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Variability in Antibacterial Activity in the Caribbean Sponge Amphimedon compressa

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Variability in Antibacterial Activity in the Caribbean Sponge *Amphimedon compressa*

by
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A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

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Abstract

Coral reefs are essential ecosystems that provide an abundance of natural resources. Sponges, common reef inhabitants, produce a diversity of secondary metabolites that are known to serve as chemical defenses. Secondary metabolites often have ecological functions, such as antipredator and antibacterial activities. I studied the common Caribbean sponge species, *Amphimedon compressa,* which is known to be chemically defended. Samples were collected from three sites in Belize, two sites in Grand Cayman, and three sites in St. Croix, U.S. Virgin Islands. To determine whether antibacterial activity varied across broad or local geographic scales, sponge extracts were tested against four bacterial strains that included coral and human pathogens. Antibacterial assays compared the degree of growth inhibition by the sponge extracts. In addition, extracts from healthy tissue samples from both healthy sponges and sponges affected by sponge white patch disease were tested to assess any variation in extract concentration and antibacterial activity with sponge condition.

Extracts from *A. compressa* inhibited all the bacterial strains tested, but there was selective antibacterial activity against the different bacterial strains. In general, *A. coralicida*, a Caribbean coral pathogen, was most strongly inhibited, whereas *V. coralliilyticus,* an Indo-Pacific coral pathogen, was least inhibited. I found no variation in extract concentrations at local or broad geographic scales. Antibacterial activity differed between countries and sites within countries, and there were significant interactions between the bacterial strains and sites in Belize and St. Croix, resulting in different geographic patterns against the different bacteria. Sponge extracts from healthy and diseased sponges showed no difference in extract concentration or level of antibacterial

activity. These results indicate that *A. compressa* has strong antibacterial chemical defenses that vary geographically, and disease has no impact on the antibacterial activity of the remaining healthy tissue. Further studies must be conducted to determine the specific chemicals responsible for antibacterial activity and the factors responsible for the geographic variation.

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Introduction

Coral Reefs

Coral reefs are essential ecosystems and natural resources across the world's tropical regions. Reefs serve as habitats for a tremendous diversity of organisms, such as microbes, macroalgae, fishes, corals, sponges, and molluscs (Gittenberger et al., 2014). Coral reefs provide important economic value to humans through various means. For example, the structure of reefs shields coastlines from the harsh conditions of waves and storms (Ferrario, 2014). Many countries rely on reef resources to support their economies through the tourism and fishing industries (Selig et al., 2012). In addition, coral reefs have shown promise as sources of novel biotechnological resources. Neurotoxins that have anesthetic potential have been discovered in marine snails (Bonnemain, 2005) and sea anemones (Urbarova et al., 2012). Antimicrobial activity against certain pathogens has been found in marine microbes (Quintero et al., 2018) and jellyfish proteins have been isolated and used as molecular probes (Martin et al., 1994; Mitra et al., 1996; Niedenthal et al., 1996). However, coral reefs are experiencing declines globally as a result of increased stress from anthropogenic sources (Jackson et al., 2014).

Stressors Affecting Coral Reefs

There has been a 30-50% reduction in global coral reef cover since the 1980s due to the rise in human industry and population (National Academies of Sciences,

Engineering, and Medicine, 2019). These stressors include local pollution, climate change, and disease outbreaks. Sewage pollution has become an increasing problem, as around half of the total human population resides in coastal areas (Abaya et al., 2018), and sewage runoff can introduce large quantities of hazardous pathogens, toxins, nutrients, and other chemicals into the local environment (Wear and Vega Thurber, 2015). The excess nutrients from sewage and other sources can instigate changes in oligotrophic waters where coral reefs typically live by promoting eutrophication and disrupting the ecosystem's equilibrium (Holmes, 2000). Increases in algal growth due to eutrophication can suppress the healthy microbiome of corals and sponges and stimulate pathogenic microbial blooms (Zaneveld et al., 2016). Algal blooms reduce light penetration and negatively impact the photosynthetic symbionts that provide energy to their coral hosts (Pastorok and Bilyard, 1985). As a result, pathogens can become more prevalent, causing diseases and mortality. Another major source of stress on coral reefs is climate change. Carbon dioxide output has increased in recent decades as human populations and manufacturing have grown. Greater atmospheric CO_2 content raises global temperatures and ocean acidification, which results from the reaction between CO_2 and ocean water that decreases pH and carbonate saturation (Hoegh-Guldberg et al., 2007). Increased ocean temperatures and acidification reduce calcification and growth of corals and other calcifying organisms. The rate of these changes has intensified, hindering the abilities of organisms to adapt (Hoegh-Guldberg et al., 2007). These conditions can increase pathogen virulence and the vulnerability of reef organisms to disease (Slattery and Gochfeld, 2012). In a five-year study, Walton et al. (2018) showed that as ocean temperatures have increased, so have coral bleaching and disease incidence.

Disease outbreaks, particularly among corals, have caused great shifts in structure and diversity on Caribbean reefs (Harvell et al., 1999).

Sponges

Sponges, a highly diverse marine taxon (van Soest et al., 2012), are becoming increasingly more abundant on reefs as corals are depleted (Bell, 2013). Sponges are important in stabilization of the reef framework (Wulff and Buss, 1979). However, they face the same climate stressors as corals (Bell et al., 2015). Sponges are immobile benthic filter feeders that serve a variety of functions for their surrounding environment (Bell, 2008; van Soest et al., 2012). They can be used as reef health bioindicators due to their proficient filtering of bacteria and debris from the water column (Potens, 2016). They house diverse endosymbiont populations that vary between individuals and species and can contribute up to 40% of the total volume of a sponge (Webster and Taylor, 2012). Like corals, the mutualistic relationship between microbes and sponges provides the host with the necessary nutrients and energy for survival (Freeman and Thacker, 2011). There are two broad classifications applied to sponge species when evaluating their microbial symbiont content: high microbial abundance (HMA) and low microbial abundance (LMA) species. HMA sponges contain large microbial populations, while LMA sponges contain smaller, less diverse populations (Hentschel et al., 2006; Vacelet and Donadey, 1977).

Sponges are ecologically beneficial to the reef ecosystems in which they reside. Marine sponges aid in nutrient cycling, provide structural support, and food and habitats for other species (Diaz and Rutzler, 2001). However, since they are unable to move away from harm, predators and competitors could easily overcome these sessile species if they

did not have mechanisms to protect themselves. Consequently, they are major producers of secondary metabolites (Rohde et al., 2012).

Chemical Defenses

Secondary metabolites are organic compounds produced by organisms that are not involved in their primary metabolic pathways (Verpoorte, 2000). Although not essential to survival, secondary metabolites serve important roles in interacting with the surrounding environment (Verpoorte, 2000). Secondary metabolites are used as chemical defenses by many sponges, and other non-motile organisms, to provide protection from ecological threats (Braekman and Daloze, 1986). However, not all sponges are chemically defended. The production of chemical defenses is energetically expensive, so some species have invested their energy elsewhere. For instance, undefended sponges have higher growth, healing, and recruitment rates (Leong and Pawlik, 2010; Pawlik et al., 2008;Walters and Pawlik, 2005). The source of secondary metabolites (i.e., whether they are produced by the host sponge or its microbial symbionts) is an ongoing topic of debate (Gochfeld et al., 2012, 2019). Despite this, a wide array of compounds, including alkaloids, terpenes, terpenoids, and fatty acids, have been identified within sponges (Thomas et al., 2010). These compounds play many ecological roles (Thoms and Schupp, 2007), including having feeding deterrent (Pawlik et al., 1995), antifoulant (Qi and Ma, 2017), allelopathic (Slattery and Gochfeld, 2012), and antimicrobial properties (Helber et al., 2018; Rohde et al., 2015).

The types and concentrations of secondary metabolites vary between sponge species and individual populations. Specifically, the antimicrobial activity of different species has shown varying levels of selectivity, likely due to variability in the types or

concentrations of compounds produced (Newbold et al., 1999). Within species of *Aplysina*, there are significant differences in chemical composition and antimicrobial activity between geographic locations in the Caribbean (Stockton, 2016; Vickers, 2017), and between healthy individuals and those affected by disease (Gochfeld et al. 2012; Vickers 2017). Likewise, evidence has shown that feeding deterrence within sponge species varies across countries (Rohde et al. 2012; Slattery et al., 2015). This variability has been attributed to the specific stressors that sponge populations encounter within their local environments.

