation of corals into thermally-variable environments demonstrated that colonies previously adapted to more variable temperature regimes were more tolerant to thermal stress events and therefore exhibited minimal change in their microbiome when exposed to higher temperatures (Ziegler et al. 2017). However, numerous other factors can influence the structure of the holobiont, including nutrient pollution or high macroalgal cover due to low herbivorous fish populations (Ziegler et al. 2016; Haas et al. 2016).

Increases in *Vibrio* and other pathogenic bacteria are seen not only as a result of thermal stress, but due to increased dissolved organic carbon (DOC) (Kline et al. 2006; Nelson et al. 2013). Land-based nutrient pollution or removal of herbivores can lead to increased DOC through macro and turf algal overgrowth of coral colonies, thus stimulating bacterial growth (Haas et al. 2011). To isolate the response of the coral microbial community to a thermal stress event, potentially confounding stressors need to be removed. Although easy to manipulate in a laboratory setting, these conditions are more difficult to replicate *in situ.* In order to understand how the coral microbial communities respond to and change following a thermal anomaly, more research is needed in locations that lack confounding stressors that could lead to bacterial overgrowth, such as overfishing or nutrient pollution.

This study aimed to examine how coral microbial communities responded to thermal stress events *in situ* by focusing on a location with minimal additional stressors. To do so, we conducted a field-based sampling campaign across multiple taxa during and after the 2015-2016

bleaching event on the remote coral reefs of Palmyra Atoll. The atoll has never had a permanent human population, resulting in a largely intact ecosystem with high herbivore abundance and coral cover on both the fore reef and reef terrace (Sandin et al. 2008; Smith et al. 2016). Destabilization of the coral-associated bacterial community is often associated with increased microbial richness or alpha diversity (Morrow et al. 2012; Ziegler et al. 2016). An additional marker of stressed microbial communities is greater spread across the coral microbiome between colonies within a coral species (Zanveld et al. 2016, 2017). We looked for evidence of these shifts on both unbleached and visually bleached colonies during the 2015 global coral bleaching event across multiple coral taxa at Palmyra Atoll. Our goals were to a) characterize differences in the microbiome of unbleached and bleached coral tissue across multiple coral taxa and b) explore how these patterns changed one year following the thermal anomaly.

METHODS

Study location and sampling sites

Samples were collected on the western reef terrace (~5 m depth) of Palmyra Atoll in the Northern Line Islands (05°52' N, 162°06' W) in the Central Pacific (Figure 4.1). Palmyra is a National Fish and Wildlife Refuge and part of the Pacific Remote Islands Marine National Monument. The first round of samples was collected in September-October of 2015, during the peak of the thermal anomaly for Palmyra (Figure 4.1). Tagged corals were re-sampled in 2016, one year after the initial sampling and ~9 months after water temperature had dropped below the Palmyra-specific bleaching threshold (Fox et al. 2019).

Coral sampling

To examine changes in alpha and beta diversity of coral-associated microbial communities, eight colonies of five species of coral were sampled in both 2015 and 2016. All colonies were tagged in 2015 (*Acropora acuminata, Acropora cytherea, Montipora aequituberculata, Montipora capitata, and Pocillopora meandrina*) and revisited in 2016. This sampling design allowed for analysis of bacterial alpha diversity within and across coral species and dispersion of microbial communities (beta diversity) from colony to colony during and after the bleaching event. In 2015, 4 unbleached and 4 bleached but living colonies (no algal overgrowth) were sampled. Six (6) replicate samples were taken from each colony to provide an average bacterial community for each. Each colony was then re-sampled in 2016, with previously unbleached colonies recorded as "Unbleached 2016" and previously bleached but recovered colonies labeled as "Recovered 2016." Fragments of coral were collected using bone cutters or a small chisel and placed into individual whirl-pak bags. Samples were stored on ice for transport back to the field station where they were transferred to RNA later for 16S rRNA amplicon sequencing.

DNA extraction and 16s rRNA gene amplicon sequencing and processing

Coral fragments in RNA later were stored in a –20 °C freezer at Scripps Institution of Oceanography prior to DNA extraction performed in conjunction with the Knight Lab and Center for Microbial Innovation at UC San Diego. The coral fragments were processed using the Earth Microbiome Project protocols (www.earthmicrobiome.org), which have been previously described for DNA extraction (Marotz et al. 2017) and amplification and sequencing of the 16S rRNA gene (http://doi.org/10.17504/protocols.io.nuudeww).

Sequence data was processed using QIIME 2 (Bolyen et al. 2018). Primary data processing of the amplicon sequence data was prepared for bacterial community analysis following the QIIME 2 methodology described in Chapter 3.

Data analysis

Replicates for each time point and status were combined to create 4 categories for each species: Bleached 2015, Unbleached 2015, Recovered (previously bleached) 2016, and Unbleached 2016 (n=240). Sequences were classified to the lowest taxonomic resolution possible and analyzed as described below. The relative abundance of bacterial families were made using observation tables that were normalized to relative abundance, grouped and averaged by treatment/year, and taxa (family level, "D5"). Taxa having average relative abundance >1% across all groups were plotted with boxplots, and taxa not in these groups were placed in "Others".

Changes in Alpha Diversity for each species

To assess change in sample alpha diversity (species richness), ASV counts were rarefied to 1000 observations per sample. For each species, a two-way analysis of variance (ANOVA) was used to assess how alpha diversity changed across bleached/unbleached tissue and between the two years.

