The microbiome of the staghorn coral *Acropora pulchra* from West Hagåtña Bay

BY

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Coral reefs around the world are in decline due to various environmental stressors, including rising sea surface temperatures and pollution. These affect the coral-associated bacterial microbiome. Shifts in microbiome community composition and function can stress corals and ultimately cause mortality, but how the aforementioned environmental factors influence the coral bacterial microbiome is unknown. This study tested three hypotheses: 1) community diversity of the bacterial microbiome in *A. pulchra* did not differ between populations close to shore and populations far from shore; 2) the bacterial microbiomes of coral tissue, coral mucus, and seawater were equally diverse; and 3) the community diversity of the bacterial microbiome of *A. pulchra* did not change with seasonal changes associated with wet and dry seasons in Guam. To test these hypotheses, *A. pulchra* colonies growing near the Hagåtña sewage treatment plant were sampled over a period of eight months spanning the wet and dry seasons. To examine the bacterial microbiome, samples of seawater, coral tissue, and coral mucus of *A. pulchra* was collected during the following time periods in 2021: in early May, in early July, at the end of September, and in late December. In addition, water samples were collected and analysed for fecal indicator bacteria (FIB). The bacterial community diversity of the coral tissue remained stable throughout the project and was dominated by Endozoicomonadaceae, a proposed obligate symbiont and indicator of coral health. Coral tissue in colonies growing close to shore contained greater abundances of Simkaniaceae, another proposed obligate symbiont, than colonies growing near the reef crest. The coral mucus bacterial microbiome varied with the changing seasons and was not specific to the microhabitat. This study not only shows how these coral bacterial microbiomes respond to different environmental conditions, but also displays the importance of considering separate microbial compartments when analysing microbiomes.

Key words: *Acropora pulchra, microbiome, bacteria, season, environment, habitat*

TO THE OFFICE OF GRADUATE STUDIES

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1. Introduction

1.1 Environmental Impacts on Coral Reefs

Coral reefs around the world are restricted to shallow zones that are strongly affected by changing environmental conditions such as different rates of water flow, extreme low tides, and variable sea surface temperatures (Guilcher, 1988). These zones are also most susceptible to anthropogenic impacts such as pollution and nutrient runoff (Hughes, 1994). These factors, working separately, additively, or synergistically, can cause coral bleaching, which is a sign of the breakdown of the symbiotic relationship between the coral host and its dinoflagellate symbiont community (LaJeunesse et al., 2018).

Island-wide coral bleaching was first recorded in Guam (Mariana Islands, Micronesia) in 1982-1984 (Coffroth, Lasker, and Oliver, 1990) and in 1994 but resulted in little coral mortality. At the time, this bleaching was thought to be unrelated to ocean warming or anthropogenic influence (Paulay and Benayahu, 1999). However, from 2013 to 2017, Guam was affected by four severe coral bleaching events (Raymundo et al., 2019). These events were associated with heat stress caused by increased sea surface temperatures and extreme low tides driven by an El Niño Southern Oscillation (ENSO) as well as repeated coral disease outbreaks. Between 2013 and 2015 alone, a reduction between 40 and 60% of staghorn *Acropora* corals from Guam's reef flats was documented (Raymundo et al., 2017). Some sites around Guam lost more than 50% of staghorn *Acropora* coral cover (Raymundo et al., 2019) and other sites lost more than 90% (Raymundo et al., 2017). Variability in mortality was likely attributable to differences in environmental attributes such as depth and water flow. For instance, *A. pulchra* that grew in environments further from the shore suffered less mortality than populations at the same site (West Hagåtña Bay) closer to the shore (Raymundo et al., 2019). Evidence suggests water flow was one direct cause for this difference in mortality, as

hot, stagnant water stresses coral and flushing from wave action relieves stress (Fifer et al., 2021).

The severity of impact from environmental factors varies with changing seasons throughout the year. The combined effects from acute stressors can damage coral by affecting the cnidarian host as well as its micro-associates, trigger coral bleaching and/or disease, and ultimately cause death (Bourne, Morrow, and Webster, 2016). Exactly how these differing environmental factors affect the coral and its associates requires more in-depth study. Bacteria are one of these micro-associates that researchers have found can be indicative of reef health and condition. An individual examination of each physical stressor can shed light on how it impacts the coral bacterial microbiome and which environmental shifts overall negatively impact coral health. To do this, my study looked at environmental variables including temperature, precipitation, and concentrations of human fecal indicator bacteria (FIB) to assess site-specific differences across seasons and how they vary in correlation with the community diversity of bacteria living in coral tissue, coral mucus, and seawater.

1.2 Water Flow in Coral Reefs

Water flow and flushing can be highly variable across coral reefs (Johansen, 2014) and between coral colonies (Hench and Rosman, 2013). Mechanistically, higher flow rates cause a thinning of the diffusive boundary layer, which allows for greater respiration, increased uptake of nutrients, increased endosymbiont respiration, and reduced photoinhibition (Finelli et al., 2007; Atkinson and Bilger, 1992; Sebens et al., 2003; Nakamura, van Woesik, and Yamasaki, 2005). Furthermore, high flow rates reduce bleaching susceptibility (Fujimura and Riegl, 2017). Fifer et al. (2021) found that *A. pulchra* colonies under higher water flow conditions near the reef margin experienced an upregulation of

stress-related genes as well as increased calcification, greater uptake of nutrients, more productive endosymbionts, and a trend of higher abundances of endosymbionts.

1.3 Extreme Tides

The tidal zone is an area of periodic aerial exposure (Anthony and Kerswell, 2007), leaving sessile organisms in this zone, such as anemones and corals, vulnerable when water levels fall below mean sea level (Yamaguchi, 1975). Historically, extreme low tides around the world have been linked to coral bleaching and increased mortality (Anthony and Kerswell, 2007; Loya, 1976). Aerial exposure causes coral tissue to retract, leaving the skeleton exposed (Brown, Le Tissier, and Dunne, 1994; Moorhouse, 1936). Previous studies on the effects of low tide exposure on Pacific corals showed that acroporids were more susceptible to negative effects from low tides than poritids, faviids, musiids, and soft corals (Anthony and Kerswell, 2007). Faviids and musiids can retract their tissues into their skeletons, allowing them to withstand aerial exposure for longer periods of time (Brown, Le Tissier, and Dunne, 1994). In 1970, the Gulf of Eilat experienced a six-day period in which water levels were 40 cm lower than predicted; reef flat corals were fully exposed to the air and sun for three to four hours daily. Observed coral mortality was 81-85% (Loya, 1976). An extensive low tide event in 1972 killed all observed corals living on Guam's Pago Bay reef flat after two weeks of aerial exposure (Yamaguchi, 1975). A 2007 study (Anthony and Kerswell, 2007) on the Great Barrier Reef showed that 79% of pocilloporids and over 50% of acroporids experienced mortality after three days of aerial exposure combined with high solar radiance. Better understanding of these extreme tides have allowed researchers to develop models to predict both high tide (Sweet and Park, 2014) and low tide events (Heron et al., 2020).

From late 2014 to 2016, extreme low tides linked with ENSO conditions were recorded in Guam (Heron et al., 2020). These tides, together with anomalously high sea surface temperatures and increased irradiance, triggered a severe and prolonged bleaching event resulting in a reduction in island-wide staghorn coral populations of roughly 50%, with some sites seeing as much as 100% mortality of staghorn *Acropora* (Raymundo et. al, 2017). Understanding the effects of low tides on corals is critical to assessing their vulnerability, especially when combined with other compounding factors such as pollution.

1.4 Nutrient and Bacterial Pollution on Coral Reefs

Pollution is one of several stressors that negatively impacts coral reefs by disrupting the balance between various microbes within the coral microbiome (Wooldridge and Done, 2009). Anthropogenic pollution and nutrient runoff have increased significantly over the last century; anthropogenically-derived nutrients outstrip natural nutrient sources (Vitousek et al., 1997). Excess nitrate from runoff causes eutrophication, which can lead to algal blooms and large-scale die-off of corals (Hughes et al., 2007). Eutrophication is associated with benthic phase shifts from coral to macroalgal dominated reefs (Hunter and Evans, 1995), as well as negative impacts to coral reproduction, growth, and survivorship (D'Angelo and Wiedenmann 2014; Shantz and Burkepile, 2014). Naturally derived nitrogen comes primarily in the forms of ammonium and urea, whereas anthropogenically derived nitrogen occurs in the form of nitrate (Donovan et al., 2020). According to Burkepile et al. (2020), Pacific corals exposed to nitrate exhibited more frequent bleaching, bleached for a longer period of time, and were more likely to die than those in conditions with low concentrations of nitrogen. Marchioro et al. (2020) found that abundances of Endozoicomonadaceae in the tissue of *A. millepora* were negatively correlated with concentrations of NO_2^-/NO_3^-. A long-term study

conducted on the Great Barrier Reef showed that reefs with chronically elevated levels of dissolved inorganic nitrogen (DIN) had bleaching thresholds 2.5°C lower than those without nutrient enrichment (Wooldridge and Done, 2009). Corals in the genera *Pocillopora* and *Acropora* exposed to high inputs of nitrogen in conjunction with low heat stress bleached as severely as those without nitrogen inputs in high levels of heat stress (Donovan et al., 2020). One study conducted in the Caribbean (Vega Thurber et al., 2014) found that increased concentrations of phosphorous and nitrogen in seawater were positively correlated with coral bleaching and disease. In conjunction with increased temperatures, heightened nitrogen loads caused bacterial community alterations, and increased coral disease as well as mortality (Zaneveld et al., 2016). In French Polynesia, *Acropora* exposed to nitrate pollution were up to twice as likely to bleach as control corals and experienced a prolonged bleaching period (Burkepile et al., 2020). This susceptibility to anthropogenic nitrogen inputs makes *Acropora* corals useful indicators of nutrient pollution (Guzner, Novoplanksy and Chadwick, 2007).

Sewage is highly enriched in δ15N and therefore has a distinct stable isotope composition in comparison to other nitrogen sources, and it can be absorbed by—and later detected in— macroalgae (Abaya et al., 2018). Studies in Hawaii (Abaya et al., 2018) have shown that macroalgal δ15N, %N, and FIB all have a significant negative correlation to percent coral cover. A positive correlation between macroalgal δ15N and percentage of dead coral was observed on the west coast of the island of Hawaii (Parsons et al., 2008), suggesting that sewage pollution may contribute to declining coral cover. In Guam, Redding et al. (2013) found a positive correlation between sewage-derived nitrogen and severity of coral disease in *Porites* spp. as well as correlations between precipitation and δ15N. A study by LaPointe, Barile, and Matzie (2004) showed increases in δ15N following periods of rain, suggesting transfer of sewage-derived nitrogen with rain events.

Many agents within sewage, including nutrients, bacteria, suspended solids, and sediments, can interact with each other to escalate the impacts of coral reef stressors and impair coral growth and reproduction (Wear and Vega Thurber, 2015). Sewage pollution releases bacteria and nutrients that alter coral growth, calcification rates, species distribution, species abundance, and coral community diversity (Reopanichkul et al., 2009). These bacteria are used to monitor fecal waste contamination in places such as Guam, where beaches with high concentrations of *E. coli* are deemed unfit for recreational swimming (Howe, 2022). Marine waters affected by sewage pollution will have an *Enterococcus* signature at a geometric mean of at least 35 colony forming units (cfu) per 100 mL and a statistical threshold value of 130 cfu/100 mL (Guam EPA, 2015). Enterococci are considered the best FIB for monitoring contamination in saltwater (Guam EPA, 2015), as they can survive in water up to 6.5% NaCl at temperatures between 10 and 45° C, and are specific to human feces (Murray, 1990). *Escherichia coli* (E. coli) is a thermotolerant coliform bacteria (Albuquerque de Assis Costa, Mano Pessoa, and da Silva Carreira, 2018), and is primarily used as an indicator of fecal contamination in freshwater as it does not survive long term in saltwater. Total coliform count includes *E.coli* and is a widely-used biological indicator due to simplicity and low cost (Albuquerque de Assis Costa, Mano Pessoa, and da Silva Carreira, 2018). Given how sewage and nutrient inputs negatively impact coral cover, the presence of FIB in water may alter coral microbiomes.

1.5 The Coral Microbiome

Bacteria are members of the coral holobiont, a term that collectively refers to the coral host and its micro-associates such as archaea, fungi, protists, endosymbiotic algae, and viruses (Maher, Epstein, and Vega Thurber, 2022; Bourne, Morrow, and Webster, 2016).

These micro-associates supply the coral host with nutrients and protect it from pathogens. In return, the coral provides shelter, protection, and nutrients (Glasl, Webster, and Bourne, 2017). Micro-associates are considered to be members of either the core or dynamic microbiomes, though these communities are still poorly understood. The core microbiome refers to symbiotic microbiota selected by the host that are shared among members of the same species, whereas the dynamic microbiome refers to a variable microbial community that is responsive to biotic and abiotic processes (Hernandez-Agreda, Gates and Ainsworth, 2016).

Microbial interactions are not exclusive to microorganism interactions with the coral host, but also include interactions between the symbionts (Peixoto et al., 2017). For example, Symbiodiniaceae produce sulfur compounds which are consumed and metabolized by bacteria which, in turn, generate antimicrobial compounds that can inhibit the growth of coral pathogens such as *Vibrio coralliilyticus* and *V. owensii* (Raina et al., 2016). Pathogenic control can also be facilitated by viruses and protists, which function as biological control of bacteria in the open ocean (Chow et al., 2014). Bacterial colonization can be regulated and manipulated to protect coral against disease (Bourne, Morrow, and Webster, 2016; Glasl, Herndl, and Frade, 2016). Microbes also have the potential to maintain or return a coral holobiont to a healthy state of symbiosis, or eubiosis, after dysbiosis, a state in which the microbiomes are disturbed and/or unhealthy (Peixoto et al., 2017; Boilard et al., 2020). Dysbiosis results from stressors such as pollution, sedimentation, rising sea surface temperatures, ocean acidification, and other forms of environmental degradation (Sweet and Bulling, 2017).