Amphimedon compressa

Amphimedon compressa is one of the most common sponge species found on Caribbean reefs (Wulff, 1991). It is classified as an LMA species as its microbial count is virtually analogous to seawater (Angermeier et al., 2012). The microbiome of *A. compressa* includes mostly unclassified bacteria, as well as unclassified Gammaproteobacteria and Acidomicrobidae, but also includes some coral disease-associated bacteria (Negandhi et al., 2010). Microbial abundance in *A. compressa* has been shown to vary among locations in South Florida and Panama (Negandhi et al., 2010; Potens, 2016), so it would be expected that secondary metabolite production would vary over those geographic scales as well. The genus *Amphimedon* is known to produce various alkaloids and fatty acids, some of which have anticancer and antimicrobial properties, as well as ecological activities. *A. compressa* is chemically defended against natural threats, including predators (Pawlik et al., 1995), marine fouling bacteria, and pathogens (Newbold et al., 1999). Specifically, amphitoxin, which has ichthyotoxic and

insecticidal activity, and cyclostellettamine, which has antifungal and antibacterial activity, have been isolated from *A. compressa* (Shady, 2019).

Objectives

The focus of this study was to determine whether *A. compressa* is chemically defended against human and marine pathogens, as well as whether extracts from *A. compressa* exhibit selectivity of inhibition against certain bacteria. Specifically, this study evaluated selectivity of sponge extracts against *Serratia marcescens* and *Yersinia enterocolitica,* human pathogens (Gochfeld and Aeby, 2008) that are increasingly present on reefs as pollution increases. In addition, since no known sponge pathogens have been cultured to date, selectivity of antibacterial activity was also assessed against two common coral pathogens, *Aurantimonas coralicida* and *Vibrio coralliilyticus*. Both *A. coralicida* and *S. marcescens* have been found to occur in the microbiomes of *A. compressa* and at least one other Caribbean sponge (Negandhi et al. 2010). Geographic variability in levels of antibacterial activity between sponges from different countries and from different reefs within countries across the Caribbean basin were tested. In addition, variability in antibacterial activity in extracts from healthy *A. compressa* samples were compared to those from *A. compressa* affected by sponge white patch disease (Angermeier et al. 2012) to assess the effects of disease on the sponge's ability to produce antibacterial chemical defenses.

Methods

Sample Collection

Samples from individuals of the sponge *A. compressa* (Figure 1) were collected from a depth of 15 m at three sites in Belize (Carrie Bow Cay, Southwater Cay, and Curlew Cay), three sites in St. Croix, U.S. Virgin Islands (Cane Bay, Eagle Ray, Salt River), and two sites in Grand Cayman (Kittiwake Anchor Buoy, Wall Street) (Figure 2). The samples for the healthy and diseased assays were collected from healthy individuals and healthy tissue on diseased individuals exhibiting sponge white patch disease (Angermeier et al. 2012; Figure 1B), respectively, at a depth of 12 m at Perseverance Point in St. Thomas, U.S. Virgin Islands. Sponge samples were harvested and placed in individual plastic bags underwater, and frozen following collection. Frozen samples were then transported to the National Center for Natural Products Research at the University of Mississippi.

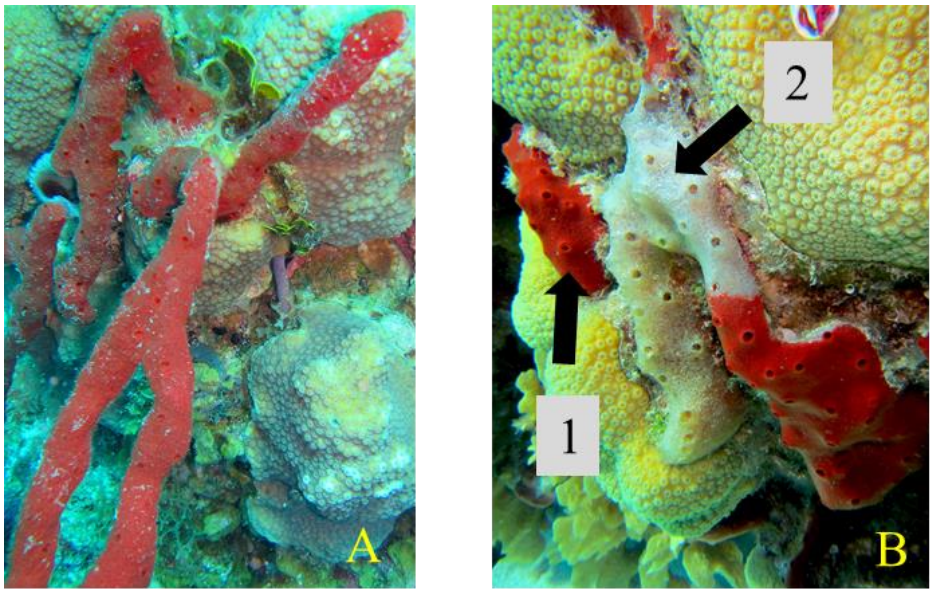

Figure 1. (A) *A. compressa* healthy individual, (B) *A. compressa* affected by sponge white patch disease; arrows indicate (1) healthy and (2) diseased tissues (Photos by D. Gochfeld).

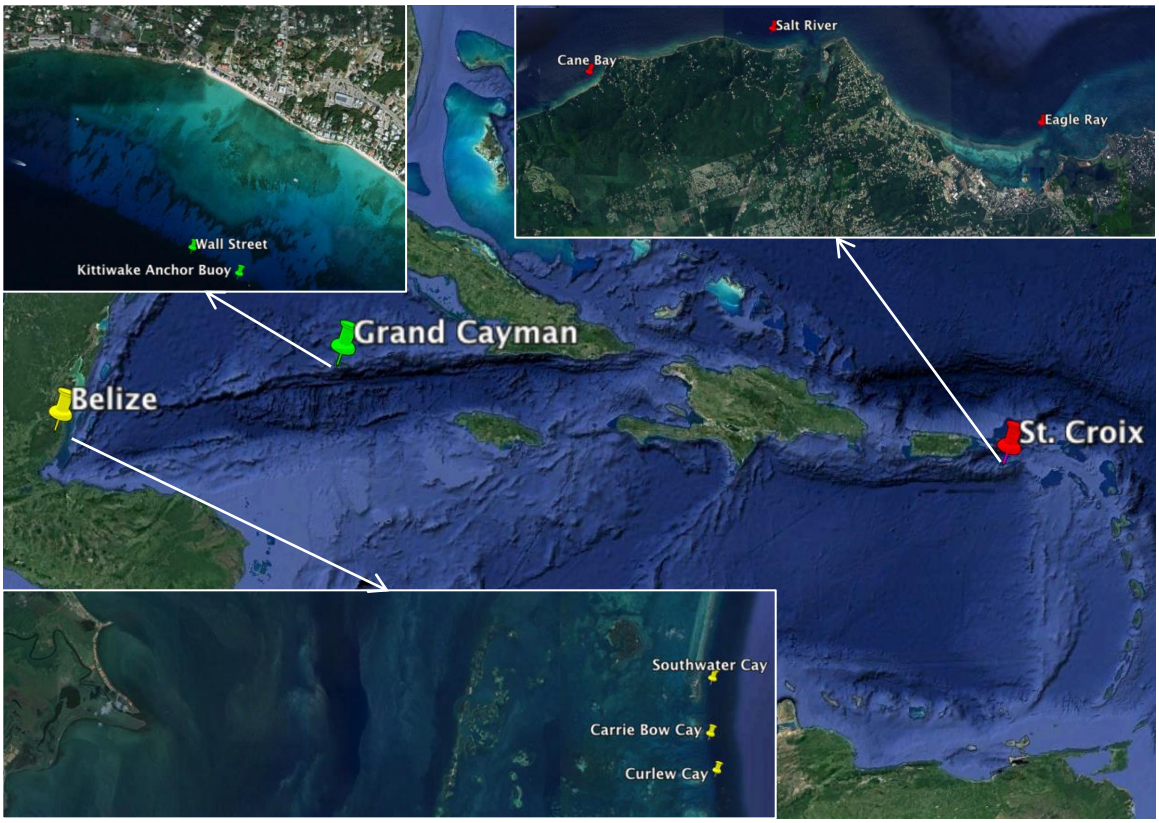

Figure 2. Map of collection sites showing locations of the countries. Arrows point to insets showing locations of collection sites within Belize, Grand Cayman and St. Croix. (Map courtesy of D. Gochfeld)

Extract Preparation

A 5.0 cm piece was excised from each frozen sponge sample and placed in a pre-weighed Whirl-Pak® bag. After freeze-drying for 24 hours, the sponge dry weight was documented, and the samples were crushed into a fine powder. For each sample, 300 mg of powder were deposited into a 50-mL centrifuge tube in order to perform organic extractions. Next, 15 mL of high-performance liquid chromatography-grade methanol were added to each sample tube. Following 15 min of sonication, extracts were decanted into a corresponding pre-weighed vial. The process was repeated twice more before the final combined extract underwent vacuum centrifugation to remove the solvent. Lastly, the final weight of the extract was logged.