Species-specific coral microbial community response to stress over time

To assess how bacterial community composition varied across the same two factors (bleaching condition and year), we conducted a 2-way permutation-based analysis of variance

(PERMANVOA, partial sum of squares, 9999 permutations) on Bray Curtis similarity values calculated from log (x+1) transformed data of unique ASV counts. The contribution of each bacterial family to the community composition and the dissimilarity between status and year for each species was calculated using Similarity Percentage (SIMPER) analysis. To visualize differences in beta diversity between bleached versus unbleached tissue and over year we used a Canonical Analysis of Principal coordinates (CAP) ordination for each species.

RESULTS

Here we investigated the response of coral-associated bacterial communities to the 2015 global coral bleaching event in 5 reef-building coral species over 2 years. Paired-end sequencing (2 x 151 bp) on the Illumina MiSeq platform was carried out by the UC San Diego Center for Microbiome Innovation. Sequencing resulted in 8,209,420 paired reads (16,418,840 reads) total for 240 samples. The DADA2 feature tables contained 21,006 unique ASVs (median length: 253 bp) totaling 5,191,726 observations over 288 samples. Sequence data and metadata are available from the Qiita database (González et al. 2018) with study number 10798 and from the European Nucleotide Archive. Observation (ASV) and metadata tables are available from Zenodo.

Changes in Alpha Diversity for each species

The cutoff of 1000 ASV counts per sample eliminated the *Acropora acuminata* (AA) Recovered 2016 (previously bleached) time point. As a result, we could not test across all 4 bleaching status/year combinations for AA. There was no significant difference in sample alpha diversity across status or between years for *Acropora cytherea* (AC), *Montipora aequituberculata* (MA), or *Montipora capitata* (MC). Only *Pocillopora meandrina* (PM)

exhibited a significant difference in status for Recovered tissue in 2016 (two-way ANOVA, $p < 0.0001$, Figure 4.2, Table 4.1). There were no significant interactions between status and year for any species.

Species-specific coral microbial community response to stress over time

Beta diversity of *Acropora acuminata* bacterial communities shifted in response to the thermal stress event in 2015 (Figure 4.4). However, bleaching status in 2015 did not have a significant effect on bacterial community composition (PERMANOVA, F 3.34, $p > 0.05$), but there was a significant difference between years (PERMANOVA, F 4.96, $p < 0.005$) (two-factor PERMANOVA, Table 4.2). The interaction between status and year was not significant ($p > 0.05$). Similarity percentage (SIMPER) analysis showed that the unbleached coral-associated bacterial community in 2015 was characterized by the bacterial families Enterobacteriaceae, Endozoicomonoadaceae, Burkholderiaceae, and Moraxellaceae. The bleached community in 2015 was also characterized by Enterobacteriaceae and Burkholderiaceae, but with the additional of Bacillaceae, Pseudomoadaceae, and Thermaceae (a thermally-tolerant bacterial family). Unbleached communities in 2016 were also dominated by Enterobacteriaceae, Burkholderiaceae, Endozoicomonoadaceae, and Moraxellaceae. The recovered bacterial communities in 2016 (previously bleached) were also dominated by Enterobacteriaceae, Burkholderiaceae, and Endozoicomonoadaceae. Although many of the same bacterial families were present in high abundance across the statuses for AA, there were less common families that drove the dissimilarity between groups. For example, unbleached communities in 2015 and 2016 were 71% dissimilar and this difference was driven by various species of Endozoicomonoadaceae, Thermaceae, and unidentified Bacteroidia that were more abundant in 2015 (Figure 4.3).

Unbleached and bleached communities in 2015 were 63.35% dissimilar, which was again driven by species of Endozoicomonoadaceae, Moraxellaceae, unidentified Bacteroidia, Thermaceae, and Rhodobactereaceae, all of which were higher in abundance in the unbleached tissue samples from 2015.

Analysis of the *Acropora cytherea* (AC) bacterial communities showed that both bleaching status in 2015 ($p < 0.005$) and year ($p < 0.0001$) were significant, as was the interaction between the two ($p < 0.05$) (two-factor PERMANOVA, Table 4.2). Percent similarities (SIMPER) showed that unbleached communities in 2015 were characterized by Endozoicomonoadaceae, Myxococcales P30B-42, Enterobactereaceae, and Moraxellaceae (Figure 4.3). Bleached microbial communities in 2015 were also dominated by Endozoicomonoadaceae, Enterobactereaceae, and Moraxellaceae, as well as an unidentified proteobacteria. Unbleached bacterial communities in 2016 were primarily dominated by Gammaproteobacteria: Endozoicomonoadaceae, Enterobactereaceae, Moraxellaceae, and Alteromonadaceae. The recovered communities in 2016 (previously bleached) were characterized by Enterobactereaceae, Burkholderiaceae, Thermaceae, and Bacillaceae. Although there were similar families present in unbleached tissues in both 2015 and 2016, the communities were 60.63% dissimilar (SIMPER). This dissimilarity was primarily driven by the abundance of Myxococcales P30B-42 and Kiloniellaceae in the 2015 samples. In contrast, Idiomarinaceae, Alteromonadaceae, and Vibrionaceae were all elevated in the 2016 unbleached bacterial communities (Figure 4.3). Unbleached communities in 2015 were higher in abundance of P30B-42, Kiloniellaceae, Rhodospirillales, and an unidentified proteobacteria when compared to bleached communities in 2015 (SIMPER, 62.56% dissimilarity).