The integrity of the microbiome can also be disrupted by the types of bacteria living in and invading it. Some bacteria are beneficial to coral health and are important symbionts, such as *Endozoicomonas* (Neave et al., 2017). *E. acroporae* is vital to the health of acroporid

corals by metabolizing dimethylsulfoniopropionate (DMSP) into dimethylsulfide (DMS) and can metabolize DMSP as a carbon source (Tandon et al., 2020). In *Porites astreoides*, the loss of *Endozoicomonas* bacteria was seen coupled with deterioration of coral health (Meyer, Paul, and Teplitski, 2014). Conversely, some bacteria are harmful to coral health. *V. shiloi* is a coral pathogen that inhibits photosynthesis of symbiotic zooxanthellae (Banin et al., 2001), and can lead to coral bleaching. Similarly, *V. coralliilyticus* has been linked to coral tissue lesions and white syndrome (Wilson et al., 2013).

How microbes impact corals also depends on where the microbes are located within the holobiont. Coral microhabitats, also called compartments, are divided into the skeleton, the tissue, and the surface mucopoly-saccharide layer (SML), each with distinct bacterial communities of their own (Glasl, Herndle, and Frade, 2016; Hernandez-Agreda, Gates, and Ainsworth, 2016; Peixoto et al., 2017). Algal endosymbionts reside in the coral tissue, and each algal cell is surrounded by a host-derived symbiosome membrane (Yellowlees, Rees, and Leggat, 2008). Nutrient and metabolite transfer between the host and symbiont occurs in the pocket created by this membrane, referred to as the peri-algal space (Kazandjian et al., 2008; Hernandez-Agreda, Gates and Ainsworth, 2017). Some bacteria, such as those from the genera *Propionibacterium* and *Ralstonia* are reported as potential universal symbionts that inhabit this space in relatively high abundance (Ainsworth et al., 2015). How these bacteria are able to inhabit this space, how they affect the coral host and what role they play in the coral's functioning can lead to further understanding of how the bacterial microbiome operates in coral holobiont maintenance.

As it is directly exposed to the environment, the SML is a first line of defense for corals and plays an important role in disease mitigation by means of antibiotic activity and microorganismal population control (Hernandez-Agreda, Gates, and Ainsworth, 2017; Ritchie, 2006). Field experiments that depleted SML microbes negatively impacted the

holobiont, resulting in an increase in *Vibrio* spp. and other pathogenic bacteria (Glasl, Herndle, and Frade, 2016). Disturbing the mucus microbiome may thus open the way for pathogens and disease, potentially leading to coral death (Glasl, Webster, and Bourne, 2017). The SML is a dynamic environment and the micro-associates within are constantly exposed to biotic and abiotic variation, suggesting that the community of this environment may be highly variable (Hernandez-Agreda, Gates, and Ainsworth, 2016).

One source of biotic variation in the SML could originate from the water column itself. The microbes associated with the SML could be representative of those found in the surrounding water column, as SML and seawater samples can share bacterial species due to exchange between the sloughed SML and surrounding water (Sweet, Croquer and Bythell, 2010; Ritchie, 2006). Conversely, microbes found in seawater may be transferred and incorporated into the SML. One study observed that bacteria found in the seawater and coral samples of anthropogenically impacted sites were significantly different from those found in unimpacted sites (Ziegler et al., 2019). Because the SML acts as a protective layer between the coral tissue and seawater, the microbiome of coral tissue is more stable and less dynamic than that of mucus and seawater.

1.6 Microbiome Regulators vs. Conformers

Previous studies (Camp et al., 2020) show that bacterial profiles of different corals are site-specific depending on the habitat. Coral microbiomes with higher bacterial biodiversity have been observed when exposed to human impacts such as sewage and municipal wastewater (Ziegler et al., 2016). According to Voolstra and Ziegler (2020), “microbiome conformers” refer to species that show microbial adaptation to their surrounding environments, whereas “microbiome regulators” maintain a consistent microbiome regardless

of the differences in their external environment. Garren et al. (2009) showed that *Porites cylindrica* specimens transplanted under high deposits of fish farm effluent had elevated abundances of *Vibrio* spp. characterizing their microbiomes after five days of exposure, but their microbiomes restored to their original states after 22 days of exposure to effluent, showing a characteristic expected of a "microbiome regulator."

"Microbiome conformers" often refer to taxa such as *Acropora* spp., which absorb constituents from their surrounding environment and incorporate them into their holobiont (Greer et al., 2009; DeVantier et al., 2006). When examining corals living in environments under different degrees of anthropogenic impact, Ziegler et al. (2019) found that the bacterial communities in *A. hemprichii* reflected external conditions more than those found in *P. verrucosa*. This behavior is why "microbiome flexibility" is used to describe the potential for species such as those in the genus *Acropora* to alter their microbiome as their environments change (Ziegler et al., 2019). Whether or not staghorn *Acropora* on Guam's reef flats restructure their bacterial communities seasonally or across microhabitats, and how these changes would be reflected in different compartments of the coral holobiont, has yet to be explored. This study examined the spatiotemporal dynamics of the microbiome of *A. pulchra* in West Hagåtña Bay on the island of Guam.

1.7. Research Objectives

The objectives of this study were to (1) characterize bacterial communities of coral tissue and coral mucus in *A. pulchra* populations as well as seawater in the near-shore and far-shore zones of West Hagåtña Bay, and (2) elucidate impacts of habitat and seasonal change on the coral bacterial microbiome.

1.8. Hypotheses

This study tested three hypotheses to address spatial, compartmental, and temporal variability of the microbiome of *Acropora pulchra*.

$H_0$1: Community diversity of the bacterial microbiome in *A. pulchra* does not differ between near-shore and far-shore populations.

H_a1: Bacterial community diversity does differ between populations near shore and far from shore.

$H_0$2: The bacterial microbiomes of coral tissue, coral mucus, and seawater are equally diverse.

H_a2: The microbiomes of the coral tissue, coral mucus, and seawater each have different bacterial diversity.

$H_0$3: The community diversity of the bacterial microbiome of *A. pulchra* does not change with seasonal changes associated with wet and dry seasons in Guam.

H_a3: The bacterial microbiome of *A. pulchra* reflects changing environmental conditions of the wet and dry seasons.

2. Materials and Methods

2.1 Characterization of Study Site

West Hagåtña Bay, Guam (Figure 1) has two main environmental zones: outer (closer to the reef crest) and inner (closer to the shore). Within each zone, three sites were targeted for sampling. Because West Hagåtña Bay has two distinct environmental zones with different rates of water flow, and as staghorn *Acropora* within this bay have previously been studied, it was deemed suitable for carrying out this experiment to study how the bacterial microbiome of staghorn *Acropora* reflects different environmental conditions associated with established habitats as well as changing seasons.

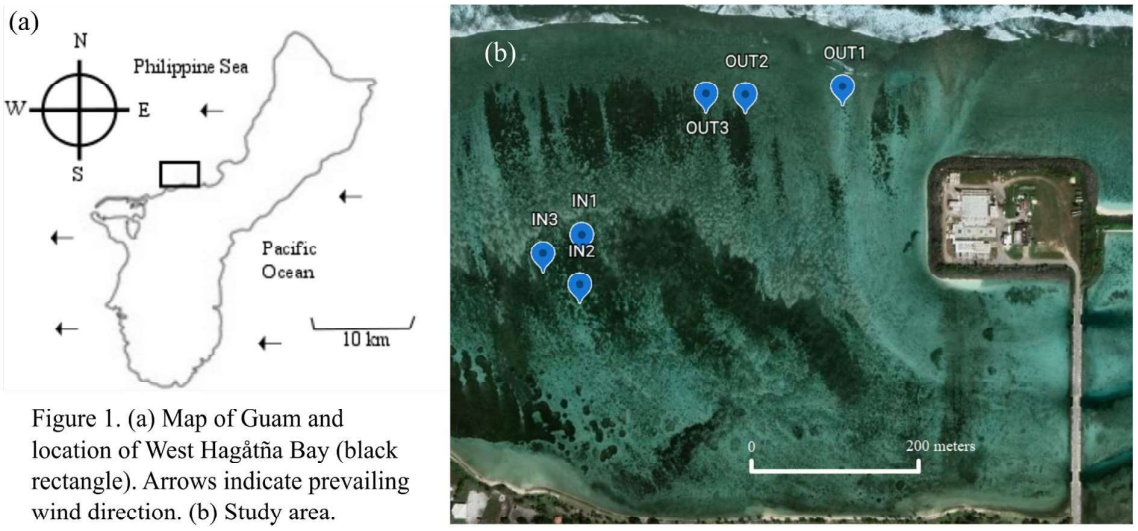

Figure 1. (a) Map of Guam and location of West Hagåtña Bay (black rectangle). Arrows indicate prevailing wind direction. (b) Study area.

West Hagåtña Bay lies on the west coast of the island of Guam, adjacent to the Hagåtña sewage treatment plant. The sewage outfall was renovated and repaired in 2008 to discharge 100 m further away from shore than it previously did; it currently sits 366 m beyond the reef line at a depth of 84 m (GWA, 2019). The outfall pipe is 107 cm in diameter with a single-port diffuser (GWA, 2019). The treatment process was enhanced in 2014 and the plant treats an average of 5.8 million gallons daily (MGD) (GWA, 2022). In 2017, the

average daily design flow capacity and discharge effluent flow was 12.0 and 6.11 MGD, respectively (GWA, 2019).

Guam Environmental Protection Agency (Guam EPA) lists treatment standards for municipal wastewater treatments for total suspended solids (TSS) and biochemical oxygen demand (BOD) as 30 mg/L per day for each (Guam EPA, 2010). In Guam, the geometric mean (GM) of *E.coli* in freshwater for recreational use at an acceptable number is 126 cfu/100mL with a statistical threshold value (STV) of 410; GM of *Enterococcus* is not to exceed 35 cfu/100mL with an STV of 130. The pH of the water must be within the range of 6.5 to 9. Arithmetic mean of total fecal coliform samples is not to exceed 200 cfu/mL over a 30-day collection (Guam EPA, 2015). The most recent collection of monitoring data was from 2015 – 2018; water pH in West Hagåtña Bay was 6.7-8.1. Maximum BOD was 166 mg/L, TSS was 130 mg/L, and the maximum detected MPN/100 mL of *Enterococci* was 1,109,898 (GWA, 2019). This bay periodically reaches bacteriological levels deemed too high for recreational standards (Howe, 2022).

When tested for δ15N content, soft corals in this bay showed twice as much δ15N as those found in areas further removed from human impacts such as Luminao and Piti (Redding et al., 2013). Fifer et al. (2021) found trace amounts of ammonia in this bay's water (0.013–0.10 mg/L). The land adjacent to West Hagåtña Bay is paved with a main road, several stores, and housing units. According to EPA (2022), rainwater does not soak into paved surfaces and will instead flow into bodies of water. As a result, West Hagåtña Bay receives large influxes of runoff from land after rainfall events. This runoff can discharge nutrients and pollutants, harm fish and wildlife populations, and make waters unsafe for recreational activity. This bay is influenced by a longshore current flowing from northeast to southwest (Wolanski et al., 2003). Water currents within this bay have not been modeled.

West Hagåtña Bay contains three extensive staghorn coral thickets. The surface area of the thickets covers 121,439 m^2, 48.4% of which represents live coral cover. The dominant coral is *A. pulchra* and the estimated mean coral cover for this species is 23,991.6 m^2 (Raymundo et al., 2022). This bay's vulnerability to aerial exposure from extreme low tides is considered moderate-to-high (Heron et al., 2020). As a result of the 2014 mortality events, this area experienced an estimated 55% mortality of staghorn coral (Raymundo et. al, 2017), and live coral cover was reduced from 29% to 7% after the 2015 extreme low tide event (Raymundo et al., 2019). Overall, this bay saw a reduction in roughly one-third of areal extent of staghorn coral cover from 2013 to 2017 (Raymundo et al, 2019). Raymundo et al. (2019) found that mortality of staghorn *Acropora* populations increased with proximity to the shoreline and that, closer to the reef margin, mortality was not observed. A study by Fifer et al. (2021) that focused on the same area as the 2019 study found a significant difference in water flow speeds between a site close to the reef margin (far shore) and a site close to shore (near shore). This was tested using clod card dissolution rates (Doty, 1971) and an acoustic doppler current profiler (ADCP). The "near shore" site corresponded with low water flow rates and large amounts of dead coral colonies, and the "far shore" site was associated with high water flow rates and no dead coral colonies were observed (Fifer et al., 2021; Raymundo et al., 2019).

2.2 Environmental Data

Six temperature loggers were placed in the field, one at each sampling site, to establish temperature dynamics throughout the course of this study, from April until December 2021. Temperature was monitored using HOBO TidBit loggers (Onset Corp., Bourne, MA) attached to the margin of coral colonies at a depth of 1 meter. Temperatures are

generally highest during the wet season and extreme low tides coincide with high temperatures annually.

Wave action is usually the calmest during the height of temperatures in Guam's dry season, and this often coincides with the lowest tides. To quantify water level range, two pressure loggers were deployed at the inner and outer zones. One additional pressure logger was placed outside the water at the sewage treatment plant on the eastern end of the bay to record atmospheric pressure and thus calibrate water pressure. From water pressure, water level range was calculated by converting kiloPascals to meters. This was accomplished using the formula:

$$Water\ level\ range\ (meters) = \frac{\left(1000 * pressure(kPA)\right)}{(saltwater\ density * gravity)}$$

With saltwater density at 1,023.6 kg/m^3 (Brown, 2016) and gravity at 9.8 m/s^2.