Natural extract concentrations were determined for each sponge by dividing the dried extract mass by the dried sponge mass that was extracted (300 mg). Next, the total dried sponge mass was divided by the sponge's displacement volume, and the two products were multiplied to give the natural volumetric concentration for each crude sponge extract in mg/mL.

Bacterial Growth Assays

There are currently no identified sponge pathogens in culture, so known coral and human pathogens were selected for the antibacterial assays due to their presence in the ocean and potential to become pathogenic to marine organisms. The selected bacterial strains included known coral pathogens, *Aurantimonas coralicida* and *Vibrio coralliilyticus* (Ben-Haim et al., 2003; Denner et al., 2003), and known human pathogens, *Serratia marcescens* and *Yersinia enterocolitica* (Gochfeld and Aeby, 2008). *Serratia marcescens* has also been associated with a Caribbean coral disease (Patterson et al. 2002).

A. coralicida and *V. coralliilyticus* were cultured using marine broth at 28°C for 24 hours. *S. marcescens* and *Y. enterocolitica* were also cultured for 24 hours; however, trypticase soy broth (28°C) and tryptose media (37°C) were required, respectively. These conditions were outlined by the vendors from which they were obtained, American Type Culture Collection (ATCC, Manassas, VA, USA) or Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) (Gochfeld and Aeby 2008). Healthy and diseased sponge extracts were only tested against three of the bacterial strains (*A. coralicida, V. coralliilyticus,* and *S. marcescens)*.

The extracts were diluted to a standard concentration of 100 mg/mL in dimethyl sulfoxide (DMSO). The bacterial cultures were diluted to an absorbance reading between 0.09 and 0.11 at an optical density of 600 nm (OD600) using an Eppendorf BioPhotometer. Each assay was conducted using a 96 well plate and run in triplicate. Each well contained a total volume of 200 µl. Experimental wells for each sponge extract held 190 µl of the bacterial culture and 10 µl of sponge extract. Three controls were also run in triplicate. The first held 200 µl of media alone, the second had 200 µl of the bacterial culture alone, and the third included 195 µl of the bacterial culture plus 5 µl of 1 mg/mL ciprofloxacin. Extract controls were also necessary to account for the extracts' color (i.e., pink, red or orange). These wells contained 190 µl of media and 10 µl of extract. Following plate preparation (0 hours), OD600 absorbance readings were taken on a BioTek Synergy HT Multi-Detection Microplate Reader. The plates were incubated on a shaker at the bacteria's specified temperature. After a 24 hour incubation period, absorbance readings were taken again.

Data Analysis

The natural concentration of each crude sponge extract was analyzed using one-way analyses of variance (ANOVAs) to compare among countries and among sites within each country, except for Grand Cayman (which only had two sites), where t-tests were performed. The slope of the growth curve for each extract, tested against each bacterial strain, was calculated to assess whether there was significant antibacterial activity between locations, and between healthy and diseased sponges. For all assays, the more negative the slope, the more the extract inhibited bacterial growth. For extracts from each site or condition against each of the bacteria tested, unpaired t-tests were used to determine whether the slopes differed from zero; that is, whether they had a significant effect on bacterial growth. The slopes of the growth curves for each bacterial strain were then analyzed using one-way ANOVAs to compare the levels of antibacterial activity between sponge extracts from Belize, Grand Cayman, and St. Croix. For each bacterial strain, one-way ANOVAs were also used to compare activity among the three sites within each country for Belize and St. Croix, whereas a t-test was used to compare between the two sites in Grand Cayman. One-way ANOVAs were also used to compare the slopes of the growth curves among bacterial strains across countries, and two-way ANOVAs were performed to assess the antibacterial activity among bacterial strains across sites within each country. Extract concentrations from healthy and diseased sponges were compared using t-tests. Antibacterial activity of healthy and diseased extracts was compared across the three bacterial strains using two-way ANOVAs. The results from significant ANOVAs were further analyzed using Fisher's Partial Least-Squares Difference post-hoc tests.

Results

Mean natural concentration of all *A. compressa* crude extracts from Belize, Grand Cayman and St. Croix was 24.98 ± 0.96 mg/mL. The natural concentrations of the *A. compressa* extracts were compared to assess variability across geographic locations. Extract concentrations among countries were not significantly different from each other (1-way ANOVA, P=0.14; Figure 3A). Likewise, within each country, extract concentrations were also not significantly different among sites (1-way ANOVA: Belize, P=0.43; St. Croix, P=0.80; t-test: Grand Cayman, P=0.22; Figure 3B-D, respectively). The final concentration of extracts used in the antibacterial assays was 5 mg/mL, which is approximately 1/5 of the mean natural concentration of *A. compressa* crude extracts.

The degree of inhibition of bacterial growth (i.e., antibacterial activity) by sponge extracts in the antibacterial assays was determined by comparing the slopes of the bacterial growth curves. All of the sponge extracts inhibited bacterial growth significantly relative to controls without extracts (unpaired t-test, $P<0.05$ for all extracts). Antibacterial activity of sponge extracts tested against *A. coralicida* exhibited significant differences among the three countries (1-way ANOVA, P=0.0007; Figure 4A). Within Belize and St. Croix, antibacterial activity against *A. coralicida* varied between sites (1-way ANOVA: Belize, P=0.0021; St. Croix, P=0.0002; Figure 4B,D), while activity of sponge extracts from the two sites in Grand Cayman did not differ (t-test: P=0.65; Figure 4C). Specifically, in Belize, sponge extracts from Southwater Cay and Curlew Cay did not differ in bioactivity (Fisher's PLSD, P=0.95; Figure 4B), whereas sponge extracts from

Carrie Bow Cay had significantly greater inhibitory effects than those from the other two sites (Fisher's PLSD, $P<0.05$; Figure 4B). Among St. Croix sites, sponge extracts from Salt River had significantly greater inhibitory activity than those from Cane Bay or Eagle Ray (Fisher's PLSD, $P<0.05$; Figure 4D), whereas extracts from Cane Bay and Eagle Ray were similar (Fisher's PLSD, $P=0.69$; Figure 4D).

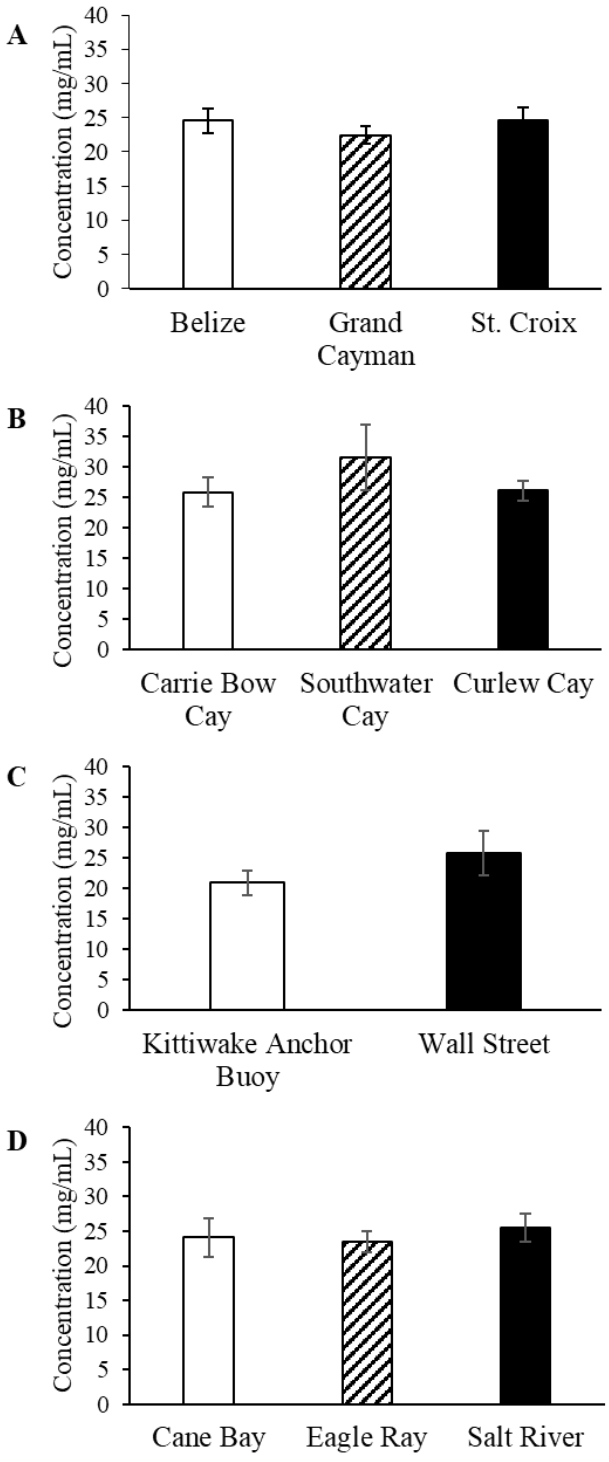

Figure 3. Natural concentrations of crude extracts (mean ± SE) from *A. compressa* among countries (A) and across sites within Belize (B), Grand Cayman (C), and St. Croix (D). There were no significant differences between extract concentrations among countries or among sites within countries.