Pocillopora meandrina (PM) bacterial communities were also significantly different across bleaching status ($p < 0.0001$), year ($p < 0.0001$), and by the interaction between status x year ($p < 0.0005$) (two-factor PERMANOVA, Table 4.2). SIMPER showed that the most abundant bacterial families in unbleached bacterial communities in 2015 were Endozoicomonadaceae, Enterobactereaceae, Rhodobactereaceae, and Amoebophilaceae. Bleached communities in 2015 were also dominated by the same families, although an unidentified bacteria was also present in high abundance. Unbleached communities in 2016 were characterized by the same families from 2015 with the exception of Rhodobactereacae, which was not present in high levels in 2016. Recovered bacterial communities in 2016 had high levels of Rhodobactereaceae, as well as Flavobactereaceae, unidentified Alphaproteobacteria, and P30B-42 (Myxococcales). Dissimilarity between unbleached communities in 2015 and 2016 was driven by Flavobactereaceae, unidentified alpaproteobactereaceae, and Cryomorphaceae (all of which were more abundant in 2016). Prevotellaceae was only present in the unbleached coral tissue from 2015. Presence of Deltaproteobacteria (P30B-42), Cyclobacteriaceae, Chroococcidiopsaceae, and Amoebophilaceae in bleached PM corals in 2015 drove the dissimilarity between unbleached and bleached colonies.

Analysis of *Montipora aequituberculata* bacterial communities were significant for both factors (Status $p < 0.05$ and Year $p < 0.0001$), but not for the interaction between the two ($p > 0.05$) (two-factor PERMANOVA, Table 4.2). SIMPER showed that unbleached communities in 2015 were dominated by Rhodobacteraceae, Flavobacteriaceae, Endozoicomonadaceae, and Sphingomonadaceae. Bleached communities in 2015 were also characterized by Rhodobacteraceae, and Flavobacteriaceae along with Saprospiraceae and an unidentified Alphaproteobacteria. Unbleached communities in 2016 also had high levels of

Rhodobacteraceae, Flavobacteriaceae, Endozoicomonadaceae, and Enterobactereaceae. Recovered communities in 2016 were characterized by Enterobactereaceae, Rhodobactereaceae, Bacillaeceae, and Flavobacteriaceae (Figure 4.3). Differences between unbleached 2015 and 2016 were driven by Rhizobiaceae and Arcobacteraceae in 2016 and Endozoicomonadaceae and Microtrichaceae in 2015 (SIMPER, 65.26% dissimilarity). Differences between bleached and unbleached communities in 2015 were driven by the abundance of Phormidesmiaceae, Phycisphaeraceae, P30B-42, and unidentified proteobacteria that were abundant in bleached corals (Figure 4.3). Bacterial communities for unbleached and recovered corals in 2016 were highly dissimilar (SIMPER, 76.95%). Unbleached corals had higher levels of Rhizobiaceae, Arcobacteraceae, uncultured Thalassobaculales and unidentified Gamma- and alphaproteobacterial.

Montipora capitata (MC) bacterial communities were also significantly different across all factors and interactions (two-way PERMANOVA, Table 4.2). SIMPER showed that unbleached communities in 2015 were dominated by gammaproteobacterial and firmicutes (Enterobacteriaceae, Moraxellaceae, Bacillaceae, Pseudomonadaceae). In contrast, bleached MC corals in 2015 had high levels of the Myxococcales P30B-42, Halomoadaceae, Rhodobactereaceae, and Preotellaceae. The unbleached MC communities in 2016 were characterized by Alteromonadaceae, Vibrionaceae, Idiomarinaceae, and Myxococcales. The previously bleached (recovered) communities in 2016 were dominated by Vibrionaceae, followed by Alteromonadaceae, Enterobactereaceae, and Flavobacteriaceae. The dissimilarity between unbleached bacterial communities in 2015 vs. 2016 was 69.36% and driven by Idiomarinaceae, Pseudoaltermonoadaceae, Vibrionaceae, and Altermonadaceae, all of which were elevated in the 2016 samples. The difference between unbleached and bleached

communities in 2015 was driven my Halomonadaceae, Myxococcales, and Colwelliaceae in the bleached communities.

DISCUSSION

Palmyra experienced widespread bleaching across both the fore reef and reef terrace habitats, but exhibited remarkably low mortality, particularly on the reef terrace, with no overall change in hard coral cover (Fox et al. 2019). Out of the 40 colonies that were tagged and sampled during this study we observed no full colony mortality, with partial mortality occurring primarily in *A. cytherea* (AC) and *P. meandrina* (PM). With the exception of *A. acuminata*, all species of coral exhibited significantly different bacterial communities as a result of bleaching status in 2015 and by year (Figure 4.4, Table 4.2). *A. acuminata* (AA) bacterial communities were not significantly affected by their bleaching status in 2015, but year did have a significant effect (Figure 4.4). CAP ordination showed strong grouping by year for AA, regardless of status for each colony, suggesting that bleached and unbleached communities were more similar to one another in 2015 than to communities in 2016. AC, PM, and MC all had significant interactions between their bleaching status in 2015 and years, suggesting that all bacterial communities recovered. Unbleached colonies for 2015 and 2016 were more similar to one another than colonies in the same year (Figure 4.4).