Benthic cover was characterized at each inner and outer zone by laying six ten-meter line intercept transects (LITs) in each zone for a total of 60 meters at each zone. LITs were used to determine the abundance of hard coral cover, coral rubble, fleshy macroalgal cover, pavement, and sand. Daily precipitation data were collected by the National Oceanic and Atmospheric Administration (NOAA) at the Guam International Airport and retrieved from the NOAA website (NOAA, 2022) and was then averaged by month. To corroborate findings from previous studies, water flow rates were quantified by using Tilt Current Meters (TCM) (Lowell Instruments, LLC, Falmouth, MA). One TCM was placed at the inner zone, and two TCMs were placed in the outer zone: one at site OUT1, near the Hagåtña sewage treatment plant and the other at OUT2 (Figure 1). TCMs were deployed for a total of five weeks from the start of November through the first week of December 2022, and calculated water flow velocities to establish speed of water flow as well as direction.

2.3 Sample Collection

Samples were collected twice in Guam's dry season, at the beginning of May and beginning of July, and twice in Guam's wet season, at the end of September and the end of December. One coral nubbin (4.5 – 5 cm) was taken from each of nine *A. pulchra* colonies near the reef crest and nine colonies from the inner zone four times throughout the year for a total of 72 coral tissue samples (Figure 2). Colonies were tagged to allow for sampling from the same colonies repeatedly. The terminal end of each cut coral fragment was removed to avoid sequencing biases since new tissue may not be fully colonized by the coral microbiome community. Mucus was collected from the same specimens by exposing the cut coral fragment to air and using sterilized cotton swabs (Lampert et al., 2008). 3L of seawater were collected from each site by using containers sterilized with 10% bleach. Seawater was pre-filtered through a wire mesh to remove large particles and then filtered through a 1.2 µm nylon filter (Sigma-Aldrich, St Louis, MO) to collect bacteria. Coral tissue, coral mucus, and seawater samples were frozen immediately in liquid nitrogen and then stored at -80°C until further processing.

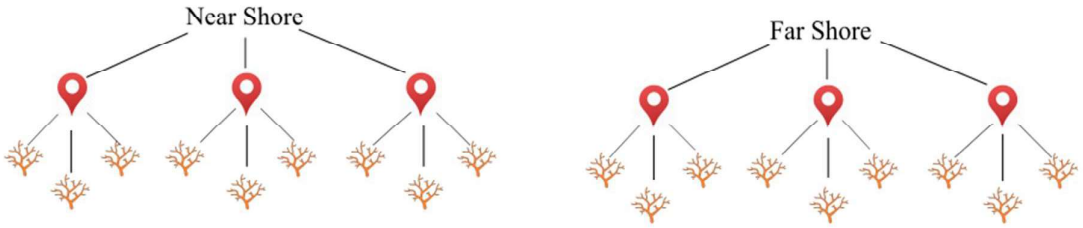

Figure 2. Experimental design of sampling from different colonies within each zone. Each zone has three sites, and each site has three colonies.

To acquire nutrient and bacterial load data from seawater, additional seawater samples were taken during each sampling event and submitted to the Water and Environmental Research Institute (WERI) at the University of Guam (UOG) to determine FIB concentrations of *E.coli*, *Enterococcus*, and total coliform. To test for amounts of nitrate/nitrite (NO_3/NO_2^-N)

and ortho-phosphate (PO_4^-P), additional seawater samples were collected in 50 mL falcon tubes at each site for a total of six falcon tubes at each sampling date and submitted to WERI at UOG.

2.4 Environmental Data Analysis

Normality of each environmental dataset was estimated using a Shapiro-Wilk test. To test for significant differences in benthic cover between zones, a one-way ANOVA was used. Two-way ANOVAs incorporating zones and months as factors were used to test for significant differences in FIB concentrations between inner and outer zones as well as between months. Significant differences between inner and outer zones as well as different months were calculated using Kruskal-Wallis Chi^2 tests on temperature and water level range. Kruskal-Wallis Chi^2 tests were also used to test for significant differences between inner and outer sites in water flow rate followed by a post-hoc Dunn's Test using the DescTools package (Signorell, 2023). Because water flow rates were not monitored over the same time scale as the other environmental variables, they were tested only for significant differences between zones and not over time.

2.5 DNA Extraction and Metabarcoding

DNA was extracted from coral tissue, coral mucus, and seawater samples using a DNEasy Powersoil kit (Qiagen, Hildenheim, Germany) following the manufacturer's protocol. DNA extracts were quantified using Qubit Fluorometric Quantification (Thermo Fisher Scientific, Waltham, MA). Each DNA extract was diluted to a concentration of 10 ng/µl. The V4 hypervariable region of 16S ribosomal DNA from each sample was amplified using 515F and 806R universal bacterial primers (Walters et al., 2016) via PCR. The 30-µl

PCR reactions included 3 µl of template DNA, PCR-grade water, 1x ExTaq buffer (Takara Bio, San Jose, CA), 2.5 mM dNTPs, 10 µM forward and 10 µM reverse primers, and 0.75 U ExTaq DNA Polymerase (Takara Bio, San Jose, CA). The thermocycler protocol had an initial denaturation step of 95°C for 40 seconds, an annealing step at 58°C for two minutes, and an extension at 72°C for one minute. This cycle happened thirty times with a final step at 72°C for 5 min. A negative control using PCR-grade water instead of DNA was included in every PCR run. All PCR products were checked on a 1% agarose gel stained with GelRed (Sigma-Aldrich, St Louis, MO). 2 µl of PCR product and 2 µl of loading dye were added to each well of the gel and product sizes checked using 1 Kb DNA ladder (New England BioLabs, Ipswich, MA). PCR products that showed up as bright fluorescent bands were considered successful. Samples that produced faint bands had low yields from the initial round of PCR and were re-amplified using another PCR round with a new negative control. Each successfully amplified 16S sample was purified using GeneJet PCR Purification Kit (Thermo Fisher Scientific, Waltham, MA) following the manufacturer's protocol. The DNA concentration of each purified sample was checked again using Qubit Fluorometric Quantification. Each purified sample was barcoded using custom barcode indexes and MiSeq adapters (Macrogen, Seoul, South Korea) in a PCR reaction. Each reaction used 2 µl of PCR product, 1 mM MiSeq adapter, PCR-grade water, 1x ExTaq buffer, 2.5 mM dNTPs, and 0.5 U ExTaq DNA Polymerase per 20 µl reaction. The adapters were attached to the PCR product using the following PCR protocol: denaturing at 95°C for 40 seconds, annealing at 59°C for two minutes, then an extension at 72°C for one minute. This cycle was repeated four times for a total of five cycles. After five cycles, there was a step at 72°C for seven minutes. After attaching the adapters, they were each checked on a 1% agarose gel stained with GelRed.

All successfully amplified PCR products were pooled after indexing with barcodes. Samples that yielded bright PCR bands had 3 µl added to the pool, samples with faint bands had 10 µl added, and samples that were of moderate brightness had 5 µl added to the pool. 40 µl of each pooled sequencing library were purified using GeneJet PCR Purification Kit and resuspended with 40 µl of elution buffer. The purified pooled products were checked on a 2% TBE agarose gel. 10 µl of pooled product were added to a well along with 5 µl of loading dye and another well was loaded with 3 µl of 1 Kb DNA ladder and 3 µl of loading dye. The target band representing the sequencing library was extracted using a sterilized scalpel. 200 µl of nuclease-free water were added to the excised gel and incubated overnight at 4°C. DNA in the resulting solution was purified using GeneJet PCR Purification Kit following the manufacturers protocol and resuspended in 20 µl of elution buffer. DNA concentration was again checked using Qubit Fluorometric Quantification. The size distribution of the final purified sequencing library was verified using an Agilent 4150 TapeStation (Agilent, Santa Clara, CA) with a D1000 ScreenTape assay. Libraries were sequenced at CD Genomics (Shirley, NY) using an Illumina MiSeq. Each sequencing pool contained a mixture of sampling timepoints to prevent batch effect.

2.6 Sequence Analysis

All 16S data processing followed the protocols of Fifer et al. (2022). The R package DADA2 (Callahan et al., 2016) was used to remove primer sequences, truncate reads, calculate error rates, de-duplicate reads and infer sequence variants, merge paired reads, and remove chimeras. Non-bimeric amplicon sequence variants (ASVs) were assigned taxonomy using the Silva v138 dataset (Glöckner et al., 2017) using a naive Bayesian classifier (Wang et al., 2007) with a minimum bootstrap confidence of 50. Phyloseq (McMurdie and

Holmes, 2013) was used to remove ASVs matching to "Mitochondria," "Chloroplast," or "Eukaryota." MCMC.OTU (Matz, 2016) trimmed ASVs representing <0.1% of counts and identified putative outlier samples (those that had total counts falling below z-score cutoff of -2.5); one sample was removed from downstream 16S analyses due to low quality. Samples with fewer than 1000 reads (n = 2) were discarded and not used in analyses.

Rarefaction is commonly used in microbial data (Pollock et al., 2018; Pootakham et al., 2019; Marchioro et al., 2020; Fifer et al., 2022) to create an even sequencing depth and ensure that all samples contain equal amounts of reads for analyses. This technique randomly discards reads from larger samples without replacement until the total number of remaining reads is equal to an established threshold. Some researchers (Hong et al., 2022) argue that use of non-rarified data may lead to weak control of false discovery rates and that rarefaction can be used to guarantee the validity of permutation tests. Others argue that rarefying data is inadmissible due to introducing bias, giving false impression of equal richness (Willis, 2019), causing potential loss of sensitivity, and having high rates of false positives (McMurdie and Holmes, 2014). Hong et al. (2022) developed a guideline called the "rarefaction efficiency index" (REI) to determine whether rarefaction is suitable for particular datasets.

To assess whether data rarefaction would be appropriate for this study, a copy of the bacterial sequences dataframe was created with every sample rarefied to 5000 reads. Permutational analysis of variance (PERMANOVA) tests were then conducted on both rarefied and non-rarefied datasets. On both rarefied and non-rarefied datasets, PERMANOVA tests yielded the same statistical results. Given these results, the remainder of statistical analyses were calculated on the non-rarefied dataset to prevent bias and loss of sensitivity. To assess adequacy of sequencing depth, a rarefaction curve was calculated (Appendix 1) showing that the majority of microbial diversity was captured (Amend et al.,

2022). Thus, no further normalization or downsampling steps were taken to account for differential sequencing effort.

Phyloseq calculated observed ASV richness and microbial diversity based on the Shannon diversity index. Normality for each compartment was tested using a Shapiro-Wilk test. Non-parametric analysis of variance (Kruskal-Wallis test) was used to compare alpha diversity across compartments, between months, and between inner and outer zones when normality was rejected. A post-hoc Dunn's Test was then used for multiple comparisons. For compartments in which normality was detected, a two-way analysis of variance (ANOVA) was used to test for alpha diversity differences. PERMANOVA tests using the Adonis2 function in the vegan package (Oksanen et al., 2020) were run with 1,000 permutations to calculate beta diversity between months, compartments, and inner and outer zones based on weighted UniFrac distances. Weighted UniFrac distances were used to create a Principal Coordinate of Analysis (PCoA) plots for visualization of the compartments, zones, and seasons. Weighted UniFrac distances were chosen over unweighted because they consider the presence and absence of taxonomic groups while incorporating the abundance of ASVs (Lozupone and Knight, 2005). *Post hoc* pairwise PERMANOVAs were conducted to detect significant differences between individual months and compartments.

Because environmental factors are interrelated and their effect on microbial diversity may be indirect, structural equation models (SEMs) were created using the piecewise SEM package (Lefcheck, 2016) to examine the relationships between precipitation, temperature, FIB concentrations, and bacterial diversity. These SEMs were made by creating linear mixed effects models (LME) with Shannon diversity as the response variable and environmental variables as the predictors. One dataframe joined by month was created to compare precipitation, temperature, FIB and microbial diversity. These factors were included as fixed factors and zone was included as a random factor to account for significant habitat

differences between zones that likely played a role in driving diversity. This removed potential influence that zone would have and allowed for closer examination of other factors. One LME was created to examine overall microbial diversity and additional LMEs were created to isolate each compartment. If statistical significance was found from any of the fixed factors, they were tested for interaction effects with zone in a linear model (LM) to further explore environmental effects within each zone.

This study examined two exogenous variables (temperature and precipitation), three endogenous variables (concentrations of *Enterococcus*, *E.coli*, and total coliform) and one response variable (Shannon diversity values). Endogenous variables are defined as those nested within and influenced by the external environment that will potentially have an impact on the response variable. Exogenous variables are those that originate from the environment that influence nested environmental aspects as well as the response variable. SEMs tested for effects of (1) precipitation on microbial diversity and concentrations of *Enterococcus,* total coliform, and *E. coli*; (2) temperature on microbial diversity and concentrations of *Enterococcus*, total coliform, and *E. coli*; (3) concentration of *Enterococcus* on microbial diversity and concentrations of total coliform and *E. coli*; (4) concentration of total coliform on microbial diversity and concentrations of *E. coli* and *Enterococcus*; and (5) concentration of *E. coli* on microbial diversity and concentrations of total coliform and *Enterococcus*. Testing for collinearity among concentrations of FIB required inclusion of correlated error structures between each FIB. Separate SEMs that included the aforementioned tests were constructed for each compartment of the bacterial microbiome to further explore potential drivers of microbiome diversity.

3. Results

3.1 Environmental Data

Temperature ranged from ~27°C to ~35°C. From June until August, there was roughly a 0.5°C difference between the inner and outer zones. On average, the inner zone was warmer than the outer zone (Figure 3), however, the difference between the two zones was not significant (Kruskal-Wallis $Chi^2 = 3.181$, df =1, $p = 0.075$). Water temperature differed significantly by month throughout the year (Kruskal-Wallis $Chi^2 = 275.510$, df = 8, $p < 0.001$). There was no significant interaction effect between zone and month for water temperature (Kruskal-Wallis $Chi^2 = 16.000$, df = 16, $p = 0.453$).