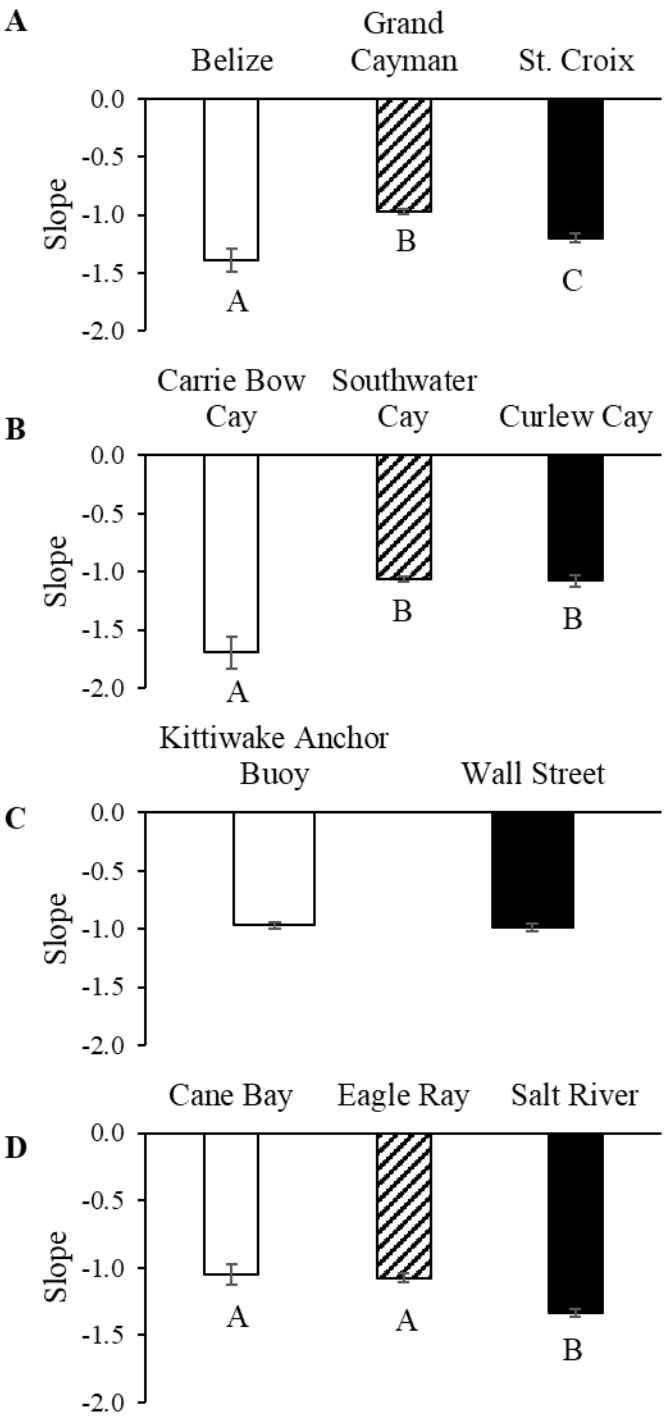

Figure 4. Slopes (mean ± SE) of the growth curves for *Aurantimonas coralicida* across the three countries (A), and across sites within Belize (B), Grand Cayman (C), and St. Croix (D). Letters identify sites that are significantly different within each graph by Fisher's PLSD post-hoc tests at $P<0.05$.

There was significant variation in growth inhibition of *V. coralliilyticus* between countries (1-way ANOVA, P=0.0012; Figure 5A). Sponge extracts from St. Croix had greater inhibitory effects than extracts from Belize and Grand Cayman (Fisher's PLSD, P<0.05; Figure 5A). Locally, variance in antibacterial activity can be seen within Belize and St. Croix (1-way ANOVA, P=0.015 for Belize, P=0.038 for St. Croix; Figure 5B,D), while there was no difference in antibacterial activity between Grand Cayman sites (t-test, P=0.35; Figure 5C). Within Belize, the antibacterial activity was similar for sponge extracts from Southwater Cay and Curlew Cay, which showed less inhibition than sponge extracts from Carrie Bow Cay (Fisher's PLSD, P<0.05; Figure 5B). The greatest inhibition within St. Croix was demonstrated by extracts from Cane Bay and Eagle Ray, both of which had notably greater activity than Salt River's sponge extracts (Fisher's PLSD: P<0.05; Figure 5D).

Sponge extracts from the three countries displayed significant dissimilarity in growth inhibition of *S. marcescens* (1-way ANOVA, P=0.017; Figure 6A). Belize sponge extracts were significantly more inhibitory than those of Grand Cayman; however, antibacterial activity of extracts from St. Croix did not differ from either of the other two countries (Fisher's PLSD: P<0.05; Figure 6A). There was no difference in bioactivity against *S. marcescens* for sponges from the three sites within Belize or St. Croix (1-way ANOVA: Belize, P=0.65; St. Croix, P=0.18; Figure 6B, D). Conversely, sponge extracts from Wall Street were more inhibitory towards *S. marcescens* than those of Kittiwake Anchor Buoy in Grand Cayman (t-test, P=0.044; Figure 6C).

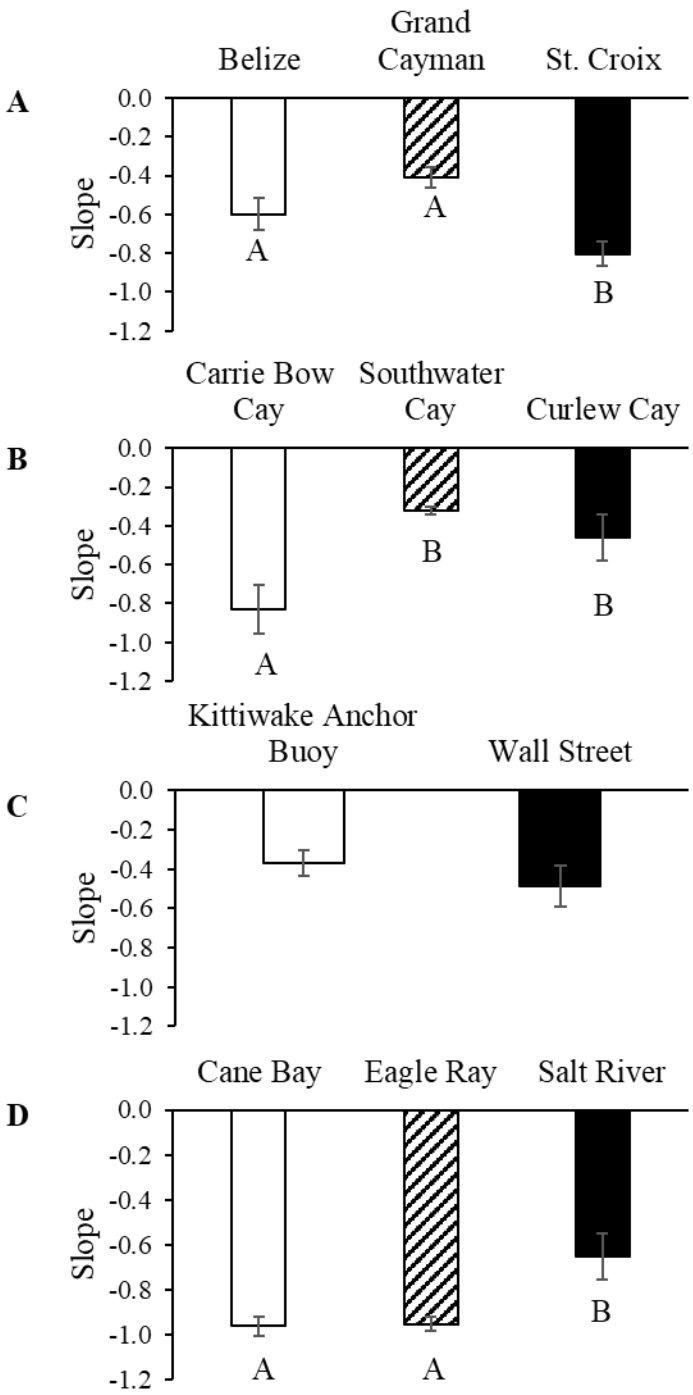

Figure 5. Slopes (mean ± SE) of the growth curves for *Vibrio coralliilyticus* across the three countries (A), and across sites within Belize (B), Grand Cayman (C), and St. Croix (D). Letters identify sites that are significantly different within each graph by Fisher's PLSD post-hoc tests at $P<0.05$.