Bacterial community composition for AC, MC, PM, and AA did not become more similar with increasing stressors (Bleaching 2015), suggesting that their microbial community were more stable despite thermal stress. Studies investigating elevated stressors and changes in the human gut microbiome have shown a decrease in the number of bacterial species present (Lozupone et al. 2012). Interestingly, the opposite is frequently seen with corals, where blooms

of bacterial species are able to invade the coral holobiont and increases in alpha diversity are observed (McDevitt-Irwin et al. 2017). It has been hypothesized that this occurs as a result of dysbiosis between the coral host and its microbial community, where the host's ability to regulate its microbiome suffers and opportunistic bacteria are able to invade (Thurber et al. 2009; Glasl et al. 2016). Coral-associated microbial communities on the reef terrace at Palmyra did not show significantly higher levels of alpha diversity (species richness) during the 2015 bleaching event, although there was a general trend that showed lower diversity in 2016 vs. 2015, with the exception of PM (Figure 4.2). However, the significantly higher alpha diversity in the Recovered 2016 time point for PM was likely the result of turf algae contamination due to partial coral mortality. *P. meandrina* was the only coral species that had high levels of turf algae in the areas of partial mortality. In contrast, *A. cytherea* also experienced partial mortality at the top of the large, plating colonies but crustose coralline algae (CCA) had already covered the area by the time samples were taken in 2016 and turf was minimal. High herbivore populations at Palmyra may have contributed to the low abundance of turf following the bleaching event, suggesting that minimal local stressors can influence the trajectory of reef recovery following large-scale disturbances (Hamilton et al. 2014; Fox et al. 2019).

McDevitt-Iriwn et al. (2017) found that specific bacterial taxa respond to stressors in a similar manner across coral species (e.g., decreasing Oceanospiralles and increasing Vibrionales, Rhodobacterales, Flavobacteriales, and Alteromonadales). Interestingly, we observed Endozoicomonadaceae, a family within the order Oceanopsiralles, in high abundance across almost all species and health statuses throughout the sampling time period. Oceanospiralles, and specifically *Endozoicomonas*, are well-known coral symbionts that are considered beneficial to overall coral health via their roles in nitrogen fixation and nutrient cycling (van de Water et al.

2016; Ziegler et al. 2016). AA, AC, MA, and PM all maintained high levels of *Endozoicomonas*, even during when visibly bleached in 2015 (Figure 4.5). *Endozoicomonas* was also present in MC throughout the sampling period, although it was not as abundant when compared to the other species of coral. These data suggest that although the coral microbial community shifted during the bleaching event, the corals sampled in this study were able to maintain some level of control over their microbiome when stressed, perhaps due to adaptation to thermal fluctuation as a result of their variable environment. Safaie et al. (2018) found that a 1º C increase in diurnal temperature variability reduced the odds of coral bleaching by a factor of 33. Interestingly, the natural daily temperature range of the reef terrace is almost exactly 1º C (Figure 4.1, Fox et al. 2019), suggesting that corals on Palmyra's reef terrace may have adapted to increased temperature variability.

Although there was minimal mortality of coral colonies, we observed elevated levels of bacterial taxa strongly associated with stressed and diseased corals, such as Rhodobacteraceae, Flavobacteriaceae, Burkholderiaceae, and Enterobacteriaceae (Sunagawa et al. 2009; Roder et al. 2014a, b). These bacterial families are strongly associated with multiple stress and virulence genes, and have been identified as key players in coral disease (Daniels et al. 2015). For example, Enterobacteriaceae has been used as a proxy for anthropogenic pollution in reef-ecosystems due to its strong association with human-sourced sewage (e.g. coliforms) and its prevalence in nitrogen-enriched environments (Leite et al. 2018). However, it has been suggested that nitrogen input from bird feces can contribute to a healthier reef system by providing nutrient inputs into otherwise oligotrophic waters (Graham et al. 2018). Given the presence of Enterobacteriaceae in both unbleached, bleached, and recovered tissue across most coral

samples, it is highly probable that the signature stems from bird fecal matter rather than any human-based pollutant, and therefore may be not be an indicator of declining coral health.

Studies suggest that bleaching can result from bacterial infection that occurs when host immunity is lowered during thermal stress events, leading to the breakdown of the symbiotic relationship of the coral holobiont (Rosenberg and Ben-Haim 2002; Rosenberg et al. 2007). Bourne et al. (2008) found that increasing temperature led to elevated levels of *Vibrio*-affiliated sequences during the 2002 bleaching event on the Great Barrier Reef. These *Vibrios* are opportunistic and frequently pathogenic bacteria that are often implicated in coral disease (e.g. *Vibrio shiloi*) (Kushmaro et al. 2001). Interestingly, we did not observe elevated levels of *Vibrios* in any of the bleached coral samples taken during the 2015 event. Conversely, we only saw an increase in *Vibrionaceae* associated with the recovered *Montipora capitata* and *Acropora cytherea* samples in 2016. These data suggest that the *Vibrionaceae* were not responsible for the observed bleaching in 2015. Additionally, given the tissue recovery of all sampled coral colonies, it appears that the *Vibrios* present in 2016 were not impacting overall coral health.

The coral microbial communities for AC, MC, and PM all had significant interactions between bleaching status and year, suggesting that community composition recovered post-bleaching (Figure 4.4). Previous studies have found similar trends where bleached communities returned to their pre-bleaching bacterial community post-stressor (Bourne et al. 2008), or that community composition did not change in thermally-tolerant corals (Ziegler et al. 2017). In contrast, AA and MA did not have significant interactions, suggesting that bleaching in 2015 led to a significantly different microbial community the following year, despite the full recovery of all bleached colonies. This holds particularly true for MA, which exhibited different bacterial

communities across all status and years (Figure 4.4). However, given the high rates of survival across all species, this did not appear to have a significant effect on coral colony survivorship.

In conclusion, coral species at Palmyra Atoll maintained high levels of beneficial symbionts during and after the bleaching event and exhibited low-levels of potentially pathogenic *Vibrios* despite experiencing ~20 degree heating weeks. Although there were changes in the coral microbial communities associated with bleaching, we observed no full mortality of coral colonies and minimal partial mortality. Collectively, these data suggest that the natural temperature variability on the reef terrace at Palmyra led to increased resilience in coral communities. This work highlights the importance of studying the effects of bleaching events and subsequent recovery in environments with minimal compounding stressors. Locations like Palmyra serve as a baseline for benthic communities, showing that not all change is negative and that coral response to bleaching is inherently dynamic, even in systems free of other stressors.