Water level range varied from as low as ~0.2 m during June to a maximum of ~1.6 m during April, with the outer zone generally having a greater range than the inner zone (Figure 4). Between the two zones, water level range was significantly different (Kruskal-Wallis $Chi^2 = 123.24$, df = 1, $p < 0.001$) and was significant between months (Kruskal-Wallis $Chi^2 = 150.980$, df = 8, $p < 0.001$). Analysis of benthic cover compared the amount of fleshy macroalgae, live hard coral, pavement, coral rubble, and sand in each zone (Figure 4). Overall substrate composition quantified from LITs showed no significant difference between inner and outer zones ($F = 0.918$, df = 1, $p = 0.348$). Water flow rates were significantly different between the two outer sites ($p < 0.001$), and between OUT2 and the inner site ($p < 0.001$), but was marginally significant between OUT1 and the inner site ($p = 0.0511$) (Figure 5). Flow rate at the inner site was lower than either of the outer sites, with an exception at the very end of the data collection period. During logger deployment, the flow rate of OUT2 was greater than that of the other two sites with an exception at the start of November, in which the flow rate of OUT1 briefly was higher than that of OUT2.

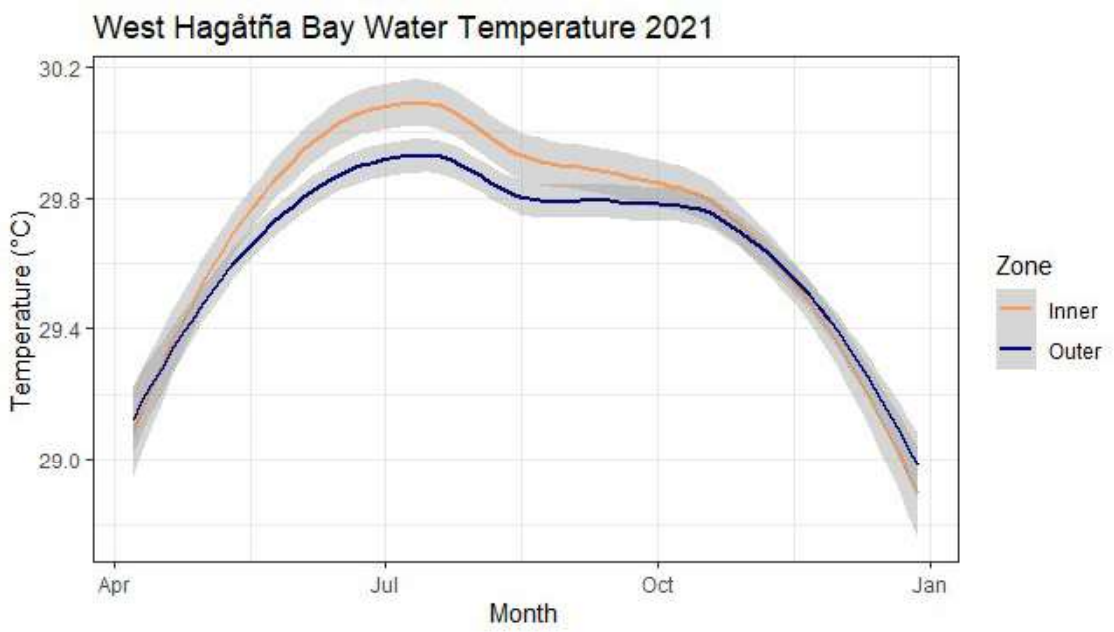

Figure 3. Average yearly temperature range in the inner (orange) and outer (blue) zones in West Hagåtña Bay. The "geom_smooth" function in R package ggplot2 (Wickham, 2016) was used to average the temperatures.

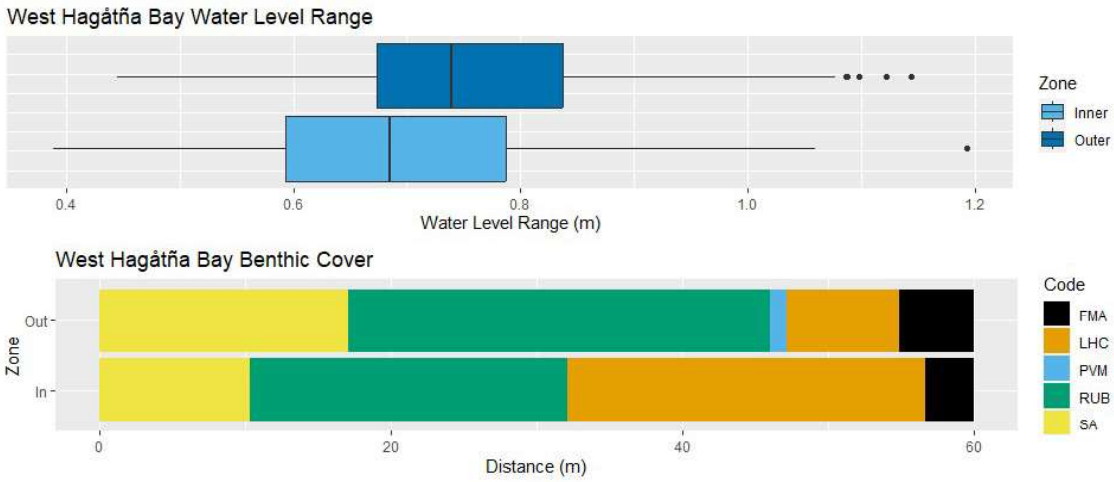

Figure 4. Tidal range and benthic cover in West Hagåtña Bay. The code for different benthic categories is as follows: FMA: fleshy macroalgae; LHC: live hard coral; PVM: pavement; RUB: rubble; SA: sand.

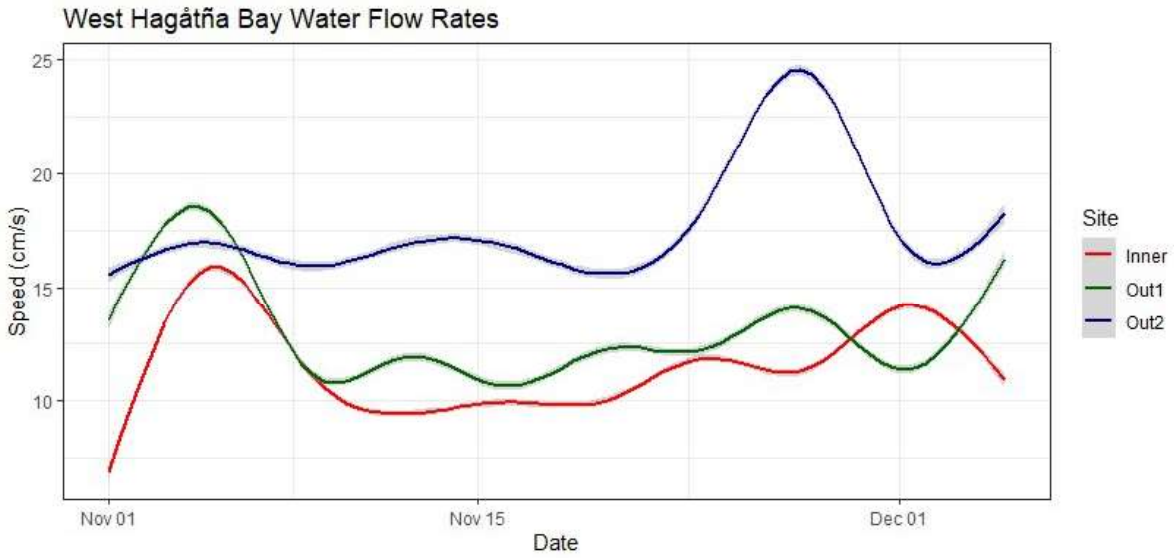

Figure 5. Water flow rates for the inner site (red), outer site near the sewage treatment plant (OUT1; green) and outer site roughly 150 m west (OUT2; blue). Flow rates are displayed in cm/s.

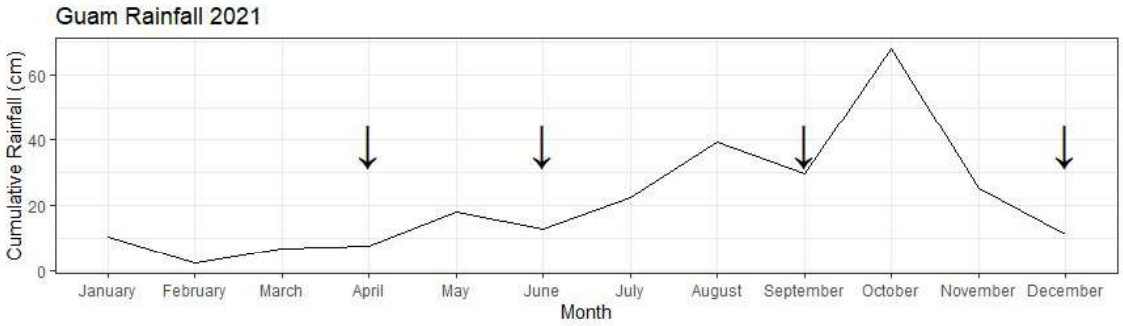

Figure 6. Cumulative rainfall throughout 2021. Arrows indicate sampling periods: the end of April, the end of June, the end of September, and the end of December.

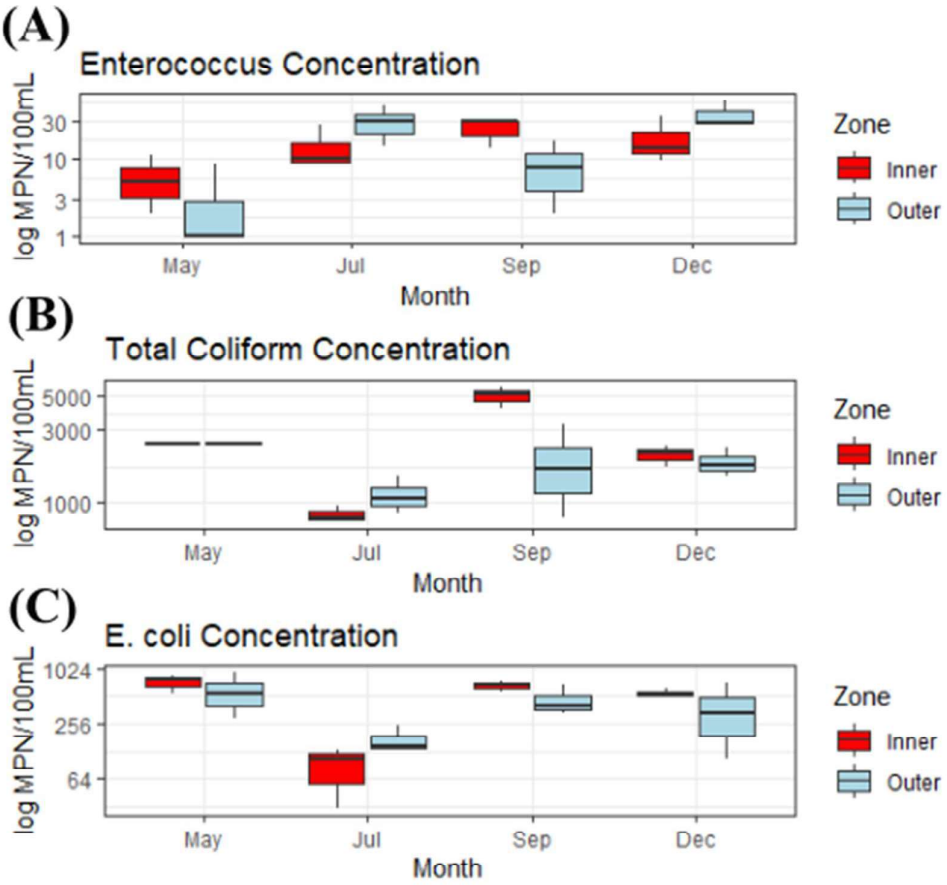

Figure 7. Concentrations of FIB from each sampling point. The y-axes are scaled by log10. (A) Concentrations of *Enterococcus*. (B) Concentrations of total coliform. Total coliform concentrations for the month of May were given as an undefined range greater than 2419.6 MPN/100 mL. (C) Concentrations of *E. coli*.

Average concentration of each FIB (Fig. 7) did not significantly differ between zones (*Enterococcus*: $F = 0.216$, $df = 1$, $p = 0.667$; total coliform: $F = 0.47$, $df = 1$, $p = 0.531$; *E. coli*: $F = 0.258$, $df = 1$, $p = 0.638$) or between sampling months (*Enterococcus:* $F = 3.717$, $df = 1$, $p = 0.126$; total coliform: $F = 0.01$, $df = 1$, $p = 0.927$; *E. coli*: $F = 0.068$, $df = 1$, $p = 0.807$) or between the interaction of zones and months (*Enterococcus:* $F = 0.300$, $df = 1$, $p = 0.613$; total coliform: $F = 0.11$, $df = 1$, $p = 0.756$; *E. coli*: $F = 0.025$, $df = 1$, $p = 0.882$). No significant amounts of nitrite/nitrate or orthophosphate above a threshold of 0.01 mg/L were detected.

3.2 Composition of Coral Tissue, Coral Mucus, and Seawater Microbiomes

Bacterial 16S gene amplicons derived from 161 samples (Table 1; n = 70 for tissue; n = 68 for mucus; n = 23 for seawater) were sequenced and a total of 16,949 ASVs were identified. The purgeOutliers function from the MCMC.OTU (Matz, 2016) trimmed ASVs present in less than 0.1% of samples, which retained 321 ASVs.

Table 1. Sample collection dates during the dry season (May 4 and July 1) and the wet season (September 21 and December 28) with number of samples from each site that yielded sequence data.