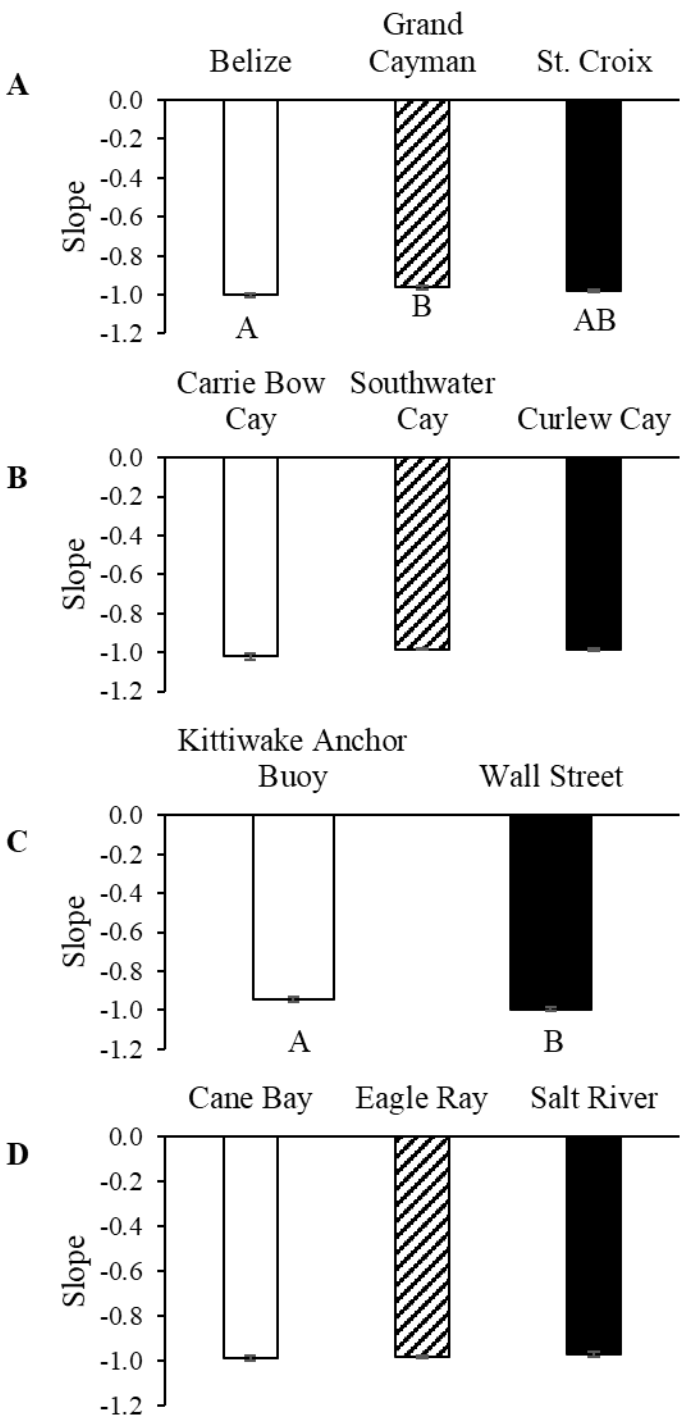

Figure 6. Slopes (mean ± SE) of the growth curves for *Serratia marcescens* across the three countries (A), and across sites within Belize (B), Grand Cayman (C), and St. Croix (D). Letters identify sites that are significantly different within each graph by Fisher's PLSD post-hoc tests at $P<0.05$.

When tested against *Y. enterocolitica*, the degree of inhibition among countries varied (1-way ANOVA, P=0.021; Figure 7A), with Grand Cayman extracts showing higher levels of inhibition than St. Croix, and Belize showing intermediate levels (Fisher's PLSD: P<0.05). Antibacterial activity against *Y. enterocolitica* did not vary among extracts from sites within Belize and Grand Cayman (1-way ANOVA, P=0.088 for Belize; t-test, P=0.35 for Grand Cayman; Figure 7B, C). However, there was variability in activity among sites within St. Croix (1-way ANOVA, P=0.035; Figure 7D). Specifically, Cane Bay and Eagle Ray extracts were less inhibitory than Salt River extracts (Fisher's PLSD: P<0.05; Figure 7D).

When antibacterial activity against all four bacterial strains was compared, there was significant variation within each of the three countries (1-way ANOVA, P<0.0001 for Belize, Grand Cayman, and St. Croix; Figure 8). Within Belize and St. Croix, inhibition of *A. coralicida* growth was significantly greater than that of *S. marcescens* and *Y. enterocolitica*, which had similar levels of inhibition, whereas *V. coralliilyticus* was least affected by sponge extracts (Fisher's PLSD, P<0.05; Figure 8A,C). Antibacterial activities of Grand Cayman's sponge extracts against *A. coralicida* was statistically similar to that of both *S. marcescens* and *Y. enterocolitica*, all of which were more strongly inhibited than *V. coralliilyticus* (Fisher's PLSD, P<0.05; Figure 8B).

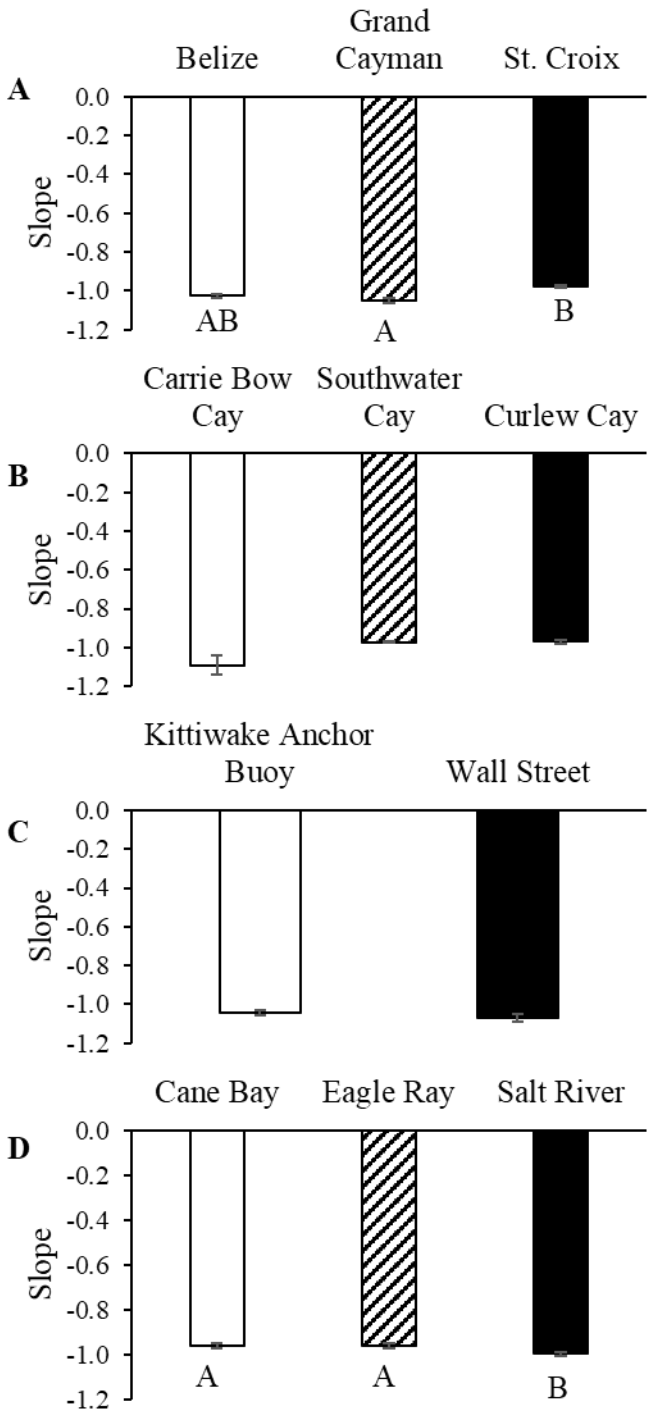

Figure 7. Slopes (mean ± SE) of the growth curves for *Yersinia enterocolitica* across the three countries (A), and across sites within Belize (B), Grand Cayman (C), and St. Croix (D). Letters identify sites that are significantly different within each graph by Fisher's PLSD post-hoc tests at $P<0.05$.