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Chapter 4, in full, is currently being prepared for submission for publication of the material. Carter, Amanda L.; Thompson, Luke R.; Fox, Michael D.; Smith, Jennifer E., Species-specific coral microbial community response to a low-mortality bleaching event at Palmyra Atoll, central Pacific Ocean. The dissertation author was the primary investigator and author of this material.

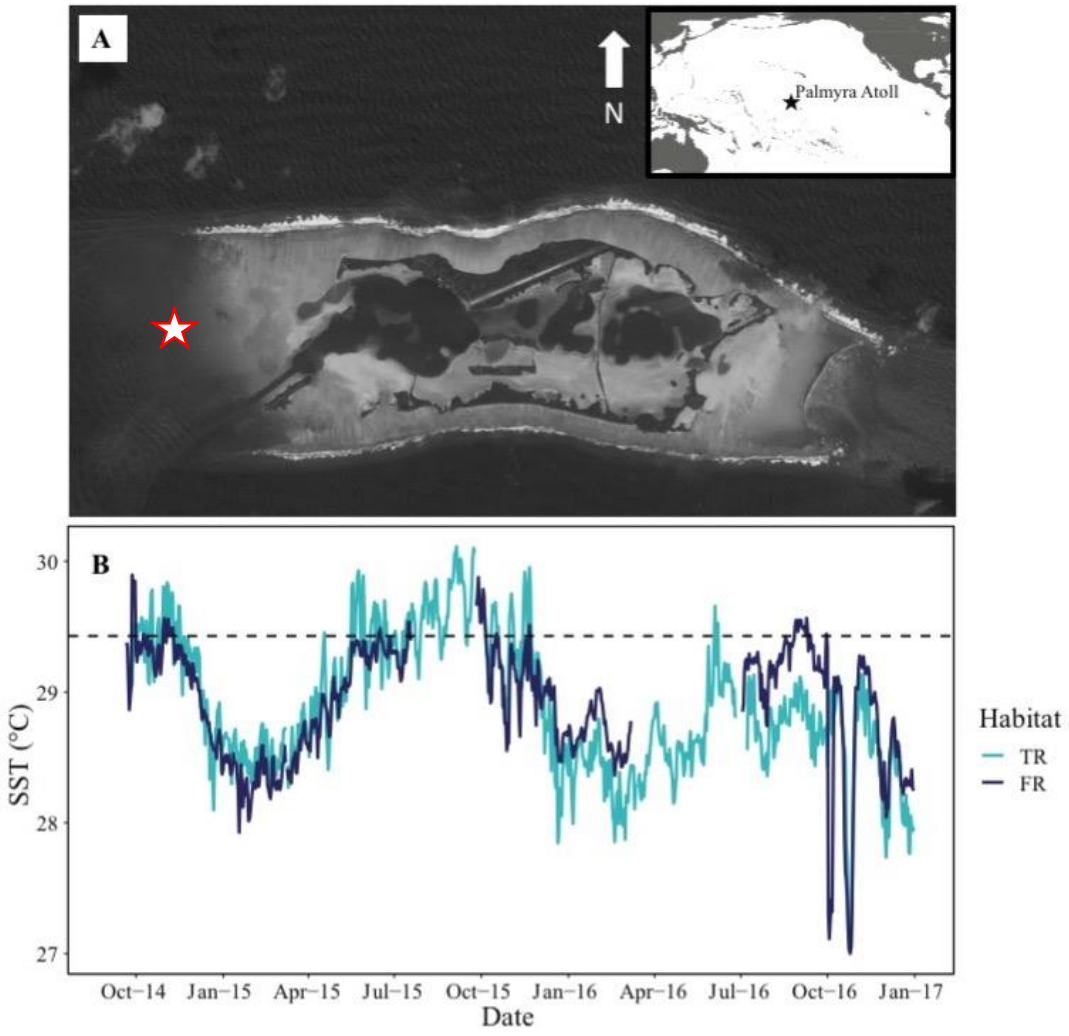

Figure 4.1. Palmyra Atoll and the Mean Sea Surface Temperature (SST) collected from *in situ* sensors spanning October 2014-January 2017. A Location of Palmyra Atoll in the Central Pacific and the sampling site (white star) for corals during the 2015 thermal anomaly. **B** Daily fluctuations on the reef terrace (TR) and fore reef (FR) collected by *in situ* CTD and Honeywell Durafet sensors from October 2014 – January 2017. The dashed line indicates the climatological bleaching threshold for Palmyra (29.47°C).