Sampling Date	Coral tissue samples with sequence data	Coral mucus samples with sequence data	Seawater samples with sequence data
May 4	18	17	6
July 1	17	16	5
September 21	18	17	6
December 28	17	18	6

When all three compartments were analyzed together, Alpha diversity based on Shannon index significantly differed between compartments (Appendix 2; Kruskal-Wallis $Chi^2 = 63.138$, $df = 2$, $p < 0.001$). Tissue had significantly lower microbial diversity than mucus ($p < 0.001$) and than seawater ($p < 0.001$). However, there was no significant difference for alpha diversity between mucus and seawater ($p = 0.286$). There was no significant difference between inner and outer zones (Kruskal-Wallis $Chi^2 = 0.310$, $df = 1$, $p = 0.310$) and marginal significance between months (Kruskal-Wallis $Chi^2 = 7.6118$, $df = 3$, $p = 0.055$). There was no significant interaction between zones and months (Kruskal-Wallis $Chi^2 = 9.2215$, $df = 7$, $p = 0.237$) but significant interactions between month and

compartment (Kruskal-Wallis Chi^2 = 73.897, df = 11, $p < 0.001$), and between zone and compartment (Kruskal-Wallis Chi^2 = 64.776, df = 5, $p < 0.001$) as well as between month, zone, and compartment (Kruskal-Wallis Chi^2 = 86.204, df = 23, $p < 0.001$). Seawater had the highest microbial diversity (Shannon index = 2.71), followed by mucus (Shannon index = 2.48) and then tissue (Shannon index = 1.69) (Figure 8).

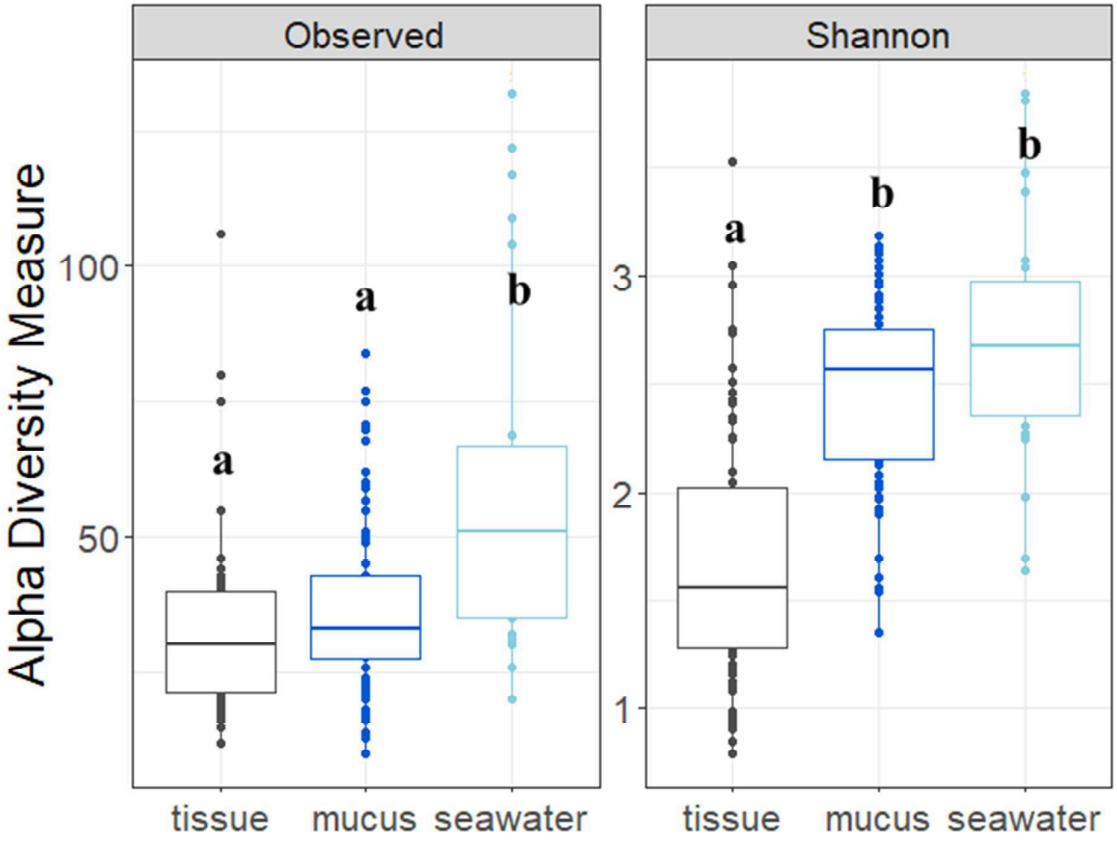

Figure 8. Calculated ASV richness for coral tissue, coral mucus, and seawater based on observed richness and Shannon diversity index. Observed: seawater microbial diversity (b) is statistically significant from coral tissue (a) and coral mucus (a) microbial diversity. Shannon: coral tissue microbial diversity (a) is significantly different from coral mucus (b) and seawater (b) diversity.

Alpha diversity based on the Shannon index for tissue and seawater compartments did not differ significantly between zone (tissue: Kruskal-Wallis Chi^2 = 0.018, df = 1, p = 0.893; seawater: F = 0.087, df = 1, p = 0.316), month (tissue: Kruskal-Wallis Chi^2 = 6.115, df = 3, p = 0.101; seawater: F = 2.967, df = 1, p = 0.100) or with an interaction between zone and

month (tissue: Kruskal-Wallis Chi^2 = 13.530, df = 7, p = 0.060; seawater: F = 0.970, df = 1, p = 0.337) (Figure 9). Mucus also did not have statistical significance between inner and outer zones (Kruskal-Wallis Chi^2 = 1.288, df = 1, p = 0.256), but did see statistical significance between months (Kruskal-Wallis Chi^2 = 8.567, df = 3, p = 0.036) as well as an interaction between zone and month (Kruskal-Wallis Chi^2 = 14.507, df = 7, p = 0.043). A Dunn's Test on mucus microbial diversity showed that there were statistically significant differences in alpha diversity between July and December (p = 0.047).

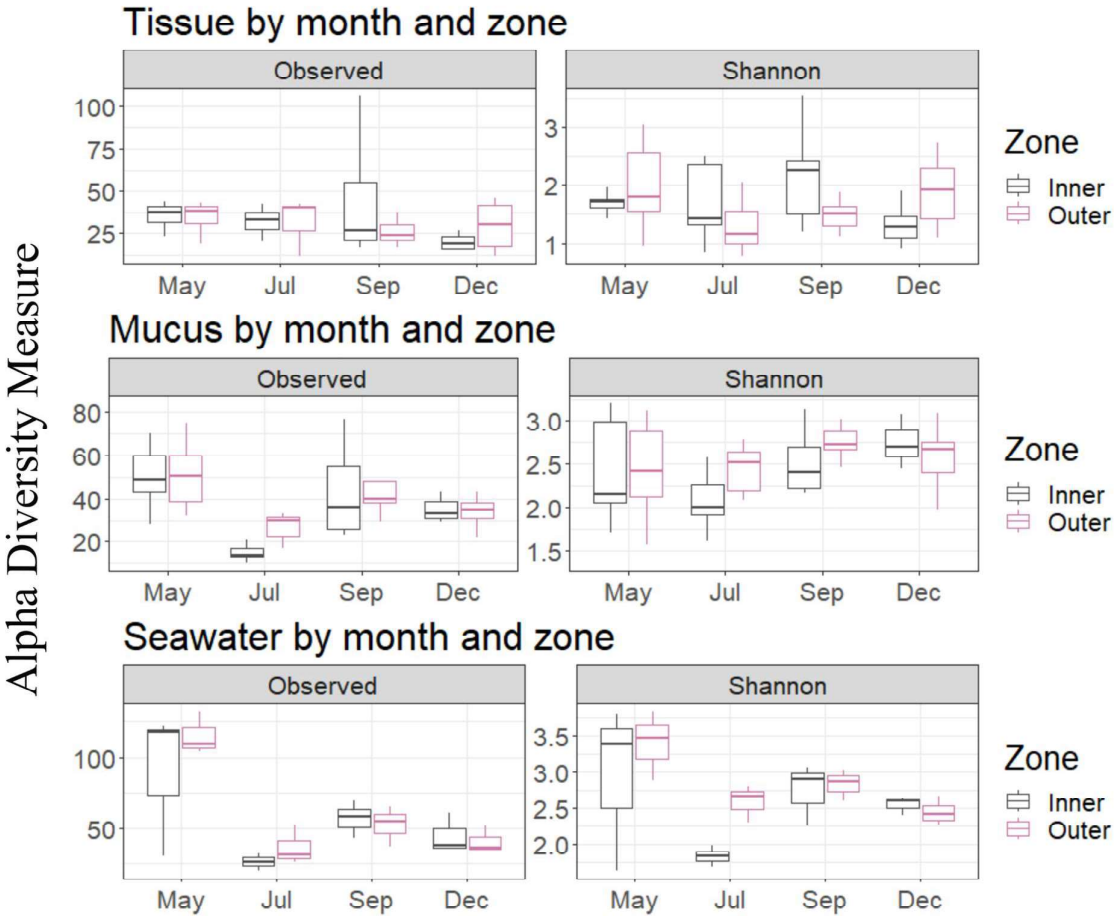

Figure 9. Boxplots were made for each compartment based on observed ASV diversity and Shannon Diversity Index. Boxplots are separated based on the month and zone in which sampling occurred.

Sequences associated with the phylum Proteobacteria dominated the microbial community overall (Table 2; average relative abundance; overall: 76.5%; tissue: 83.7%; mucus: 72.4%; seawater: 63.3%), followed by Verrucomicrobiota (overall: 9.9%; tissue: 11.7%; mucus: 9.3%; seawater: 5.2%) and Bacteroidota (overall: 5.9%; tissue: 1.6%; mucus: 6.7%; seawater: 19.3%). The family Endozoicomonadaceae was the most represented in each compartment (Table 3; overall: 51%; tissue: 76.2%; mucus: 31.1%; seawater: 23.8%). In the coral tissue, Endozoicomonadaceae dominated all months and zones (Figure 10A), followed by Simkaniaceae (11.8%), Moraxellaceae (2.5%) and Comamonadaceae (1.6%). Mucus samples (Figure 10B) were mostly characterized by Endozoicomonadaceae (31.1%), with representation from Comamonadaceae (13.6%), Moraxellaceae (11.3%), and Simkaniaceae (9.1%). Seawater samples (Figure 10C) also had representation from Endozoicomonadaceae (23.8%) followed by Cyanobiaceae (7.3%), Rhodobacteraceae (6.3%), and Chitinophagaceae (6%).

Table 2. Relative abundances of six most abundant phyla found in all compartments, in coral tissue, in coral mucus, and in seawater.

Phylum	Overall %	Tissue %	Mucus %	Seawater %
Actinobacteriota	2.6	1.1	4.5	2.3
Bacteroidota	5.9	1.6	6.7	19.3
Cyanobacteria	2.0	0.4	2.2	7.3
Firmicutes	2.3	1.2	3.9	1.6
Proteobacteria	76.5	83.7	72.4	63.3
Verrucomicrobiota	9.9	11.7	9.3	5.2

Table 3. Relative abundances of 11 most abundant families found in all compartments, in coral tissue, in coral mucus, and in seawater.

Family	Overall %	Tissue %	Mucus %	Seawater %
Alteromonadaceae	1.4	0.5	1.6	4.2
Chitinophageacae	3.2	0.9	5.0	6.0
Comamonadaceae	6.8	1.6	13.6	5.1
Cryomorphaceae	0.8	0.1	0.4	4.7
Cyanobiaceae	1.7	0.3	1.6	7.3
Endozoicomonadaceae	51.0	76.2	31.1	23.8
Moraxellaceae	6.2	2.5	11.3	4.3
Pseudomonadaceae	1.1	0.2	2.5	0.5
Rhodobacteraceae	1.3	0.1	1.2	6.3
Simkaniaceae	9.8	11.8	9.1	4.8
Sphingomonadaceae	2.1	0.5	3.8	2.8

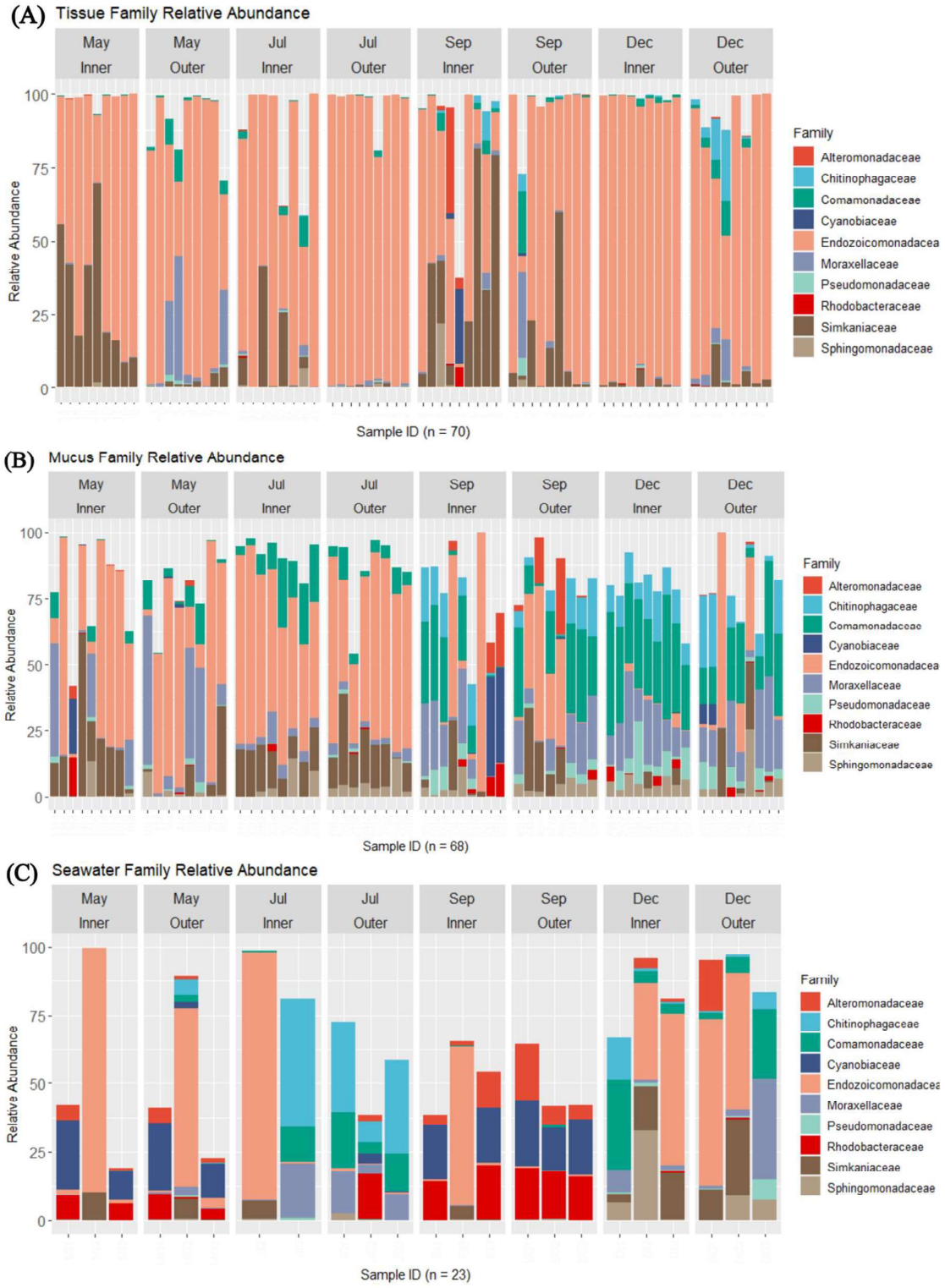

Figure 10. Relative abundances of the overall ten most abundant families found in the coral tissue (A), coral mucus (B), and seawater (C). Samples were separated by the months and zones in which they were collected. May and July are in Guam's dry season, and September and December are in Guam's wet season.