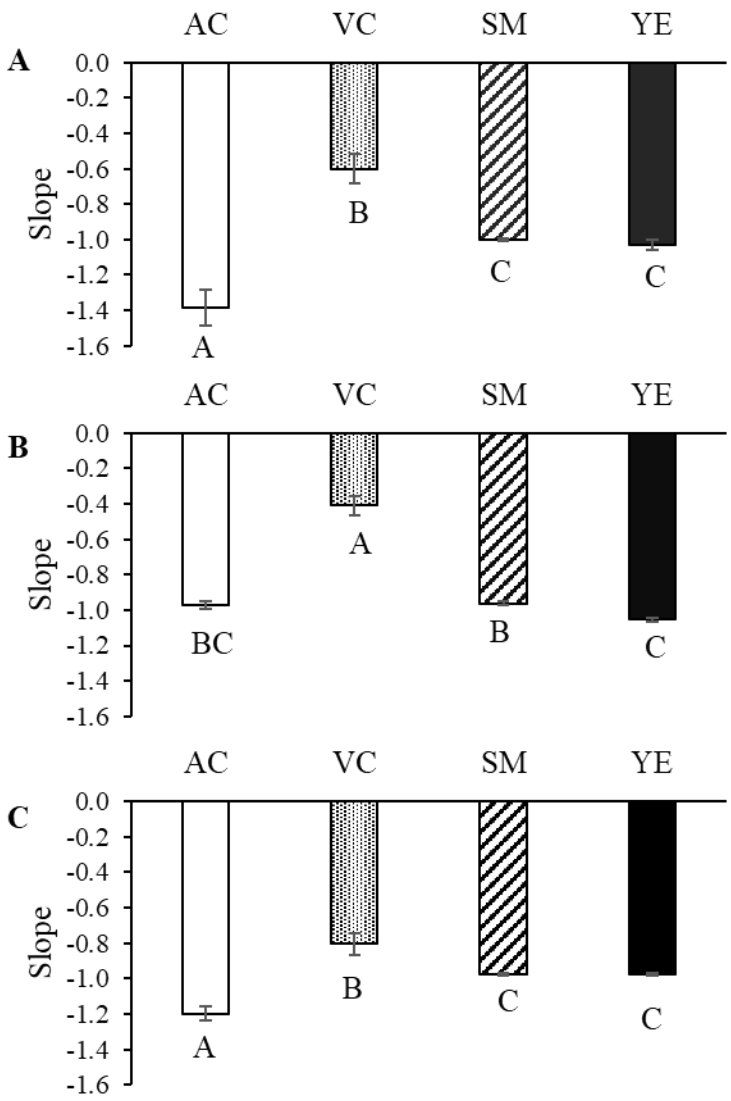

Figure 8. Slopes (mean ± SE) of the growth curves for the four bacterial strains (AC = *Aurantimonas coralicida*, VC = *Vibrio coralliilyticus,* SM = *Serratia marcescens*, YE = *Yersinia enterocolitica*) across Belize (A), Grand Cayman (B), and St. Croix (C). Letters identify sites that are significantly different within each graph by Fisher's PLSD post-hoc tests ($P<0.05$).

Variability in antibacterial activities of sponge extracts from the sites within each country against the four bacterial strains was compared using two-way ANOVAs. For all three countries, there were significant differences in antibacterial activity among bacterial strains (2-way ANOVA, $P<0.0001$ for all countries; Figure 9), with the greatest levels of inhibition against *A. coralicida* in all three countries and similar levels of inhibition against *S. marcescens* and *Y. enterocolitica* in Grand Cayman, whereas *V. coralliilyticus* was least affected by the sponge extracts in all three countries (Fisher's PLSD, $P<0.05$). Antibacterial activity also varied among sites in Belize (2-way ANOVA, $P<0.0001$), with Carrie Bow Cay sponges having higher levels of activity overall compared with the other two sites (Fisher's PLSD, $P<0.05$). There was no effect of site on antibacterial activity in Grand Cayman or St. Croix (2-way ANOVA, $P=0.12$, $P=0.99$, respectively; Figure 9B, C). There were also significant interactions between bacterial strain and site for Belize (2-way ANOVA, $P<0.0001$) and St. Croix ($P<0.0001$). In Belize, sponge extracts from Carrie Bow Cay had the greatest inhibitory effect on *A. coralicida* and those from Southwater Cay and Curlew Cay had the lowest levels of inhibition on *V. coralliilyticus*, while all other extract-bacterial combinations showed intermediate levels of activity (Figure 9A, Table 1). In St. Croix, sponge extracts from Salt River had the greatest levels of inhibition against *A. coralidica* and the least inhibition against *V. coralliilyticus*, whereas there were no differences in activity of all other site-bacteria combinations (Figure 9C).

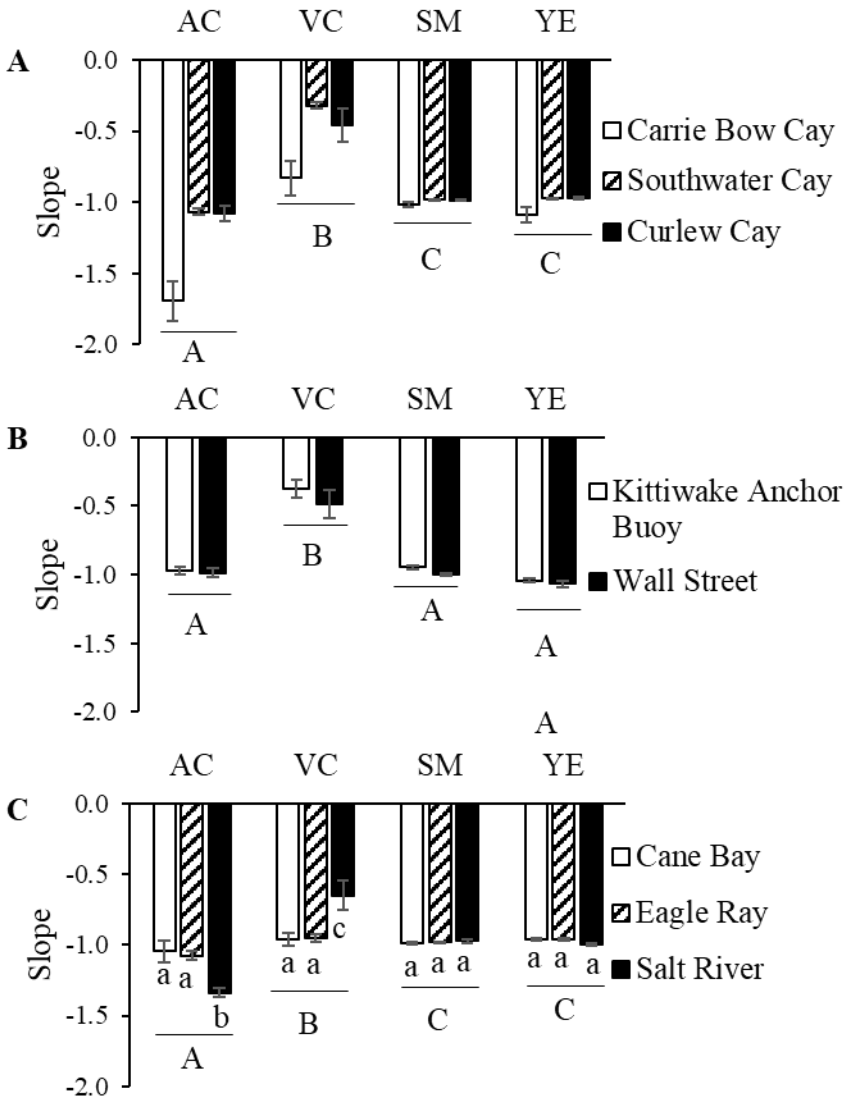

Figure 9. Slope (mean ± SE) of the growth curves for the four bacterial strains (AC = *Aurantimonas coralicida*, VC = *Vibrio coralliilyticus*, SM = *Serratia marcescens*, YE = *Yersinia enterocolitica*) by site in Belize (A), Grand Cayman (B), and St. Croix (C). Lines with capital letters indicate bacterial strains that are significantly different within each graph by Fisher's PLSD post-hoc tests. Lower case letters indicate assays that vary as a result of a significant interaction between site and bacterial strain by Fisher's PLSD post-hoc tests for St. Croix (C). Post-hoc tests for the significant interaction between site and bacterial strain in Belize (A) are listed in Table 1. There was no significant interaction for Grand Cayman (B).

Table 1. Results of pairwise Fisher's PLSD post-hoc tests for significant bacterial strain*site interactions based on two-way ANOVAs for Belize extracts. Letters indicate groups that differ at $P<0.05$. Bacterial strains: AC = *Aurantimonas coralicida*, VC = *Vibrio coralliilyticus,* SM = *Serratia marcescens,* YE = *Yersinia enterocolitica.* Sites: CBC = Carrie Bow Cay, SWC = Southwater Cay, CUR= Curlew Cay.

Bacterial Strain	Site	Groups
AC	CBC	A
AC	SWC	B
AC	CUR	B
VC	CBC	BC
VC	SWC	D
VC	CUR	BD
SM	CBC	C
SM	SWC	C
SM	CUR	C
YE	CBC	C
YE	SWC	C
YE	CUR	C

A comparison of natural concentrations of crude extracts from healthy tissue on both healthy and diseased sponges was performed in order to quantify any variation given the different health condition of the sponges. The results of the unpaired t-test revealed that there is no statistical difference in overall production of extracts between healthy and diseased *A. compressa* ($P=0.07$; Figure 10A).