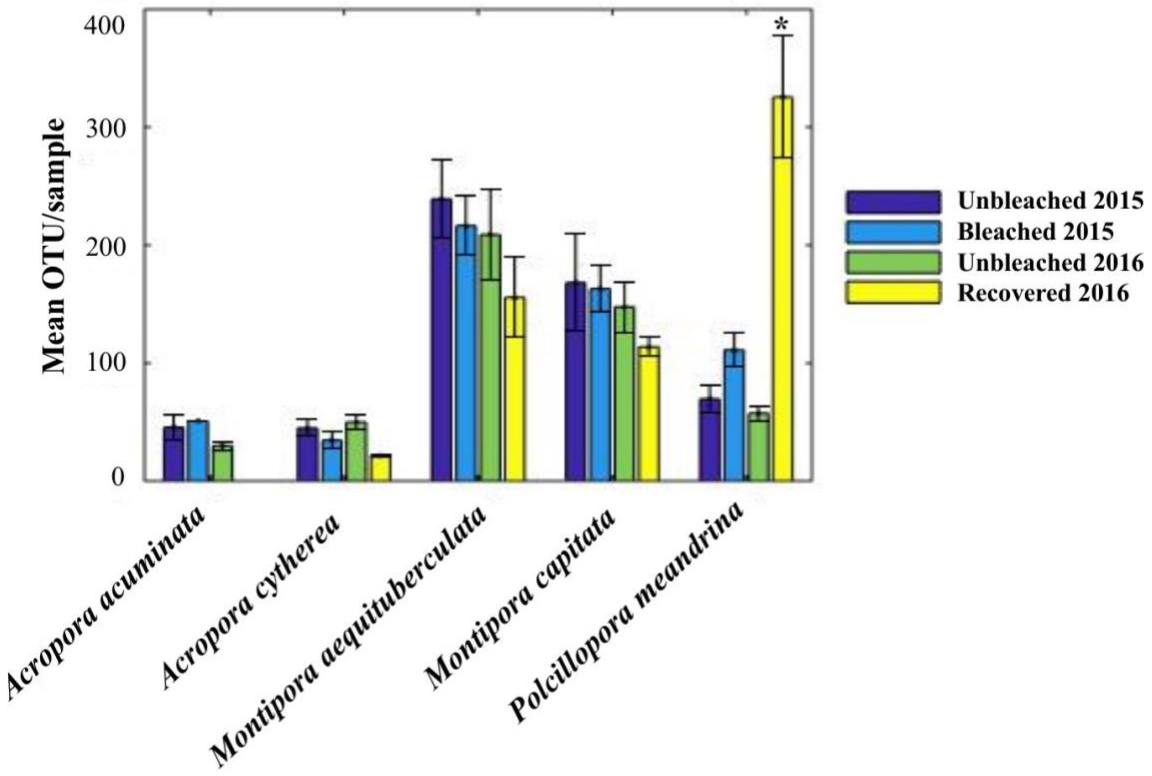

Figure 4.2. Bar graph of alpha diversity as measured by unique ASVs/sample (means ± SE) for each species across health status/year. Species associated alpha diversity measurements for all 5 species during and after the bleaching event. Unbleached 2015 and Unbleached 2016 sequences were taken from the same colonies, as were Bleached 2015 and Recovered 2016. Each sample was rarefied to a minimum of 1000 sequence reads per sample before means ± SE calculation. Significant difference in health status for species denoted by * as calculated by a two-way ANOVA ($p<0.0001$).

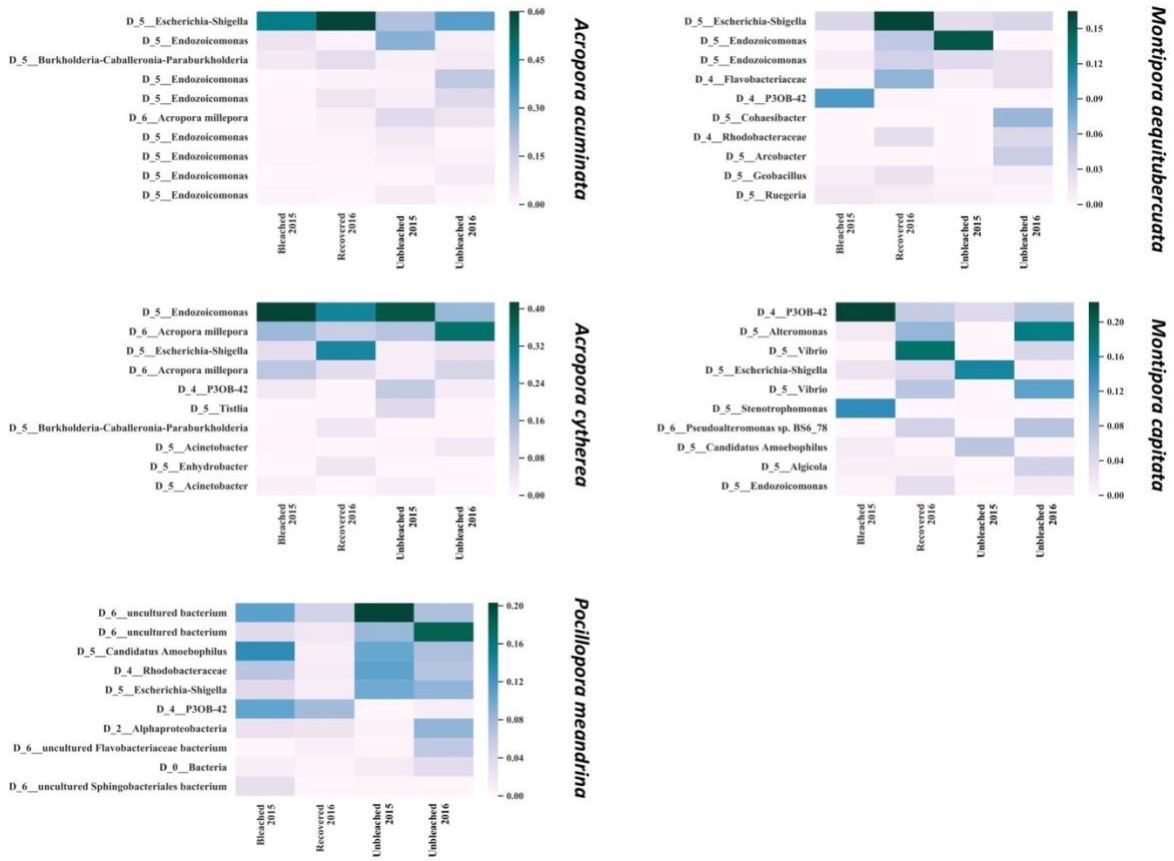

Figure 4.3. Heatmaps showing the top-10 amplicon sequence variants (ASVs) for each species. Heatmaps showing top-10 ASVs for all coral species. X-axis organized by Health Status/Year combinations from 2015 to 2016.