Microbial community composition (beta diversity) significantly differed between coral tissue, coral mucus, and seawater compartments (Appendix 3; $F = 18.955$, $df = 2$, $p < 0.001$), between months ($F = 6.713$, $df = 1$, $p < 0.001$), and between inner and outer zones ($F = 2.492$, $df = 1$, $p = 0.043$). There was also a significant interaction effect between zone and month ($F = 3.729$, $df = 1$, $p = 0.017$), compartment and month ($F = 5.552$, $df = 2$, $p < 0.001$), but no significant difference was found between compartment and zone ($F = 1.768$, $df = 1$, $p = 0.085$) or between compartment, zone, and month ($F = 1.278$, $df = 2$, $p = 0.230$). Within tissue, there was a significant difference in beta diversity between inner and outer zones ($F = 5.422$, $df = 1$, $p = 0.002$), as well as an interaction effect of zones and months ($F = 4.832$, $df = 1$, $p = 0.003$), but not between months ($F = 1.644$, $df = 1$, $p = 0.153$). For the mucus, there was no significant difference between inner and outer zones ($F = 0.8771$, $df = 1$, $p = 0.483$), or between the interaction of zones and months ($F = 1.178$, $df = 1$, $p = 0.254$), but months differed significantly ($F = 7.851$, $df = 1$, $p < 0.001$). In the seawater, there was no significant difference between inner and outer zones ($F = 0.477$, $df = 1$, $p = 0.892$), between months ($F = 1.100$, $df = 1$, $p = 0.346$) or interaction of zones and months ($F = 0.1893$, $df = 1$, $p = 0.998$).

Pairwise PERMANOVAs (Appendix 4) revealed significant differences in beta diversity between tissue and mucus ($F = 14.640$, $df = 1$, $p = 0.003$), between tissue and seawater ($F = 12.340$, $df = 1$, $p = 0.003$) as well as mucus and seawater ($F = 4.116$, $df = 1$, $p = 0.003$). Overall, significant differences were also seen between months: May and December ($F = 4.409$, $df = 1$, $p = 0.018$), and July and September ($F = 44.377$, $df = 1$, $p = 0.006$). In mucus samples, significant differences were seen between each month except for September and December ($F = 1.410$, $df = 1$, $p = 1.000$). Two PCoA plots based on weighted UniFrac distances were constructed to show differences between compartments, zones, and months (Figure 11).

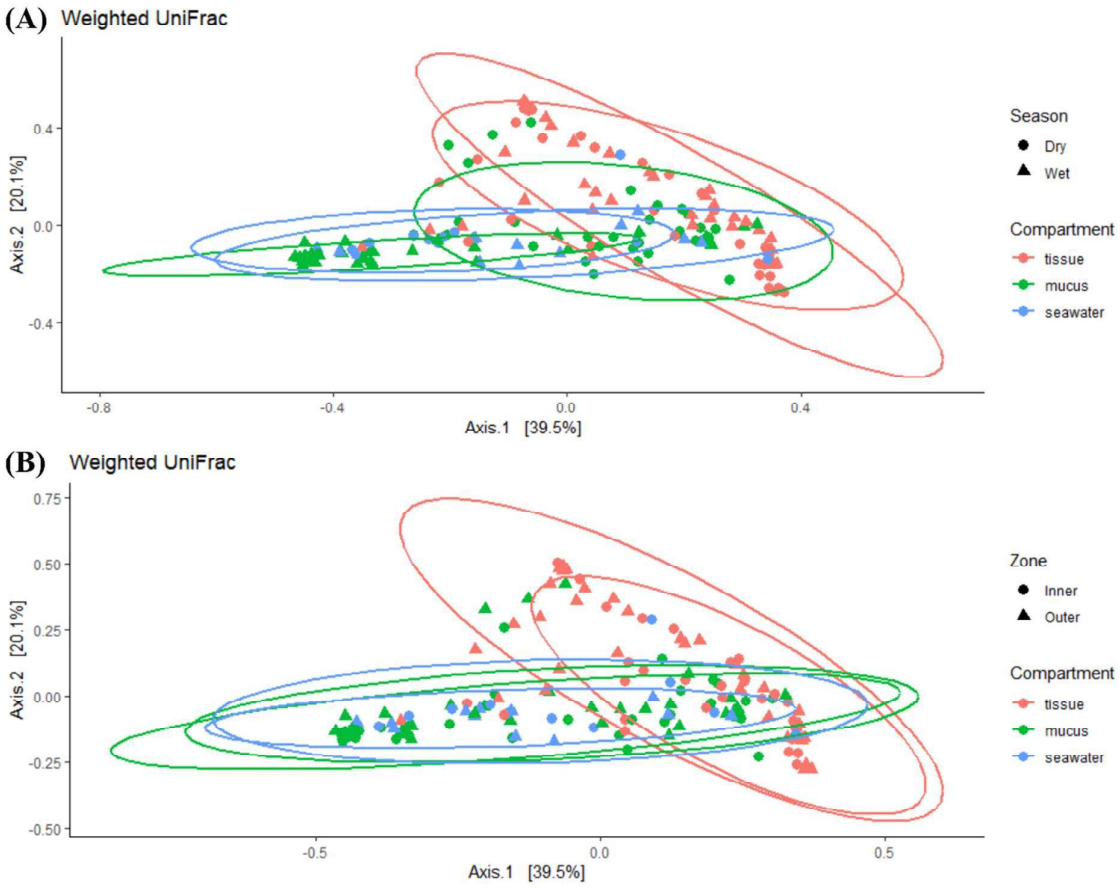

Figure 11. Principal Coordinate of Analysis (PcoA) of weighted UniFrac distances visualizing beta diversity. Each point on the plots represents a sample from the study based on abundance of bacterial ASVs. In each plot, the compartments are represented by color. Red: tissue; green: mucus; blue: seawater. (A) Shapes represent the season (circle for dry season, triangle for wet season). (B) Shapes represent zone (circle for inner zone, triangle for outer zone).

Linear mixed effects model analyses for SEMs found the following statistically significant ($p < 0.05$) linkages: (1) an effect of temperature on concentrations of total coliform, *E.coli*, and *Enterococcus*; (2) an effect of precipitation on microbial diversity of mucus and of concentrations of total coliform, *E.coli*, and *Enterococcus;* (3) an effect of *E. coli* concentration on total coliform concentration and *Enterococcus* concentration; (4) an effect of total coliform concentration on tissue microbial diversity and *E. coli* concentration; and (5) an effect of *Enterococcus* concentration on *E. coli* concentration (Figure 12). Correlations between temperature and concentrations of FIB, as well as between

concentrations of *E. coli* and *Enterococcus*, were negative; all other correlations were positive. In the SEMs examining tissue and mucus diversity, rainfall became a marginally significant predictor for concentrations of *E.coli* ($p = 0.0539$; $p = 0.0644$). Total coliform concentration was a statistically significant predictor of tissue diversity ($p = 0.0373$, $R^2 = 0.13$). No significant interaction effect was found between total coliform and zone, however ($t = 1.858$, $p = 0.068$). Precipitation was the most statistically significant predictor of mucus diversity ($p = 0.0142$, $R^2 = 0.16$) but no statistical significance was seen between rainfall and zone ($t = 1.880$, $p = 0.065$). There was no statistically significant predictor for seawater diversity ($R^2 = 0.50$) in this model.

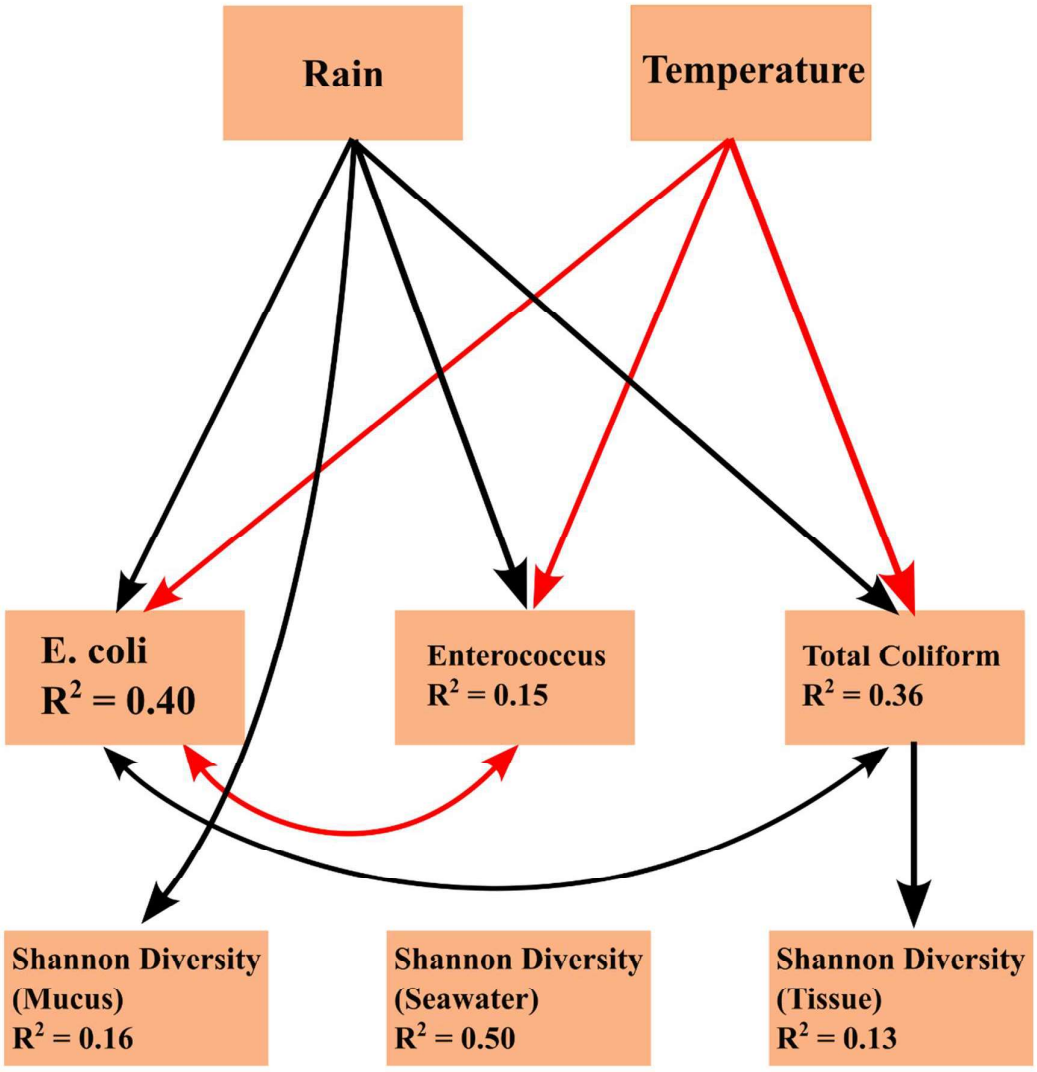

Figure 12. Structural Equation Model (SEM) showing impacts of exogenous variables (Rain and Temperature) on endogenous variables (concentrations of *E. coli*, *Enterococcus*, and Total Coliform) and response variables (Shannon Diversity of each compartment). R^2 values are given under each endogenous and response variable. Arrows indicate statistically significant ($p < 0.05$) linkages. Red lines indicate a negative correlation. Black lines indicate a positive correlation.

4. Discussion

Microbes are an essential component of the coral holobiont and confer health benefits to the coral to ensure its survival in dynamic ocean environments. Conversely, microbes can disrupt the holobiont and introduce disease, leaving the coral host vulnerable to environmental stressors. As oceans continue to change in the Anthropocene, the ability for coral hosts to regulate their own microbiome in response to the environment can be crucial for their survival. Characterizing bacterial community profiles of coral microbial compartments and how they respond to differing spatial and temporal conditions gives researchers the ability to identify which taxa of bacteria are vital to maintaining the holobiont of *A. pulchra*. Unexpectedly, overall bacterial alpha diversity did not significantly differ between the inner and outer zones of West Hagåtña Bay, but only when the coral tissue compartment was isolated. Coral tissue also had the lowest bacterial diversity, and seawater had the highest. Seasonal changes associated with the wet and dry seasons in Guam saw no significant changes in coral tissue taxonomic composition, but seasonal bacterial community shifts were seen in coral mucus.