The effects of sponge health on levels of antibacterial activity were assessed against three of the bacterial strains using 2-way ANOVAS. There was no significant difference in growth inhibition of any of the bacterial strains between healthy and diseased extracts (2-way ANOVA, $P=0.15$; Figure 10B). There was also no significant interaction between extract type and bacterial strain (2-way ANOVA, $P=0.11$; Figure 10B). However, there was significant variability in activity against the different bacterial strains (2-way ANOVA, $P<0.0001$; Figure 10B). Sponge extracts were most inhibitory against *A. coralicida* and showed the least inhibition of *S. marcescens* (Fisher's PLSD: $P<0.05$; Figure 10B).

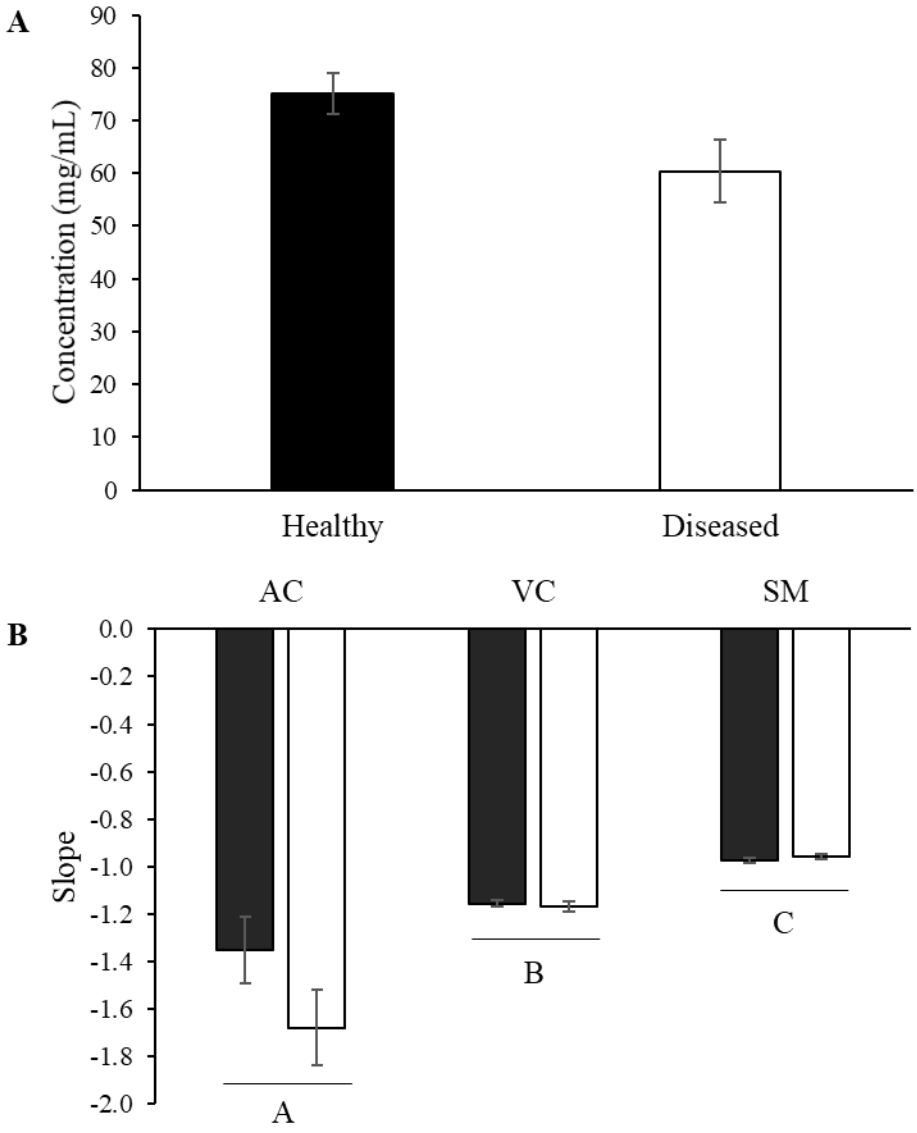

Figure 10. (A) Natural concentrations of crude extracts (mean ± SE) of *A. compressa* from healthy tissues from healthy and diseased sponges did not differ significantly (t-test, P=0.07). (B) Slope (mean ± SE) of the growth curves across three bacterial strains (AC = *Aurantimonas coralicida*, VC = *Vibrio coralliilyticus*, SM = *Serratia marcescens*) and tissue type (Healthy = black, Diseased = white). There was no difference between healthy and diseased extracts. Letters and lines indicate significant differences between bacterial strains by Fisher's PLSD post hoc tests at P<0.05.

Discussion

Sponges are an integral part of coral reef communities (Wulff and Buss, 1979). Much like plants, their sessile life cycle limits their ability to move away from stressors. One adaptation to overcome this limitation is the production of chemical defenses to combat certain stressors, particularly biological stressors, such as predation, competition, and biofouling (Braekman and Daloze, 1986; Pawlik, 2011). The species *A. compressa* is one of the most abundant and conspicuous sponges on Caribbean reefs, and it is chemically defended (Loh and Pawlik, 2014). Thus far, studies have shown that extracts from *A. compressa* provide protection against predators (Loh and Pawlik, 2014; Pawlik et al. 1995; Thompson et al., 2010) and from bacterial colonization (Galeano and Martinez, 2010; Kelly et al., 2005). However, there is variation in its antibacterial activity. For instance, Kelly et al. (2005), showed significant variation in inhibition against different bacterial strains collected from the surrounding site.

In this study, I exposed four bacterial strains to extracts from *A. compressa* in an antibacterial assay (Gochfeld & Aeby 2008; Sisson, 2019; Stockton, 2016) to determine whether there was evidence of: antibacterial chemical defenses against bacterial pathogens, selectivity in chemical defenses against different pathogens, geographic variability in antibacterial chemical defense, and variation in chemical defense production between healthy and diseased samples. The results show that these samples of

A. compressa were chemically defended, supporting data from previous studies (Kelly et al., 2003, 2005; Loh and Pawlik, 2014; Thompson et al., 2010). In fact, extracts in all assays exhibited significant inhibition of bacterial growth. The assays were conducted with extract concentrations that were approximately 20% of the natural concentration. Therefore, if tested at natural concentrations, the antibacterial activity of these extracts could be even greater than observed in this study.

Antibacterial Activity and Selectivity

Although sponges are exposed to a multitude of pathogens in the seawater, it is necessary to have specific activity. For sponges to remain healthy and thrive, their community of bacterial symbionts must be maintained, because they are key to the sponge's physiological processes (Taylor et al., 2007). Broad-spectrum defenses may harm these endosymbionts, indirectly hurting the sponge itself. Thus, it would be beneficial for sponges to have specific defenses that limit the growth of potential pathogens without affecting beneficial microbes.

The extracts from *A. compressa* tested in this study exhibited selectivity in their antibacterial activity, as indicated by the significant differences in the slopes of the growth curves between strains. For instance, Belize and St. Croix extracts were the most inhibitory against *A. coralicida* growth, whereas extracts from all three countries showed the lowest levels of inhibition against the growth of *V. coralliilyticus*. Selectivity in antibacterial activity has been previously shown in sponges. For example, extracts from the sponge, *Xestospongia muta*, were more inhibitory against *S. marcescens* growth than against *V. coralliilyticus* (Sisson, 2019). Sisson's sponge samples were collected from the same countries and sites as in my study with the addition of Curacao. Both of our

studies showed less inhibition of *V. coralliilyticus*, a pathogen associated with coral disease in the Red Sea (Ben-Haim et al. 2003), and stronger inhibition towards *A. coralicida* and *S. marcescens*, pathogens of Caribbean corals (Denner et al., 2003; Patterson et al. 2002). Another study of *Aplysina fulva* and *Aplysina cauliformis* found both significant inhibitory and stimulatory activity against the same bacterial strains used in my study (Stockton, 2016). Stockton (2016) found stimulation of *Y. enterocolitica* growth by several extracts. She concluded sponges may benefit from a symbiotic relationship with these bacterial strains and, therefore, enhance the bacterial growth. Conversely, there were no stimulatory effects on the growth of any of these bacterial strains by *A. compressa* extracts.

Species in the genera *Aplysina* and *Xestospongia* are HMA sponges, whereas *A. compressa* is an LMA species (Gloeckner et al. 2014). LMA species have relatively fewer endosymbionts compared to HMA species, and their microbial communities tend to resemble that of the surrounding seawater. HMA sponges tend to have more unique microbial communities, which limits their ability to produce broad-spectrum chemical defenses against microbes. Therefore, it is likely that LMA sponges may have greater overall chemical defenses against microbes than HMA sponges, which may be able to inhibit or stimulate the growth of certain microbial taxa. This concept is demonstrated here with the significant inhibition of all strains by *A. compressa* as compared to *Aplysina* spp. and *X. muta*, which had a combination of inhibitory and stimulatory activities.