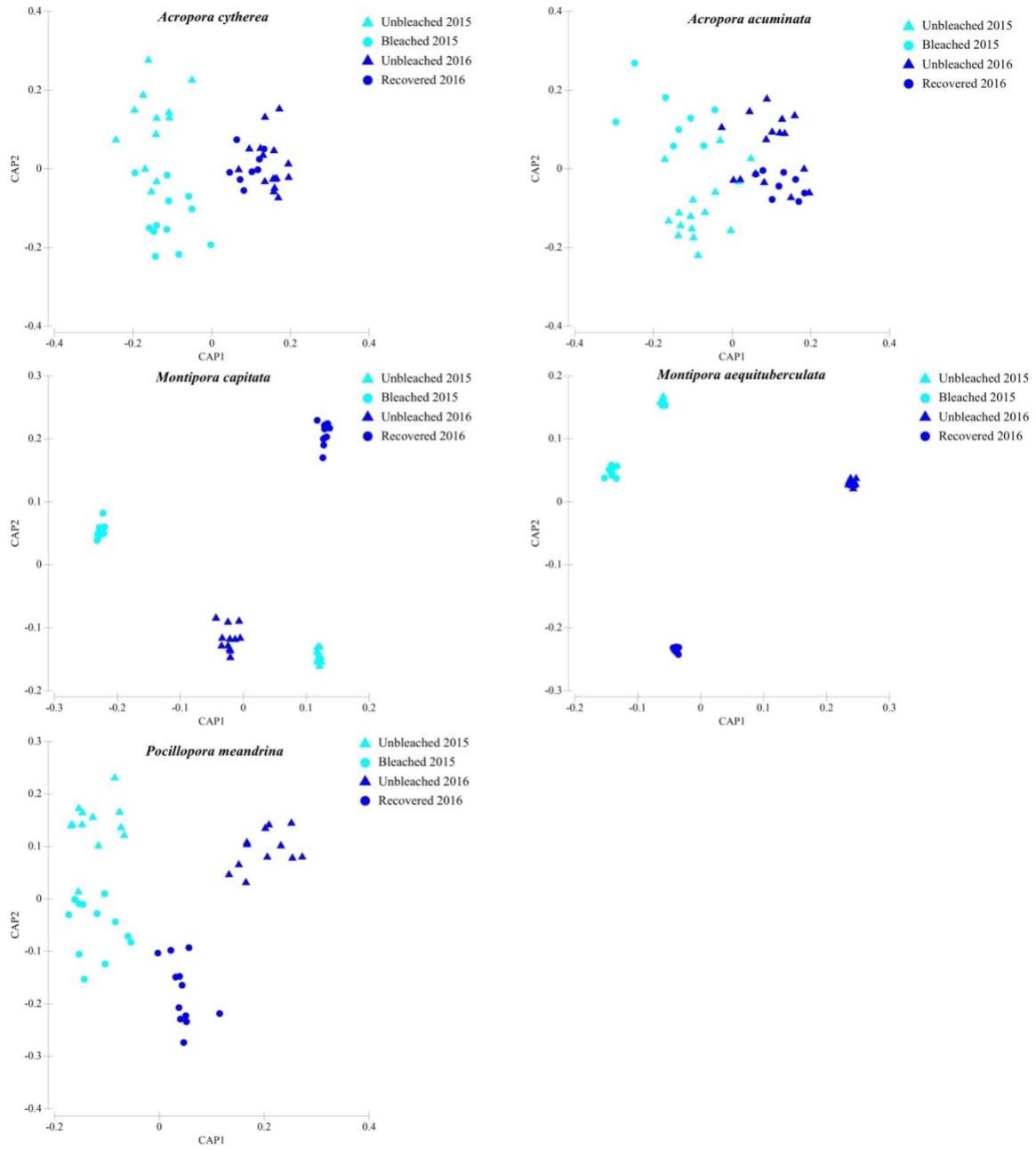

Figure 4.4. Canonical analysis of principal coordinates (CAP) of microbial community composition for each species across status and year. CAP ordinations showing similarity of microbial communities as a measurement of beta diversity for each health status and across years.

Figure 4.5. Mean proportions of bacterial families contributing to the microbial assemblage of 5 species of coral at Palmyra Atoll, Northern Line Islands. Species names (n=5 species) are shown along the left of the graph with each column corresponding to a specific status and sampling year. Unbleached and Bleached 2015 refer to samples taken during the 2015 bleaching event. Unbleached and Recovered 2016 were sampled from the 2015 colonies to track changes in microbial community for unbleached and bleached corals over time.

Table 4.1. Two-way ANOVA results examining change in alpha diversity across bleaching status and year for each species.

Species	Factor	F	*P* value
AC	Status	3.123	0.0562
	Year	0.349	0.5584
MA	Status	0.518	0.6003
	Year	0.463	0.5010
MC	Status	0.581	0.5645
	Year	0.372	0.5457
PM	Status	18.838	0.0000
	Year	0.069	0.7950

Table 4.2. Results from a two-factor PERMANOVA examining changes in bacterial community composition as a result of bleaching status and year for each coral species. Interactions between factors are listed as "Status x Year."