4.1 Environmental Differences in West Hagåtña Bay

This study aimed to examine two environmental zones with significantly different water flow rates. However, because the outer sites from which coral was sampled were dispersed, significant flow rate differences were found not only between the near shore and far shore habitats, but also between the far shore sites (Figure 5). Because the water flow rates between the two outer sites were significantly different, this could have had an effect on the statistical analyses. High water flow rates are associated with increased uptake of nutrients (Atkinson and Bilger, 1992) and endosymbiont abundances (Fifer et al., 2021).

Because the bacterial microbiome responds to nutrient inputs and interacts with algal endosymbionts (Vega Thurber et al., 2014; Raina et al., 2016), different water flow rates likely caused variable responses in the bacterial community composition of these corals. For future studies, researchers should avoid collecting samples near the sewage treatment plant due to water flow being more similar to that of the inner zone.

Originally, water level range was going to be included in analyses for SEM. However, the logger at the inner zone went missing partway through the study, rendering us unable to incorporate it in comparisons of other temporal variables.

Though rainfall dropped in December (Figure 6), concentrations of FIB remained relatively high (Figure 7) and the microbiome of the mucus more closely resembled that of September samples when compared to samples from May and July (Figure 10B). This suggests that increased amounts of rain may have lasting effects on the mucus microbiome. Taxa of bacteria that increased during the wet season (Chitinophagaceae, Comamonadaceae, Moraxellaceae, and Pseudomonadaceae) are often found in assemblages of healthy corals (Chu and Vollmer, 2016; McKew et al., 2012; Vijay et al., 2021), though their functions are largely unknown, and so they may benefit the coral microbiome when it receives an influx of nutrients and bacteria. Interestingly, the microbiome of seawater in December looked vastly different from that of the September samples (Figure 10C), showing that the seawater microbiome changes more rapidly in response to the environment than that of the mucus.

The outer zone experienced a greater range in water levels than the inner zone (Figure 4), likely caused by greater wave amplitude at the outer site, signifying that corals living in the outer zone receive a greater degree of flushing. This coincided with increased relative abundances of Endozoicomonadaceae in the tissue samples (Figure 10A). Corals in the inner zone experienced a lower range in water levels as well as increased relative abundances of

Simkaniaceae. The inner zone also experienced higher temperatures than the outer zone (Figure 3), and previous studies (Raymundo et al., 2019) have shown that corals within this zone experienced higher degrees of bleaching and coral colony mortality than those in the outer zone. Because Simkaniaceae and Endozoicomonadaceae can potentially be an energy source for their host (Maire et al., 2022) increased abundances of Simkaniaceae is likely indicative of the coral host's ability to regulate its own microbiome and produce its own energy in stressful environmental conditions.

FIB concentrations typically correlated with rainfall patterns. The concentrations of total coliform are greater at the inner zone and in the wet season than at the outer zone and dry season (Figure 6; Figure 7). *E. coli* follow a similar trend, as they are at their highest concentration in early May and during the first wet season sampling timepoint in September. *E. coli* concentrations remain fairly high in December despite having a much lower amount of rainfall than September (Figure 7). The high concentrations of FIB at the first sampling timepoint are likely because of a rainfall event on the day preceding sampling. Total coliform shows a sharp increase in concentration at the inner site during the month of September (Figure 7). Though the total concentration of *Enterococcus* was roughly the same between July and September, the inner zone in September had a much higher concentration than the outer zone (Figure 7).

4.2 Differences in Microbiome Community

Previous studies on the coral bacterial microbiome have compared different compartments (Pollock et al., 2018; Marchioro et al., 2020; Sweet, Croquer and Bythell, 2011), microhabitats (Camp et al., 2020; Fifer at al., 2022) or examined microbiome shifts over time (Dunphy et al., 2019; Chu and Vollmer, 2016; Sweet, Croquer and Bythell, 2010).

This study conducted comparisons of the coral microbiome by incorporating compartmental, spatial, and temporal elements.

The overlap seen between the mucus and seawater communities demonstrates that the mucus is more susceptible to influence from the surrounding environment, and that the tissue microbiome experiences a greater degree of regulation from the host. Despite *Acropora* being considered a "microbiome conformer" (Ziegler et al., 2019), the microbiome of *A. pulchra* tissue remained surprisingly stable throughout the duration of this study. Results from this study support that seawater and mucus microbiomes are richer and more diverse than that of the tissue, which has been shown previously (Marchioro et al., 2020; Sweet, Croquer and Bythell, 2011; Apprill, Weber and Santoro, 2016).

Overall, microbial beta diversity was significantly different between the inner and outer zones of West Hagåtña Bay as well as between months. Unweighted UniFrac was also constructed to examine if there were significant differences when not considering abundance (Appendix 5). In both weighted and unweighted UniFrac PCoA plots, mucus samples in the wet season always clustered together. This highlights the seasonal change in the mucus microbiome as it responds to Guam's wet and dry seasons. The wet season brings turbidity, freshwater, nutrients, and bacteria to West Hagåtña Bay, and because the mucus is in direct contact with each of these agents, it is intuitive that the microbiome would respond accordingly. Mucus samples from the dry season heavily overlapped with tissue samples from both seasons, indicating that, without the influx of freshwater and associated contaminants, the mucus microbiome receives a greater degree of regulation by the coral host.

4.3 Microbiome Regulation

To what degree the species *A. pulchra* regulates its own microbiome is largely unexplored. One previous study evaluating whether corals within the genus *Acropora* were considered "conformers" or "regulators" used *A. hemprichii* (Ziegler et al., 2019) and deemed it to be a "microbiome conformer" due to its bacterial community diversity reflecting shifts in the external environment. However, the species *A. hyacinthus* is proposed to be a heat tolerant coral whose microbiome can acclimate to stressful conditions after transplantation (Ziegler et al., 2017). Likewise, *A. millepora* has shown to be resistant to infectious strains of *Vibrio coralliilyticus* (Ushijima et al., 2014), when other corals such as *Montipora* were affected, though *V. coralliilyticus* is known to show host specificity (Brown, Bourne, and Rodriguez-Lanetty, 2013). Studies that examined bleaching susceptibility of *Acropora* to anthropogenic nitrogen inputs (Burkepile et al., 2020; Donovan et al., 2020) looked at *A. retusa, A. hyacinthus, A. globiceps*, and *A. lutkeni*. Some *Acropora* species, such as *A. millepora*, are able to regulate their own microbiome so that seasonal changes in the environment do not have as much of an effect the coral holobiont, whereas the coral tissue of species such as *A. tenuis* is highly susceptible to its microbiome being influenced by the environment (Marchioro et al., 2020). In Guam as well as the Hawaiian archipelago, corals from the genus *Porites* were found to have a higher disease prevalence than *Acropora* and *Montipora* (Myers and Raymundo, 2009; Aeby et al., 2011), despite *Porites* being considered a microbiome "regulator". When assessed for bacterial indicator taxa, Camp et al. (2020) found that *A. pulchra* only harbored six taxa, whereas *A. muricata* harbored 27. They also found that *A. muricata* living in a lagoon had greater bacterial diversity than colonies of the same species living on the reef, or of *A. pulchra* colonies living in either site. These results suggest that, compared to *A. muricata* and potentially other acroporids, *A. pulchra* has a relatively stable microbiome. The microbial variability of corals in the genus *Acropora*

shown by the aforementioned studies indicate that whether a taxon is considered a microbiome "regulator" or "conformer" is not predictable at the genus level but requires species-level scrutiny.

Because the bacterial beta diversity of the coral tissue only showed significant differences between zones and the interaction effect of zone and month, this implies that the coral host acclimates to the microenvironment it lives in, and is relatively unaffected by environmental changes that fluctuate seasonally, such as rain and temperature. In this study, the coral tissue of *A. pulchra* is dominated by the family Endozoicomonadaceae, which is considered an essential endosymbiont for corals through production of antimicrobial compounds (Rua et al., 2014) and has been seen with strong positive correlations of Symbiodiniaceae densities (Marchioro et al., 2020). This dominance was more noticeable for colonies living near the reef crest, whereas colonies from the inner zone contained greater relative abundances of Simkaniaceae. A similar trend has been seen in New Caledonia (Camp et al., 2020), where colonies of *A. pulchra* at the reef margin were dominated by *Endozoicomonas*, but those living in lagoons were characterized by Simkaniaceae as well as Moraxellaceae. Previous studies (Vouga, Baud, and Greub, 2017) have found that Simkaniaceae are sometimes the dominant bacterial family in juvenile corals and Simkaniaceae have been described as obligate intracellular endosymbionts. *Simkania* is a genus of bacteria within the family Simkaniaceae commonly found in conjunction with the genus *Endozoicomonas*. In some corals, such as *Pocillopora acuta, Simkania* are always found in cell-associated microbial aggregates (CAMAs) adjacent to *Endozoicomonas* (Maire, 2022). *Simkania* contain genes for glycolysis, the tricarboxylic acid cycle (TCA) (Köstlbacher et al., 2021), and pentose phosphate pathway (Maire, 2022). Because of this, *Simkania* can potentially use acetate as a substrate for TCA and as a carbon source for the coral host. Because *Endozoicomonas* can produce and secrete excess acetate, neighboring

Simkania CAMAs can likely use this as a source of energy for the coral host (Maire, 2022). This signifies that coral hosts harboring *Endozoicomonas* and *Simkania* can regulate a degree of their own energy production and maintain their own microbiome. The ability to self-regulate a microbiome may signify the ability of the coral to withstand heat stress during coral bleaching, and possibly fight off pathogenic bacteria to resist disease.

Conversely, the mucus showed no significant difference in beta diversity based on zone or interaction of zone and month, but showed a significant difference based on month. Different months reflect seasonal changes, particularly the abundance of rainfall. Because rainfall brings increased amounts of nutrient and bacterial loads into West Hagåtña Bay, this intuitively causes changes to the composition of the mucus microbiome due to the mucus absorbing constituents from the environment. The clustering of mucus samples from the wet season with seawater samples as well as the clustering of mucus samples from the dry season with tissue samples in the PCoA plots displays that mucus represents a boundary layer between the coral tissue and the seawater. Endozoicomonadaceae dominated the dry season but was retained in only a few samples from the wet season. The microbiome of the mucus during the wet season had greater abundances of Pseudomonadaceae, Moraxellaceae, Comamonadaceae, and Chitinophagaceae. Some taxa within Pseudomonadaceae, such as *Pseudomonas aeruginosa,* are found in the mucus of healthy corals and are known for their antibacterial, antiviral, and antifouling fatty acid methyl esters, allowing them to fight off viruses and prevent development of biofilms (Vijay et al., 2021). Moraxellaceae include the genus *Psychrobacter*, which are often found in the coral mucus (McKew et al., 2012) and have genes affiliated with carbon and nitrogen metabolism as well as an ability to utilize organic compounds contained in coral mucus (Badhai, Ghosh and Das, 2016). Comamonadaceae has been reported in anthropogenically unimpacted colonies of *A. cervicornis* and *A. palmata* (Chu and Vollmer, 2016), *Pavona, Porites* (Barott et al., 2011), as

well as algae assemblages (Roder et al., 2014). These three bacterial taxa are commonly associated with healthy corals and possibly confer benefits to their coral host. Mucus microbial composition is partly regulated by the host's physiology (Glasl, Herndle and Frade, 2016), so it is maintained by the coral host while being subjected to influence by the environment.

Seawater had the greatest microbial diversity of each compartment as well as the most variability but the lowest sampling effort. Sampling effort was constrained due to physical feasibility. Seawater samples weighed substantially more than the tissue and mucus samples, and thus there was limited capacity to transport it from the field. This high variability and low sampling effort may be what led to lack of statistical significance in analyses. ANOVA and PERMANOVA showed no statistically significant predictor for alpha and beta diversity, respectively, and the only statistically significant pairwise interaction were between samples collected during the months of May and July ($F = 4.620$, $df = 1$, $p = 0.024$). There was no readily apparent pattern between inner and outer zones, but samples collected during the timepoint with the greatest amount of rainfall (September) had greater abundances of Rhodobacteraceae, Cyanobiaceae, and Alteromonadaceae. Samples collected at the height of temperatures in the dry season contained greater abundances of Chitinophagaceae, Comamonadaceae, and Moraxellaceae. Interestingly, the seawater composition of samples collected during the dry season, at the first sampling time period, were most similar to those collected during the wet season, at the third sampling period. These samples were most strongly characterized by Cyanobiaceae and Rhodobacteraceae, which are commonly found together (Deignan and McDougald, 2022; Botte et al., 2022). Rhodobacteraceae are considered an opportunistic heterotrophic bacterial family which rapidly grow when presented with organic-rich nutrients from terrestrial runoff (McDevitt-Irwin et al., 2017). Increased abundances of Rhodobacteraceae have been correlated with declining amounts of

Endozoicomonadaceae in colonies of *Porites lutea* undergoing heat stress in the Andaman Sea (Pootakham et al., 2019). Rhodobacteraceae fall under the order Rhodobacterales, which grow quickly in response to amino acid resources that could be supplied by cells damaged by *Vibrio* (Welsh et al., 2017). In *A. millepora*, *A. gemmifera*, and *A. muricata*, abundances of Rhodobacterales and Vibrio-related taxa have been seen increasing together during thermal bleaching (Bourne et al., 2008; Gardner et al., 2019). Cyanobiaceae, a family of Cyanobacteria, also increased in abundance in conjunction with the rainfall events and amounts of Rhodobacteraceae. Cyanobacteria can take up nitrogen so it can be utilized by Symbiodiniaceae for photosynthesis production (Yellowlees, Rees, and Leggat, 2008). Alteromonadaceae, which includes the genus *Alteromonas*, also increased with Cyanobiaceae and Rhodobacteraceae. *Alteromonas* live freely in seawater and can incorporate and translocate NH_4^+ into coral tissues and associated Symbiodiniaceae (Ceh et al., 2013).