Sponges and corals inhabit the same reefs, so it is likely that sponges will encounter the same pathogens. It is currently unknown whether sponges and corals are affected by the same pathogens, because so few sponge pathogens have been identified

(Olson et al., 2014). *A. coralicida* and *V. coralliilyticus* cause White Plague type II and coral tissue lysis in corals, respectively, while *S. marcescens* is known to infect both corals and humans (Gochfeld and Aeby, 2008). *Y. enterocolitica* is a human pathogen that survives in seawater; however, it is not known to be pathogenic to marine organisms (Gochfeld and Aeby, 2008). Interestingly, a recent study *A. coralicida* and *S. marcescens* found to occur within the microbiome of *A. compressa* sponges from Ft. Lauderdale, FL (Negandhi, et al., 2010). It is unknown whether this is a site-specific phenomenon, or whether those bacterial strains have detrimental effects on the sponges. It is more likely that sponges have evolved defenses against common coral pathogens due to the negative impacts they may face from infection. In this case, the greater inhibition against *A. coralicida* by *A. compressa* extracts could be the result of the greater threat of *A. coralicida*; however, further studies are needed to resolve this matter.

Geographic Variability

In addition to variability in the biological activity of chemical defenses within sponges, there is also evidence that chemical defenses vary among populations of sponges across geographic locations, both across countries and at specific sites (Rohde et al., 2015). Geographic variability in antibacterial activity was found on broad and local scales in this study.

Across countries, there was significant variation in the antibacterial activities against each strain. The location of each country exposes it to different environmental factors that might act as stressors and affect the sponges' activity. Grand Cayman and St. Croix are islands with much smaller human populations than Belize; however, the sites in Belize were 16 miles from shore, while Grand Cayman and St. Croix sites were close to

shore. Without water quality sampling, it is difficult to predict which of these sites might experience greater exposure to terrestrial runoff, which could contain a combination of nutrients, pollutants and potential human pathogens. Excess nutrients can cause eutrophication in the ecosystem (Holmes, 2000). As a result, algal blooms could hinder photosynthetic activity of certain sponge endosymbionts and overall health of the sponges by blocking light penetration (Zanefeld et al., 2016). In fact, there has been a long-term trend of poor water quality monitored at Carrie Bow Cay, Belize (Chollet et al., 2017). It is likely that sponges exposed to poor water quality may be stressed, which may affect their chemical defense production as they shift their energy into other resources, such as growth and wound repair. However, turbidity of Grand Cayman waters is low (Beanish and Jones, 2002), indicating low eutrophication levels and better reef health. Differences in water quality may contribute to some the differences in antibacterial activity among the countries, but those relationships are not clear based on the limited water quality data available.

Other environmental factors that could affect the production of antibacterial chemical defenses over broad geographic scales might include water temperature or ocean acidification, which can directly affect the health of marine organisms (Hoegh-Guldberg et al., 2007). There is currently no data available on pH at these sites. The average water temperatures vary minimally among Belize, Grand Cayman, and St. Croix due to their similarity in latitude. All three countries experienced water temperatures higher than average during the year prior to sample collection, but only Belize experienced water temperatures high enough to cause coral bleaching (NOAA Coral Reef Watch, 2020). Bacteria are extremely temperature sensitive, so slight differences could

greatly alter their abundance on the reef. In addition, exposure to thermal stress could affect the ability of sponges to produce chemical defenses and could contribute to a difference in the chemical diversity among countries, which would affect their bioactivity.

On the local scale, variance in antibacterial activity against specific bacterial strains was found within each country. Belize sites had significant variation in inhibition against *A. coralicida* and *V. coralliilyticus*, while Grand Cayman sites only showed significant differences between sites in activity against *S. marcescens*. St. Croix sites differed in antibacterial activity against *A. coralicida, V. coralliilyticus,* and *Y. enterocolitica.* Differences in antibacterial activity may be the consequence of local stressors. For instance, fishing may remove sponge predators from some reefs, which could cause sponges to shift their energy to produce chemicals for a more relevant local threat, such as fouling bacteria. Similarly, stress from runoff of nutrients, pollution or sediment could also affect sponges' ability to produce chemical defenses. For instance, the Belize sites are offshore where there is less contact between human populations and reefs. Consequently, they all have similar limited exposure to the mainland and runoff from human populations, which is the main source of *Y. enterocolitica* and *S. marcescens* to reefs (Gochfeld and Aeby 2008). Thus, it is more likely that local factors are responsible for differences between the sites in Belize. However, Southwater Cay, which houses several small resorts, and Curlew Cay, which has no land mass at all (Gochfeld, personal communication), are most similar in antibacterial activity, and there is no clear explanation for this. Even over short distances, these reefs could be exposed to different pollutant levels, which could provide an influx of nutrients and pathogens. Added

nutrients may cause a shift in the reef population, creating more competition by increasing algal abundance, resulting in more competition for space, so *A. compressa* may shift production to secondary metabolites that combat overcrowding from other algae as opposed to bacterial pathogens. While there was less site-specific variability in St. Croix overall, the only site that differed was Salt River, which is near the mouth of an estuary (Gochfeld, personal communication). Sponges at that site are likely exposed to periodic pulses of freshwater and terrestrial runoff that could affect their ability to produce chemical defenses and their exposure to pathogens.

A similar study conducted the same antibacterial assays using extracts of *X. muta* from some of the same sites as in the present study; however, they found different results, with little to no local variability in antibacterial activity against the same bacterial strains (Sisson, 2019). Conversely, his results indicated variability across countries. This indicates that sponge species from the same reefs may face the same stressors but have different responses. Thus, the production of chemical defenses is both species-specific, as well as location dependent. Stockton (2016) found geographic variation at the site level in her study of *A. cauliformis*. Her study was conducted with the same bacterial strains, although samples were from different countries and sites. She included a comparison of the secondary metabolite profiles of the sponges from her study, and variability in these profiles corresponded to the differences in antibacterial activity, although she did not identify the specific compounds responsibility for the activity.

Sponge Condition

There is an expectation that there would be a difference between the activity levels of healthy samples from healthy and diseased sponges. A diseased sponge would

be expected to shift its energy to produce greater amounts of secondary metabolites in order to combat the infection (Gochfeld et al., 2012). However, there was no significant difference in antibacterial activity between healthy and diseased samples of *A. compressa*. Sponge White Patch disease causes irregular bleaching along the branches of *A. compressa*, and the exact cause remains unidentified (Angermeier et al., 2012). This disease causes tissue necrosis, with distinct white patches that have a sharp contrast with the surrounding healthy red tissue. Angermeier et al. (2012) found that this healthy tissue was identical to that of healthy individuals under scanning electron microscope studies, whereas the white tissue was severely degraded, with few intact sponge cells. For this reason, I used the healthy tissue on the diseased sponges for my study. Unless the disease causes excessive stress to the sponge, the similarity in healthy tissue from diseased sponges suggests that they should have the same capability of secondary metabolite production as their healthy neighbors. My results support this idea by showing no difference in extract concentrations or inhibition of the bacterial strains between healthy tissue from the healthy and disease sponges.

Aplysina Red Band syndrome (ARBS) is a Caribbean sponge disease that causes lesions on *Aplysina* species; however, extracts of healthy tissue from diseased individuals were found to be more inhibitory against the four bacterial strains used in the present study than extracts from healthy individuals (Vickers, 2017). The pathogen responsible for ARBS is most likely a cyanobacterium (Olson et al. 2014), while the pathogen causing Sponge White Patch disease remains unknown (Angermeier et al., 2012). Overall, it can be concluded that sponge diseases have diverse effects that are just as variable as the species affected.

Future Directions

My study demonstrated that *A. compressa* has significant and selective antibacterial activity against the pathogens tested, and that the level of activity varies over small and large geographic scales. Additional studies are needed to identify the specific *A. compressa* compounds responsible for the antibacterial activity observed. The significant antibacterial properties of *A. compressa* extracts suggest that it could produce compounds that could be used in medicine one day. For example, the *A. compressa* compound 8,8′-dienecyclostellettamine showed antibacterial activity against 6 clinical bacterial strains (Xu et al., 2007).

It is important to further research the geographic variability of sponge chemical defenses for ecological and biomedical purposes. An understanding of factors that affect production of specific compounds could help develop methods for collection of sponges to maximize concentrations of those compounds or design culture conditions to maximize yields. Furthermore, *A. compressa* is common in the Caribbean, and therefore, a key member of the reef system. In order to continue understanding reef dynamics and health, there must be further studies into *A. compressa* secondary metabolites and their interactions with surrounding organisms.

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