Species	Factor	Pseudo-F	P value
AA	Status in 2015	3.337	0.0053
	Year	4.957	0.0006
	Status x Year	1.229	0.2226
AC	Status in 2015	4.392	0.0020
	Year	4.894	0.0001
	Status x Year	2.122	0.0205
MA	Status in 2015	2.436	0.0149
	Year	7.758	0.0001
	Status x Year	1.434	0.1253
MC	Status in 2015	1.884	0.0395
	Year	7.123	0.0001
	Status x Year	4.566	0.0001
PM	Status in 2015	11.744	0.0001
	Year	8.287	0.0001
	Status x Year	5.105	0.0003

CONCLUDING REMARKS

This dissertation utilized methods from a variety of ecological fields, including spatial ecology, marine chemical ecology, and coral microbial ecology, to investigate the impacts of local and global stressors on the benthic community at Palmyra Atoll. In Chapter 1, I used a long-term and spatially robust dataset to examine benthic community change in response to invasion by the corallimorph, *Rhodactis howesii* (Carter et al. 2019). The comprehensive nature of these data proved to be key in discovering two opposing trends in the invasion at Palmyra Atoll. Although corallimorph cover showed significant decrease at the invasion site, its continued spread to sites further around the atoll were captured due to the spatial scale of the data collected. Natural variability in reef communities at Palmyra also proved to be an important component of this study. I found that sites with high coral cover and low levels of natural disturbance (RT10 and FR3) proved more resistant to invasion, with their benthic communities keeping the invader at low levels throughout the almost decade-long study. In contrast, sites with higher levels of natural disturbance due to wave energy (e.g. FR5) (Gove et al. 2015) were more susceptible to overgrowth by the corallimorph. These trends were supported by settlement preference and establishment patterns of the corallimorph. I found that *R. howesii* disproportionately settles on crustose coralline algae (CCA) and mixed turf algae, both of which are frequently found in areas with higher levels of disturbance due to their ability to rapidly colonize available space. In contrast, *R. howesii* avoided settlement on hard coral and macroalgae, possibly due to the competitive abilities and chemical protection of those functional groups. However, I found that once corallimorph established itself in a plot, it exhibited the ability to overgrow almost all functional groups, suggesting that it had competitive abilities of its own.

In Chapter 2, I investigated possible competitive mechanisms of the corallimorph by searching for evidence of allelopathy via secondary metabolites in interactions between *R. howesii* and a hard coral. I developed a bioassay to test for chemical activity of various extracts from the corallimorph and found clear evidence of an allelopathic interaction with the hard coral, *A. yongei.* Further fractions of the chemical extract were tested and I found that non-polar compounds, similar to those found in macroalgae and soft corals, were the most active extracts (Pawlik et al. 2007; Rasher et al. 2011). This work led to identification of the carotenoid, Peridinin. This was the first study to conclusively show that *R. howesii* is able to directly kill coral tissue through chemical activity and without the physical presence of the invader.

Evidence of anti-microbial compounds led to the work conducted in Chapter 3, where I investigated shifts in coral-associated microbial communities in response to overgrowth by the corallimorph. Corals harbor complex microbial communities that have been shown to play key roles in their metabolic functioning and health (Kreidiet et al 2013). Alteration of these communities through the presence of the corallimorph could be another competitive mechanism providing *R. howesii* with its invasive capabilities. I found that microbial communities in healthy tissue were significantly different from one another across five species of coral at Palmyra. However, proximity to the corallimorph led to varied increases in alpha and beta diversity of the coral microbial communities, a sign of coral stress. In fact, the invaded tissue of all corals was no longer significantly different across species, suggesting that the decline in coral health resulted in more similar, stressed coral microbial communities. Interestingly, coral species with higher survivorship (e.g. *Acropora cytherea*) maintained beneficial symbionts such as *Oceanospiralles*. In contrast, species that experienced high mortality and corallimorph overgrowth (e.g. *Montipora*

capitata), showed high levels of opportunistic and pathogenic bacteria colonizing tissue across their interaction zones with *R. howesii*.

In Chapter 4, I shifted away from the local stressor of the corallimorph to examine coral survivorship and response to the global bleaching in 2015-16. Palmyra experienced widespread bleaching but remarkable low mortality despite over 20 weeks of temperatures above the bleaching threshold (Fox et al. 2019). Previous studies have shown that microbial communities can provide long-lived corals with the ability to rapidly respond and adjust to changing environmental variables (e.g. thermal stress) (Ziegler et al. 2017; McDevitt-Irwin et al. 2017; Safaie et al. 2018). At Palmyra, colony bleaching was observed and resulted in shifts in the microbial communities, but the microbial communities of all coral species stayed significantly different from one another regardless of stress (the opposite of what was observed with corallimorph overgrowth) and no colony mortality was observed. Interestingly, corals were able to maintain high levels of beneficial symbionts throughout the community shifts (e.g. *Endozoicomonas*). Perhaps most importantly, we saw no invasion of stressed tissue by pathogenic bacteria such as *Vibrios*, a trend which has been observed in other studies with higher coral mortality (Bourne et al. 2008; Thurber et al. 2009). Collectively, these data suggest that the coral microbial communities on the reef terrace at Palmyra demonstrated some level of acclimatization to the thermal stress, perhaps as a result of natural temperature fluctuations in the shallow habitat (Fox et al. 2019).

Overall, the results of this dissertation provide insight into the response of relatively intact coral communities to both biotic and abiotic stressors. These data create a baseline to which the response and recovery of more degraded ecosystems can be compared. The importance of long-term and spatially robust monitoring of species invasions is highlighted in

Chapter 1, and valuable information regarding the susceptibility of various coral communities around Palmyra to invasion was provided. Additionally, we provided some of the first evidence of chemical activity in the corallimorph, *Rhodactis howesii*, and show its direct impact on coral health as a result of allelopathy and shifts in microbial communities.

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