4.4 Potential Drivers of Bacterial Diversity

The environmental parameters that were examined in this study explained a limited amount of variation in the microbial communities of *A. pulchra* tissue, mucus, and seawater. Interestingly, tissue bacterial diversity was driven by the concentrations of total coliform bacteria (SEM: $p = 0.037$; $R^2 = 0.13$). Because coliform bacteria can survive for extended periods of time in salt water and under a wide range of temperatures, this may be an indicator of long-term environmental conditions within the bay. Previous studies (Marchioro et al., 2020) found that total suspended solids (TSS) significantly impacted the microbiome of two *Acropora* species: *A. tenuis* and *A. millepora*. TSS and total coliform concentrations are two indicators used on Guam to determine whether water has been anthropogenically impacted. TSS can limit light availability for photosynthesis as well as decrease Symbiodiniaceae densities, which can affect the coral microbial community. Given that the most recent

sampling period on Guam (2015-2018) had TSS more than four times greater than the threshold for acceptable environmental standards (GWA, 2019), TSS are likely influencing the microbial communities of corals living in West Hagåtña Bay. Several studies have demonstrated the influence of Symbiodiniaceae on the coral microbial community, showing that these different micro-associates not only have effects on each other, but work in conjunction in regulating and maintaining the coral holobiont (Glasl et al., 2017; Grottoli et al., 2018; Peixoto et al., 2017).

Rainfall was identified as a significant predictor for mucus microbial diversity (SEM: $p = 0.014$, $R^2 = 0.16$), and thus an increase in rainfall is expected to increase this diversity. This is most likely because rainfall causes the influx of nutrients and bacterial loads from land into the bay. Previous studies have established clear links between rainfall and increased nutrient loads (such as NO_2^- /NO_3^-) for inshore reefs (Fabricius, 2005). According to SEM, total coliform ($p < 0.001$, $R^2 = 0.36$), *E. coli* ($p = 0.038$, $R^2 = 0.40$) and *Enterococcus* ($p < 0.001$, $R^2 = 0.15$) also followed trends based on rain. Linear models showed a strong correlation between FIB and rainfall, but only when the inner and outer zones were separated. This could signify that bacterial loading from rainfall and runoff primarily affects the microenvironment inside West Hagåtña Bay, close to shore, though there was an insignificant interaction effect of rain and zone on mucus biodiversity ($t = 1.880$, $p = 0.065$).

The microbial community of seawater is more sensitive to the environment than coral-associated communities, as shown in the SEM (tissue: $R^2 = 0.13$; mucus: $R^2 = 0.16$; seawater: $R^2 = 0.50$). The lack of statistically significant predictors in this model indicates that seawater is influenced by factors that were not explored in this study. When visualizing beta diversity, PCoA plots based on weighted UniFrac did not show any clear clustering for seawater based on season or zone, though there was noticeable overlap with mucus samples from the wet season. Previous research (Marchioro et al., 2020) has shown a correlation between an

increase in particulate and dissolved nutrients that can occur from runoff, including TSS and NH_4^+, with an increase in mucus-associated Rhodobacteraceae. Because noticeable amounts of Rhodobacteraceae were seen in the seawater during the first wet season sampling timepoint, they were likely able to grow and reproduce from nutrient loads. This is also the most probable cause for the large increase in Cyanobiaceae, which also grows in response to increased nitrogen concentrations.

When averaged by day, temperature values were highest at the end of the dry season (35.29°C) and lowest in the middle of the wet season, when rainfall was most abundant (26.94°C). According to SEM, water temperatures had a direct influence on concentrations of FIB (total coliform: $p < 0.001$, $R^2 = 0.36$; *E. coli*: $p < 0.001$; $R^2 = 0.40$; *Enterococcus*: $p = 0.001$; $R^2 = 0.15$), and concentrations of total coliform directly impacted bacterial diversity of the tissue. Effects of water temperature on corals has been heavily studied (Jokiel and Coles, 1990; Barnhill et al., 2020; Donovan et al., 2020; Pootakham et al., 2019), and water temperature is associated with other environmental factors such as autotroph production (Battin et al., 2008), which have downstream effects on water chemical and nutrient composition (Feuchtmayr et al., 2009). A significant difference in temperature over an extended period of time is expected to cause changes in the environment and lead to downstream impacts on the corals and thus to seasonal changes in their microbiome. This is especially true since temperature and light availability directly impact Symbiodiniaceae, which provide energy for the coral host. Different algal genotypes have variable responses to environmental parameters (Little, van Oppen, and Willis, 2004), which can affect nutrient availability in the coral holobiont.

The lack of conformity by the coral tissue to seasonal environmental conditions is surprising, since *Acropora* species are considered “microbiome conformers” (Ziegler et al., 2019). The expected shifts were instead seen in the mucus. This is intuitive, as mucus is

constantly in contact with both tissue and seawater compartments and acts as a boundary between the two, as seen in the PCoA plots (Figure 12). Because different *Acropora* species exhibit a wide range of capabilities in maintaining their own microbiomes, it is possible that *A. pulchra* is a species that is less influenced by the environment than other acroporids. Another possibility is that studies that find conformity in *Acropora* species to environmental conditions may conflate tissue and mucus microbiomes. It is worth noting that some studies (Pootakham et al., 2021) explicitly do not separate coral compartments before extracting DNA from them with the claim that they aim to characterize microbiomes of the entire coral structure. Future studies should take care to thoroughly separate mucus from coral tissue to more accurately describe how the microbiome responds to environmental conditions.

4.5 Conclusions

This study looked at the bacterial microbiome of seawater, coral mucus, and coral tissue of staghorn *Acropora pulchra* in different microhabitats to examine shifts related to differences between the shore and the reef crest as well as across the wet and dry seasons. Despite *Acropora* species being considered "microbiome conformers", this study showed that the microbiome of coral tissue remained stable between spatial and temporal shifts and that variability was instead seen in coral mucus. Bacterial diversity mostly differed between compartments regardless of site or season. Within compartments, bacterial diversity of coral tissue differed most by zone, and coral mucus differed most by sampling timepoint. This study highlights the differential effects that environmental parameters have on each compartment of the coral bacterial microbiome and potential drivers of microbial diversity.

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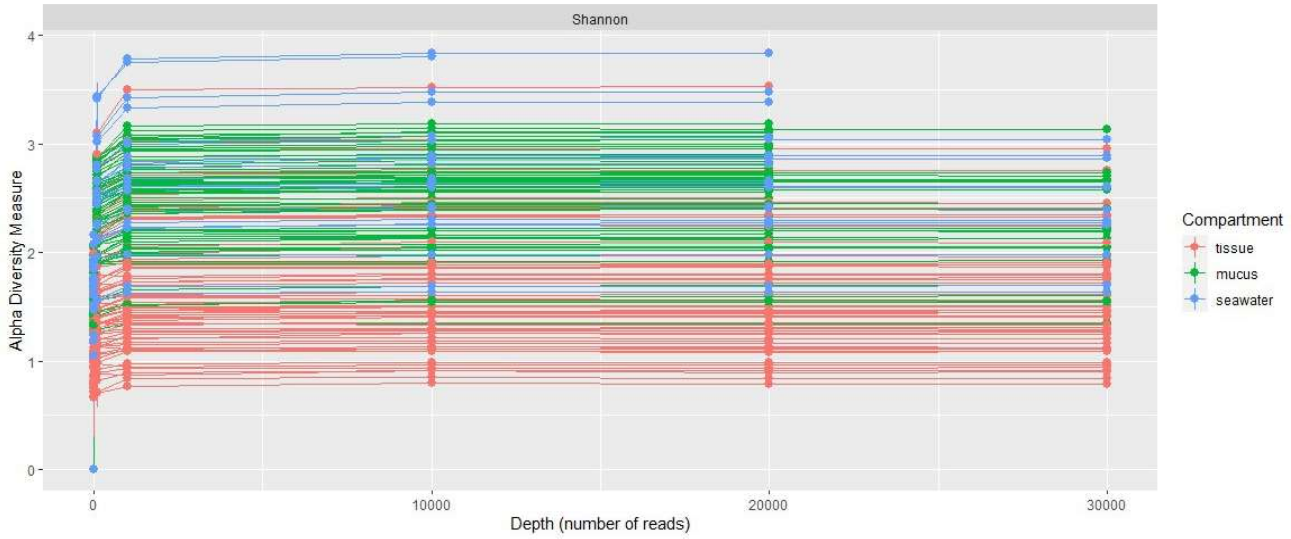
Appendices

Appendix 1. Rarefaction curve created to visualize sequencing depth of coral tissue, coral mucus, and seawater samples. With the exception of a few samples, sequencing depth plateaued around 1000 reads.



Appendix 2. Kruskal-Wallis table for interactions among microbial communities from distinct coral compartments (seawater, mucus and tissue), month (May, July, September and December) and zone (in *versus* out). Because seawater has a normal distribution, an analysis of variance (ANOVA) was used.

Overall

Source of Variation Interactions	*df*	Kruskal-Wallis Chi-squared	*p*(perm)[1]
Compartment	2	63.138	**<0.001**
Zone	1	1.029	0.310
Month	3	7.612	0.055
Zone:Month	7	9.222	0.237
Compartment:Zone	5	64.776	**<0.001**
Compartment:Month	11	73.897	**<0.001**
Compartment:Zone:Month	23	86.204	**<0.001**

Tissue

Source of Variation Interactions	*df*	Kruskal-Wallis Chi-squared	*p*(perm)[1]
Zone	1	0.018	0.893
Month	3	6.115	0.106
Zone:Month	7	13.530	0.060

Mucus

Source of Variation Interactions	*df*	Kruskal-Wallis Chi-squared	*p*(perm)[1]
Zone	1	1.288	0.256

Month	3	8.567	**0.036**
Zone:Month	7	14.507	**0.043**

Seawater

Source of Variation Interactions	*df*	*F value*	*p*[1]
Zone	1	1.056	0.316
Month	1	2.975	0.100
Zone:Month	1	0.970	0.337

[1]Significant results (p(perm) < 0.05) are highlighted in bold

Appendix 3. Permutational multivariate analysis of variance (PERMANOVA) table for interactions among microbial communities from distinct compartments (coral tissue, coral mucus, and seawater), month (May, July, September and December) and zone (in *versus* out) based on weighted UniFrac values.

Overall

Source of Variation Interactions	*df*	*F value*	*p*(perm)[1]
Compartment	2	18.955	**0.001**
Zone	1	2.492	**0.043**
Month	1	6.713	**0.001**
Zone:Month	1	3.729	**0.017**
Compartment:Zone	1	1.768	0.085
Compartment:Month	2	5.552	**0.001**
Compartment:Zone:Month	2	1.278	0.230

Tissue

Source of Variation Interactions	*df*	*F value*	*p*(perm)[1]
Zone	1	5.422	**0.002**
Month	1	1.644	0.153
Zone:Month	1	4.832	**0.003**

Mucus

Source of Variation Interactions	*df*	*F value*	*p*(perm)[1]
Zone	1	0.877	0.483

Month	1	7.851	**0.001**
Zone:Month	1	1.178	0.254

Seawater

Source of Variation Interactions	*df*	*F value*	*p*(perm)[1]
Zone	1	0.477	0.892
Month	1	1.100	0.346
Zone:Month	1	0.189	0.998

[1]Significant results (*p*(perm) <0.05) are highlighted in bold

Appendix 4. *Post hoc* permutational multivariate analysis of variance (PERMANOVA) table for pairwise comparisons among microbial communities between individual compartments (coral tissue, coral mucus, and seawater), and individual months (May, July, September and December) based on weighted UniFrac values.

Overall

Source of Variation Interactions	*df*	*F value*	*p*(perm)[1]
Tissue vs. Mucus	1	14.640	**0.003**
Tissue vs. Seawater	1	12.340	**0.003**
Mucus vs. Seawater	1	4.116	**0.003**
May vs. Jul	1	3.994	0.054
May vs. Sep	1	2.163	0.240
May vs. Dec	1	4.409	**0.018**
Jul vs. Sep	1	4.377	**0.006**
Jul vs. Dec	1	2.731	0.168
Sep vs. Dec	1	2.428	0.210

Tissue

Source of Variation Interactions	*df*	*F value*	*p*(perm)[1]
May vs. Jul	1	0812	1.000
May vs. Sep	1	0.282	1.000
May vs. Dec	1	2.168	0.701
Jul vs. Sep	1	1.178	1.000
Jul vs. Dec	1	1.188	1.000

Sep vs. Dec	1	3.051	0.138

Mucus

Source of Variation Interactions	*df*	*F value*	*p*(perm)[1]
May vs. Jul	1	7.727	**0.006**
May vs. Sep	1	3.213	**0.036**
May vs. Dec	1	8.474	**0.006**
Jul vs. Sep	1	7.236	**0.006**
Jul vs. Dec	1	17.948	**0.006**
Sep vs. Dec	1	1.410	1.000

Seawater

Source of Variation Interactions	*df*	*F value*	*p*(perm)[1]
May vs. Jul	1	4.620	**0.024**
May vs. Sep	1	1.793	1.000
May vs. Dec	1	3.152	0.216
Jul vs. Sep	1	4.292	0.054
Jul vs. Dec	1	1.886	0.486
Sep vs. Dec	1	4.506	0.114

[1]Significant results (*p*(perm) <0.05) are highlighted in bold

Appendix 5. Unweighted UniFrac to visualize differences between compartments, seasons, and zones. Each point on the plots represents a sample from the study based on phylogenetic distance of bacterial ASVs. In each plot, the compartments are represented by color. Red: tissue; green: mucus; blue: seawater. (A) Shapes represent season (circle for dry season, triangle for wet season). (B) Shapes represent zone (circle for inner zone, triangle for outer zone).